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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 December 2013 (12.12.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/185036 A2

(51) International Patent Classification:
C12N 15/82 (2006.01) *A01N 37/10* (2006.01)

(21) International Application Number:
PCT/US2013/044717

(22) International Filing Date:
7 June 2013 (07.06.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/656,546 7 June 2012 (07.06.2012) US

(72) Inventors; and
(71) Applicants : **HOFFMAN, Thomas** [US/US]; 6526 Kingsbury Way, Zionsville, Indiana 46077 (US). **CUI, Yunxing** [US/US]; 13736 Royal Saddle Drive, Carmel, Indiana 46032 (US). **OBOURN, Malcolm** [US/US]; 3210 Bowfield Way, West Lafayette, Indiana 47906 (US). **PARKHURST, Dawn M.** [US/US]; 7844 Amsterdam Court, Avon, Indiana 46123 (US). **WIGGINS, Barry** [US/US]; 617 Apollo Parkway, Westfield, Indiana 46074 (US). **VERCAUTEREN, Michael** [US/US]; 918 Sunrise Avenue, Lafayette, Indiana 47904 (US).

(74) Agents: **ADDISON, Bradford G.** et al.; Barnes & Thornburg LLP, 11 South Meridian Street, Indianapolis, Indiana 46204 (US).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: METHODS OF IMPROVING THE YIELD OF 2,4-D RESISTANT CROP PLANTS

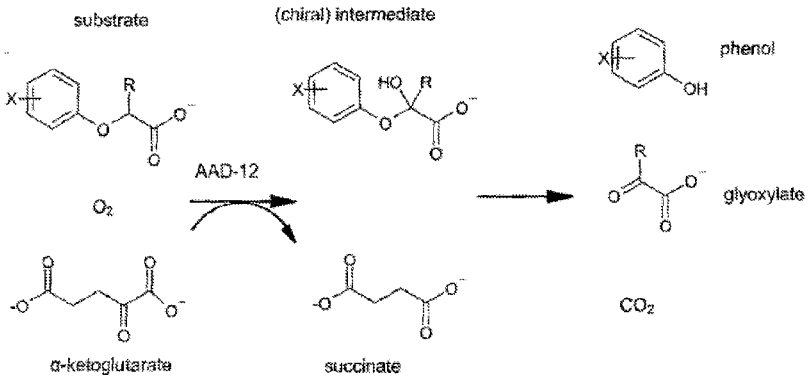

FIG. 1

(57) **Abstract**: This invention is related to methods for improving plant height and/or yield of crop plants which are resistant to herbicide 2,4-D by treating the plants with 2,4-D at application rates which are not harmful to the plants. In particular, provided is a method using 2,4-D application to increase yield of crop plants which express AAD-12 gene for 2,4-D resistance. The method provided is of particular interest for the treatment of crops plants including maize, soybean, spring and winter oil seed rape (canola), sugar beet, wheat, sunflower, barley, and rice.

WO 2013/185036 A2

METHODS OF IMPROVING THE YIELD OF 2,4-D RESISTANT CROP PLANTS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U. S. Provisional Application No. 61/656,546, filed on June 7, 2012, the disclosure of which is hereby expressly incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] Incorporated by reference in its entirety is a computer-readable sequence listing submitted concurrently herewith and identified as follows: one 11,342 byte ASCII (text) file named "72747_ST25.txt", created on May 13, 2013.

BACKGROUND OF THE INVENTION

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

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

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

can effectively compete on price and performance with glyphosate GTC systems.

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

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

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

however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

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

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

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

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

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate

dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

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

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

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

SUMMARY OF THE INVENTION

[0015] This invention is related to methods for improving plant height and/or yield of crop plants which are resistant to herbicide 2,4-D by treating the plants with 2,4-D at application rates which are not harmful to the plants. In particular, provided is a method using 2,4-D application to increase yield of crop plants which express AAD-12 gene for 2,4-D resistance. This invention further relates to the use of 2,4-D for improving the yield of crop plants which are 2,4-D resistant. The method provided is of particular interest for the treatment of crops plants including maize, soybean, spring and winter oil seed rape (canola), sugar beet, wheat, sunflower, barley, and rice.

[0016] In some embodiments, the 2,4-D resistant crop plants are transgenic crop plants transformed with an aryloxyalkanoate dioxygenase (AAD). In a further embodiment, the aryloxyalkanoate dioxygenase (AAD) is AAD-1 or AAD-12. AAD-1 has been previously

disclosed in US 2009/0093366 and AAD-12 has been previously disclosed in WO 2007/053482, the contents of which are incorporated by reference in their entireties.

[0017] The yield-improving effect of the treatment of 2,4-D can be observed at application rates from 25 g ae/ha to 5000 g/ha, or 100 g ae/ha to 2500 g ae/ha, or in particular, 1000 g ae/ha to 2000 g ae/ha. In one embodiment, 1000 g ae/ha to 1500 g ae/ha of 2,4-D is used. In another embodiment, 2000 g ae/ha to 2500 g ae/ha is used. In addition, the yield-improving effect of the treatment of 2,4-D is D is particularly pronounced when 2,4-D is applied in the 2- to 8- leaf stage of the crop plants before flowering. However, the application rate and/or leaf-stage of the crop plant required vary as a function of the plants, their height and the climate conditions.

[0018] The term increase in yield refers to that the plant yield up to 50% or more. In one embodiment, the increase in yield is at least 10%. In another embodiment, the increase in yield is at least 20%. In another embodiment, the increase in yield is from 10% to 60%. In another embodiment, the increase in yield is from 20% to 50%. In another embodiment, the increase in yield is statistically significant. The growth-enhancing activity of 2,4-D to 2,4-D resistant crop plants can be measured in field trials or pot trials. Herbicide having different mode of action are generally known to either have an adverse effect on yield or have no effect on yield.

[0019] In one aspect, provided is a method of improving yield of 2,4-D resistant crop plants, comprising treating the plants with a stimulating amount of a herbicide comprising an aryloxyalkanoate moiety.

[0020] In one embodiment, the 2,4-D resistant crop plants are transgenic plants transformed with an aryloxyalkanoate dioxygenase (AAD). In a further embodiment, the aryloxyalkanoate dioxygenase (AAD) is AAD-1 or AAD-12. In another embodiment, the herbicide comprising an aryloxyalkanoate moiety is a phenoxy herbicide or phenoxyacetic herbicide. In a further embodiment, the herbicide comprising an aryloxyalkanoate moiety is 2,4-D. In a further embodiment, the 2,4-D comprises 2,4-D choline or 2,4-D dimethylamine (DMA).

[0021] In one embodiment, the transgenic plants transformed with an aryloxyalkanoate dioxygenase (AAD) are selected from cotton, soybean, and canola. In another embodiment, the treating is performed at least once at an application rate of 2,4-D as employed also for weed control. In another embodiment, the treating is performed twice at an application rate of 2,4-D as employed also for weed control. In a further embodiment, 2,4-D is applied at the V3 and R2 growth stages of soybean with 2,4-D tolerance. In another embodiment, the treating is performed at least three times at an application rate of 2,4-D as employed also for weed control. In another embodiment, the herbicide comprising an aryloxyalkanoate moiety reaches the 2,4-D resistant crop plants via root absorption.

[0022] In another embodiment, the 2,4-D resistant crop plants are also treated with a herbicide different than 2,4-D for weed control. In a further embodiment, the herbicide different than 2,4-D is a phosphor-herbicide or aryloxyphenoxypropionic herbicide. In a further embodiment, the phosphor-herbicide comprises glyphosate, glufosinate, their derivatives, or combinations thereof. In a further embodiment, the phosphor-herbicide is in form of ammonium salt, isopropylammonium salt, isopropylamine salt, or potassium salt. In another embodiment, the phosphor-herbicide reaches the 2,4-D resistant crop plants via root absorption. In another embodiment, the aryloxyphenoxypropionic herbicide comprises chlorazifop, fenoxaprop, fluazifop, haloxyfop, quizalofop, their derivatives, or combinations thereof. In a further embodiment, the aryloxyphenoxypropionic herbicide reaches the 2,4-D resistant crop plants via root absorption.

[0023] In one embodiment, the 2,4-D resistant crop plants are treated at least once with 25 g ae/ha to 5000 g ae/ha 2,4-D. In another embodiment, the 2,4-D resistant crop plants are treated at least once with 100 g ae/ha to 2000 g ae/ha 2,4-D. In another embodiment, the 2,4-D resistant crop plants are treated at least once with 100 g ae/ha to 2500 g ae/ha 2,4-D. In another embodiment, the 2,4-D resistant crop plants are treated at least once with 1000 g ae/ha to 2000 g ae/ha 2,4-D. In a further embodiment, the 2,4-D comprises 2,4-D choline or 2,4-D dimethylamine (DMA).

[0024] In one embodiment, a method of improving yield of 2,4-D resistant crop plants is provided. The method comprises

(a) transforming plant cells with a nucleic acid molecule comprising a nucleotide sequence encoding an aryloxyalkanoate dioxygenase (AAD);

(b) selecting transformed cells;

(c) regenerating the plants from the transformed cells; and

(d) treating the plants with a stimulating amount of a herbicide comprising an aryloxyalkanoate moiety.

[0025] In one embodiment, the aryloxyalkanoate dioxygenase (AAD) is AAD-1 or AAD-12. In another embodiment, the nucleic acid molecule comprises a selectable marker which is not an aryloxyalkanoate dioxygenase (AAD). In a further embodiment or alternative embodiment, the selectable marker is phosphinothricin acetyltransferase gene (pat) or bialaphos resistance gene (bar). In another embodiment, the nucleic acid molecule is plant-optimized.

[0026] In another aspect, provided is the use of a herbicide comprising an aryloxyalkanoate moiety in the manufacture of transgenic plants with 2,4-D resistance with increased yield as compared to its non-transgenic parent plants. In one embodiment, the a herbicide comprising

an aryloxyalkanoate moiety is 2,4-D. In a further embodiment, the 2,4-D is applied at least once with 25 g ae/ha to 5000 g/ha 2,4-D. In another embodiment, the 2,4-D is applied at least once with 100 g ae/ha to 2000 g ae/ha 2,4-D. In another embodiment, the 2,4-D is applied at least once with 100 g ae/ha to 2500 g ae/ha 2,4-D. In another embodiment, the 2,4-D is applied at least once with 1000 g ae/ha to 2000 g ae/ha 2,4-D. In a further embodiment, the 2,4-D comprises 2,4-D choline or 2,4-D dimethylamine (DMA). In a further embodiment, the 2,4-D resistant crop plants are treated with 2,4-D at least two times before flowering. In another embodiment, the 2,4-D resistant crop plants are transgenic plants transformed with an aryloxyalkanoate dioxygenase (AAD). In a further embodiment, the aryloxyalkanoate dioxygenase (AAD) is AAD-1 or AAD-12.

BRIEF DESCRIPTION OF THE DRAWING AND SEQUENCES

[0027] FIG. 1 illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention. FIG. 2 shows a representative map for plasmid pDAB4468. FIG. 3 shows a representative map for plasmid pDAS1740.

[0028] SEQ ID NO: 1 is the nucleotide sequence of AAD-12 from *Delftia acidovorans*.

[0029] SEQ ID NO: 2 is the translated protein sequence encoded by SEQ ID NO: 1.

[0030] SEQ ID NO: 3 is the plant optimized nucleotide sequence of AAD-12 (v1).

[0031] SEQ ID NO: 4 is the translated protein sequence encoded by SEQ ID NO: 3.

[0032] SEQ ID NO: 5 is the *E. coli* optimized nucleotide sequence of AAD-12 (v2).

[0033] SEQ ID NO: 6 is the sequence of the M13 forward primer.

[0034] SEQ ID NO: 7 is the sequence of the M13 reverse primer.

[0035] SEQ ID NO: 8 is the sequence of the forward AAD-12 (v1) PTU primer.

[0036] SEQ ID NO: 9 is the sequence of the reverse AAD-12 (v1) PTU primer.

[0037] SEQ ID NO: 10 is the sequence of the forward AAD-12 (v1) coding PCR primer.

[0038] SEQ ID NO: 11 is the sequence of the reverse AAD-12 (v1) coding PCR primer.

[0039] SEQ ID NO: 12 shows the sequence of the "sdpacodF" AAD-12 (v1) primer.

[0040] SEQ ID NO: 13 shows the sequence of the "sdpacodR" AAD-12 (v1) primer.

[0041] SEQ ID NO: 14 shows the sequence of the "Nco1 of Brady" primer.

[0042] SEQ ID NO: 15 shows the sequence of the "Sac1 of Brady" primer.

DETAILED DESCRIPTION OF THE INVENTION

[0043] As used herein, the phrase "transformed" or "transformation" refers to the introduction of DNA into a cell. The phrases "transformant" or "transgenic" refers to plant

cells, plants, and the like that have been transformed or have undergone a transformation procedure. The introduced DNA is usually in the form of a vector containing an inserted piece of DNA.

[0044] As used herein, the phrase "selectable marker" or "selectable marker gene" refers to a gene that is optionally used in plant transformation to, for example, protect the plant cells from a selective agent or provide resistance/tolerance to a selective agent. Only those cells or plants that receive a functional selectable marker are capable of dividing or growing under conditions having a selective agent. Examples of selective agents can include, for example, antibiotics, including spectinomycin, neomycin, kanamycin, paromomycin, gentamicin, and hygromycin. These selectable markers include gene for neomycin phosphotransferase (npt II), which expresses an enzyme conferring resistance to the antibiotic kanamycin, and genes for the related antibiotics neomycin, paromomycin, gentamicin, and G418, or the gene for hygromycin phosphotransferase (hpt), which expresses an enzyme conferring resistance to hygromycin. Other selectable marker genes can include genes encoding herbicide resistance including Bar (resistance against BASTA® (glufosinate ammonium), or phosphinothricin (PPT)), acetolactate synthase (ALS, resistance against inhibitors such as sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl oxybenzoates (POBs), and sulfonylamino carbonyl triazolinones that prevent the first step in the synthesis of the branched-chain amino acids), glyphosate, 2,4-D, and metal resistance or sensitivity. The phrase "marker-positive" refers to plants that have been transformed to include the selectable marker gene.

[0045] Various selectable or detectable markers can be incorporated into the chosen expression vector to allow identification and selection of transformed plants, or transformants. Many methods are available to confirm the expression of selection markers in transformed plants, including for example DNA sequencing and PCR (polymerase chain reaction), Southern blotting, RNA blotting, immunological methods for detection of a protein expressed from the vector, *e g.*, precipitated protein that mediates phosphinothricin resistance, or other proteins such as reporter genes β-glucuronidase (GUS), luciferase, green fluorescent protein (GFP), DsRed, β-galactosidase, chloramphenicol acetyltransferase (CAT), alkaline phosphatase, and the like (See Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Press, N.Y., 2001, the content of which is incorporated herein by reference in its entirety).

[0046] Selectable marker genes are utilized for the selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT) as well as

genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. See DeBlock *et al.* (1987) EMBO J., 6:2513-2518; DeBlock *et al.* (1989) Plant Physiol., 91:691-704; Fromm *et al.* (1990) 8:833-839; Gordon-Kamm *et al.* (1990) 2:603-618). For example, resistance to glyphosate or sulfonylurea herbicides has been obtained by using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, bromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides. Enzymes/genes for 2,4-D resistance have been previously disclosed in US 2009/0093366 and WO 2007/053482, the contents of which are hereby incorporated by reference in their entireties.

[0047] Other herbicides can inhibit the growing point or meristem, including imidazolinone or sulfonylurea. Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee et al., EMBO J. 7:1241 (1988); and Miki et al., Theon. Appl. Genet. 80:449 (1990), respectively.

[0048] Glyphosate resistance genes include mutant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) genes (via the introduction of recombinant nucleic acids and/or various forms of *in vivo* mutagenesis of native EPSPs genes), aroA genes and glyphosate acetyl transferase (GAT) genes, respectively). Resistance genes for other phosphono compounds include glufosinate (phosphinothricin acetyl transferase (PAT) genes from Streptomyces species, including Streptomyces hygroscopicus and Streptomyces viridichromogenes), and pyridinoxy or phenoxy proprionic acids and cyclohexones (ACCase inhibitor-encoding genes), See, for example, U.S. Pat. No. 4,940,835 to Shah, et al. and U.S. Pat. No. 6,248,876 to Barry *et al.*, which disclose nucleotide sequences of forms of EPSPs which can confer glyphosate resistance to a plant. A DNA molecule encoding a mutant aroA gene can be obtained under ATCC accession number 39256, and the nucleotide sequence of the mutant gene is disclosed in U.S. Pat. No. 4,769,061 to Comai, European patent application No. 0 333 033 to Kumada *et al.*, and U.S. Pat. No. 4,975,374 to Goodman *et al.*, disclosing nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a PAT gene is provided in European application No. 0 242 246 to Leemans *et al.* Also DeGreef *et al.*, Bio/Technology 7:61 (1989), describes the production of transgenic plants that express chimeric bar genes coding for PAT activity. Exemplary of genes

conferring resistance to phenoxy proprionic acids and cyclohexones, including sethoxydim and haloxyfop, are the Acc1-S1, Acc1-S2 and Acc1-S3 genes described by Marshall *et al.*, Theon. Appl. Genet. 83:435 (1992). GAT genes capable of conferring glyphosate resistance are described in WO 2005012515 to Castle *et al.* Genes conferring resistance to 2,4-D, fop and pyridyloxy auxin herbicides are described in WO 2005107437 and U.S. patent application Ser. No. 11/587,893.

[0049] Other herbicides can inhibit photosynthesis, including triazine (psbA and 1s+ genes) or benzonitrile (nitrilase gene). Przibila *et al.*, Plant Cell 3:169 (1991), describes the transformation of Chlamydomonas with plasmids encoding mutant psbA genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC Accession Nos. 53435, 67441, and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes *et al.*, Biochem. J. 285:173 (1992).

[0050] For purposes of the present invention, selectable marker genes include, but are not limited to genes encoding: neomycin phosphotransferase II (Fraley *et al.* (1986) CRC Critical Reviews in Plant Science, 4:1-25); cyanamide hydratase (Maier-Greiner *et al.* (1991) Proc. Natl. Acad. Sci. USA, 88:4250-4264); aspartate kinase; dihydrodipicolinate synthase (Perl *et al.* (1993) Bio/Technology, 11:715-718); tryptophan decarboxylase (Goddijn *et al.* (1993) Plant Mol. Bio., 22:907-912); dihydrodipicolinate synthase and desensitized aspartade kinase (Perl *et al.* (1993) Bio/Technology, 11:715-718); bar gene (Toki *et al.* (1992) Plant Physiol., 100:1503-1507 and Meagher *et al.* (1996) and Crop Sci., 36:1367); tryptophan decarboxylase (Goddijn *et al.* (1993) Plant Mol. Biol., 22:907-912); neomycin phosphotransferase (NEO) (Southern *et al.* (1982) J. Mol. Appl. Gen., 1:327; hygromycin phosphotransferase (HPT or HYG) (Shimizu *et al.* (1986) Mol. Cell Biol., 6:1074); dihydrofolate reductase (DHFR) (Kwok *et al.* (1986) PNAS USA 4552); phosphinothricin acetyltransferase (DeBlock *et al.* (1987) EMBO J., 6:2513); 2,2-dichloropropionic acid dehalogenase (Buchanan-Wollatron *et al.* (1989) J. Cell. Biochem. 13D:330); acetohydroxyacid synthase (Anderson *et al.*, U.S. Pat. No. 4,761,373; Haughn *et al.* (1988) Mol. Gen. Genet. 221:266); 5-enolpyruvyl-shikimate-phosphate synthase (aroA) (Comai *et al.* (1985) Nature 317:741); haloarylnitrilase (Stalker *et al.*, published PCT application WO87/04181); acetyl-coenzyme A carboxylase (Parker *et al.* (1990) Plant Physiol. 92:1220); dihydropteroate synthase (sul I) (Guerineau *et al.* (1990) Plant Mol. Biol. 15:127); and 32 kD photosystem II polypeptide (psbA) (Hirschberg *et al.* (1983) Science, 222:1346).

[0051] Also included are genes encoding resistance to: chloramphenicol (Herrera-Estrella *et al.* (1983) EMBO J., 2:987-992); methotrexate (Herrera-Estrella *et al.* (1983) Nature, 303:209-

213; Meijer *et al.* (1991) Plant Mol Bio., 16:807-820 (1991); hygromycin (Waldron *et al.* (1985) Plant Mol. Biol., 5:103-108; Zhijian *et al.* (1995) Plant Science, 108:219-227 and Meijer *et al.* (1991) Plant Mol. Bio. 16:807-820); streptomycin (Jones *et al.* (1987) Mol. Gen. Genet., 210:86-91); spectinomycin (Bretagne-Sagnard *et al.* (1996) Transgenic Res., 5:131-137); bleomycin (Hille *et al.* (1986) Plant Mol. Biol., 7:171-176); sulfonamide (Guerineau *et al.* (1990) Plant Mol. Bio., 15:127-136); bromoxynil (Stalker *et al.* (1988) Science, 242:419-423); 2,4-D (Streber *et al.* (1989) Bio/Technology, 7:811-816); glyphosate (Shaw *et al.* (1986) Science, 233:478-481); and phosphinothricin (DeBlock *et al.* (1987) EMBO J., 6:2513-2518). All references recited in the disclosure are hereby incorporated by reference in their entireties unless stated otherwise.

[0052] The above list of selectable marker and reporter genes are not meant to be limiting. Any reporter or selectable marker gene are encompassed by the present invention. If necessary, such genes can be sequenced by methods known in the art.

[0053] The reporter and selectable marker genes are synthesized for optimal expression in the plant. That is, the coding sequence of the gene has been modified to enhance expression in plants. The synthetic marker gene is designed to be expressed in plants at a higher level resulting in higher transformation efficiency. Methods for synthetic optimization of genes are available in the art. In fact, several genes have been optimized to increase expression of the gene product in plants.

[0054] The marker gene sequence can be optimized for expression in a particular plant species or alternatively can be modified for optimal expression in plant families. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest. See, for example, EPA 0359472; EPA 0385962; WO 91/16432; Perlak *et al.* (1991) Proc. Natl. Acad. Sci. USA, 88:3324-3328; and Murray *et al.* (1989) Nucleic Acids Research, 17: 477-498; U.S. Pat. No. 5,380,831; and U.S. Pat. No. 5,436,391, herein incorporated by reference. In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, fully optimized or partially optimized sequences may also be used.

[0055] In addition, several transformation strategies utilizing the *Agrobacterium*-mediated transformation system have been developed. For example, the binary vector strategy is based on a two-plasmid system where T-DNA is in a different plasmid from the rest of the Ti plasmid. In a co-integration strategy, a small portion of the T-DNA is placed in the same vector as the foreign gene, which vector subsequently recombines with the Ti plasmid.

[0056] As used herein, the phrase "plant" includes dicotyledons plants and monocotyledons plants. Examples of dicotyledons plants include tobacco, Arabidopsis, soybean, tomato, papaya, canola, sunflower, cotton, alfalfa, potato, grapevine, pigeon pea, pea, Brassica, chickpea, sugar beet, rapeseed, watermelon, melon, pepper, peanut, pumpkin, radish, spinach, squash, broccoli, cabbage, carrot, cauliflower, celery, Chinese cabbage, cucumber, eggplant, and lettuce. Examples of monocotyledons plants include corn, rice, wheat, sugarcane, barley, rye, sorghum, orchids, bamboo, banana, cattails, lilies, oat, onion, millet, and triticale.

[0057] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the AAD-12 gene is that unlike all tfdA homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (e.g., triclopyr, fluoroxypyr) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in FIG. 1. (Addition of O.sub.2 is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in FIG. 1 illustrate the molecular backbones and that various R groups and the like (such as those shown in Table 1) are included but are not necessarily specifically illustrated in FIG. 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-12 gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled.

[0058] A single gene (AAD-12) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, AAD-12 can provide protection in planta to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing AAD-12 alone now may be

treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

[0059] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-12 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and pyridyloxyacetates auxin herbicides (e.g., triclopyr)--selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0060] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-12 stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0061] Plantlets are typically resistant throughout the entire growing cycle. Transformed

plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance, for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

[0062] Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0063] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, glyphosate oxidoreductase (GOX), GAT), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries=AHAS, Csr1, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. In vivo modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

[0064] Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonylureas (such as chlorsulfuron, halosulfuron, nicosulfuron,

sulfometuron, sulfosulfuron, trifloxysulfuron), imidazoloninones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0065] Additionally, AAD-12 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0066] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to tfdA, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "HX(D/E)X23-26(T/S)X114-183HX10-13R" motif which comprises the active site. The histidines coordinate Fe+2 ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

[0067] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0068] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol

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

[0069] The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an AAD-12 gene.

[0070] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0071] 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.

[0072] 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.

[0073] The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, sdpA, from *Delftia acidivorans* (Westendorf et al., 2002, 2003) with low homology to tfdA (31% amino acid identity). This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichlorprop (Westendorf et al., 2002 and 2003). However, no α-

ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. SdpA has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0074] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

[0075] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

[0076] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. AAD-12 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [e.g., glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide) resistance, bromoxynil resistance, HPPD-inhibitor resistance, PPO-inhibitor resistance, et al.], and insect resistance traits (Cry1F, Cry1Ab, Cry 34/45, other Bt. Proteins, or insecticidal proteins of a non-Bacillis origin, et al.) for example. Additionally, AAD-12 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0077] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). Arabidopsis, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-12-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized"

genes that encode proteins of the subject invention.

[0078] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0079] 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.

[0080] 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.

[0081] The transgenic expression of the subject AAD-12 genes is exemplified in, for example, Arabidopsis, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot crops like alfalfa, clover, tree species, et al. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

[0082] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker

technology.

[0083] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0084] One cannot easily discuss the term "resistance" and not use the verb "tolerate" or the adjective "tolerant." The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." As used herein unless otherwise indicated, herbicide "resistance" is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of The Herbicide Handbook as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered "resistant" even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term "tolerance" is broader than the term "resistance," and includes "resistance" as defined herein, as well an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

[0085] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

[0086] 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.

[0087] 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.

[0088] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0089] 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.

[0090] 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.

[0091] Polynucleotides and probes: The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides

unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0092] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-12 gene constitutively expressed in all tissues for complete protection of the plant at all growth-stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

[0093] 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.

[0094] 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.).

[0095] 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 x 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 x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1 x SSC/0.1% SDS for 30 minutes each at 55 ^{o}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 x SSC/0.1% SDS can be prepared by adding 50 ml of 20 x SSC and 5 ml of 10% SDS to 445 ml of water. 20 x 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.

[0096] 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.

[0097] 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.

[0098] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with 32P-labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25 ^{o}C. below the melting temperature (Tm) of the DNA hybrid in 6 x SSPE, 5 x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA.

[0099] Washes can typically be carried out as follows: (1) twice at room temperature for 15 minutes in 1 x SSPE, 0.1% SDS (low stringency wash); and (2) once at Tm-20 ^{o}C. for 15 minutes in 0.2 x SSPE, 0.1% SDS (moderate stringency wash).

[00100] For oligonucleotide probes, hybridization can be carried out overnight at 10-20 ^{o}C. below the melting temperature (Tm) of the hybrid in 6.times.SSPE, 5 x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA.

[00101] Washes can typically be out as follows: (1) twice at room temperature for 15 minutes 1 x SSPE, 0.1% SDS (low stringency wash); and (2) once at the hybridization temperature for 15 minutes in 1 x SSPE, 0.1% SDS (moderate stringency wash).

[00102] 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: (1) Low: 1 or 2 x SSPE, room temperature; (2) Low: 1 or 2 x SSPE, 42 ^{o}C.; (3) Moderate: 0.2 x or 1 x SSPE, 65 ^{o}C. or (4) High: 0.1 x SSPE, 65 ^{o}C.

[00103] 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.

[00104] 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 Tag 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.

[00105] 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.

[00106] 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.

[00107] The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 base pairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. See GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodoferax*) and AJ6288601.1 (44903451; Sphingomonas). *Rhodoferax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

[00108] The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[00109] 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.

[00110] "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.

[00111] Specific changes to the "active site" of the enzyme can be made to affect the inherent functionallity with respect to activity or stereospecificity. Muller et. al. (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins et al. (2002) "X-ray crystal structure of *Escerichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates," Biochemistry 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, see Chakrabarti et al., PNAS, (Aug. 23, 2005), 102(34):12035-12040.

[00112] 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.

[00113] 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.

[00114] 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.

Table 1

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

[00115] 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 1 provides a listing of examples of amino acids belonging to each class. 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.

[00116] 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."

[00117] 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."

[00118] 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.

[00119] Transgenic hosts: The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, Arabidopsis, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alfalfa and clover), pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00120] 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.

[00121] 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.

[00122] Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a Pseudomonas fluorescens (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40%

total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can yield 20 g active AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

[00123] 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.

[00124] 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.

[00125] Vectors comprising an AAD-12 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13 mp 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).

[00126] 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.

[00127] 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.

[00128] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically

can provide resistance to various herbicides such as glufosinate (e.g., PAT/bar), glyphosate (EPSPS), ALS-inhibitors (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, et al.), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00129] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using Agrobacterium technology, see U.S. Pat. Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. 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. Other direct DNA delivery transformation technology includes aerosol beam technology. See U.S. Pat. No. 6,809,232. 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.

[00130] 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.

[00131] 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.

[00132] 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.

[00133] 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.

[00134] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41; hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., ayryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

[00135] 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.

[00136] 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.

[00137] 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.

[00138] 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.

[00139] Means of further increasing tolerance or resistance levels. It is shown herein that

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

[00140] One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

[00141] Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, Arabidopsis ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression.

[00142] Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

[00143] Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See e.g. U.S. Pat. No. 7,058,515; Larson et al., Protein Sci. 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles"; Crameri et al., Nature Biotechnology 15, 436-438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling"; Stemmer, W. P. C. 1994. "DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution" Proc. Natl. Acad. Sci. USA 91: 10747-10751; Stemmer, W. P. C. 1994. "Rapid evolution of a protein in vitro by DNA shuffling" Nature 370: 389-391; Stemmer, W. P. C. 1995. Searching sequence space. Bio/Technology 13: 549-553; Crameri, A., et al. 1996. "Construction and evolution of antibody-phage libraries by DNA shuffling" Nature Medicine 2: 100-103; and Crameri, A., et al. 1996. "Improved green fluorescent protein by molecular evolution using DNA shuffling" Nature Biotechnology 14: 315-319.

[00144] The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome

for insertions. See e.g. WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application 20030232410, relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-10 x and flp-frt for example) is also known.

[00145] AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7-methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. See, e.g.: Proc. Natl. Acad. Sci. USA Vol. 74, No. 7, pp. 2734-2738 (July 1977) Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis. Protein complexes or ligand blocking groups can also be used.

[00146] Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, Wis. (see Zucker et al., Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In RNA Biochemistry and Biotechnology, 11-43, J. Barciszewski & B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker et al., Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. J. Mol. Biol. 288, 911-940 (1999); Zucker et al., RNA Secondary Structure Prediction. In Current Protocols in Nucleic Acid Chemistry S. Beaucage, D. E. Bergstrom, G. D. Glick, and R. A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v. 2.4.2 (Eddy & Durbin, Nucl. Acids Res. 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website

genetics.wust1.edu/eddy/software/, and FOLDALIGN, also freely distributed and available for downloading at the website bioinf.au.dk. FOLDALIGN/ (see Finding the most significant common sequence and structure motifs in a set of RNA sequences. J. Gorodkin, L. J. Heyer and G. D. Stormo. Nucleic Acids Research, Vol. 25, no. 18 pp 3724-3732, 1997; Finding Common Sequence and Structure Motifs in a set of RNA Sequences. J. Gorodkin, L. J. Heyer, and G. D. Stormo. ISMB 5;120-123, 1997).

[00147] Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

[00148] Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

[00149] Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.

[00150] Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenchlorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabetrinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

[00151] Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain sorghum, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione S-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios K K, Burgos N (2004) "Metabolism-based herbicide resistance: regulation

by safeners," Weed Science: Vol. 52, No. 3 pp. 454-467.

[00152] Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450 species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

[00153] One preferred embodiment of the foregoing is the use cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, FulTime® and TopNotch® herbicides) and/or trifluralin (such as Treflan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional guidance regarding such embodiments, see e.g. Inui et al., "A selectable marker using cytochrome P450 monooxygenases for Arabidopsis transformation," Plant Biotechnology 22, 281-286 (2005) (relating to a selection system for transformation of *Arabidopsis thaliana* via *Agrobacterium tumefaciens* that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprophos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," PNAS Vol. 96, Issue 4, 1750-1755, Feb. 16, 1999; Sheldon et al, Weed Science: Vol. 48, No. 3, pp. 291-295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic Nicotiana tabacum"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," J Agric Food Chem. 2006 Apr. 19; 54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetomides (acetochlor, alachlor, metoachlor, pretilachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanalines (trifluralin and pendimethalin), phosphamidates (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

[00154] There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better

substrates and better leaving groups (higher electronegativity). Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

[00155] Unless specifically indicated or implied, the terms "a," "an," and "the" signify "at least one" as used herein. 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.

[00156] 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.

EXAMPLES

Example 1

Method for Identifying Genes That Impart Resistance to 2,4-D In Planta

[00157] As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with .gtoreq.50% homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to tfdA from *Ralstonia eutropha*) can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00158] A single gene (sdpA) was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 31% amino acid identity to tfdA. Percent identity was determined by first translating both the sdpA and tfdA DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Example 2

Optimization of Sequence for Expression in Plants and Bacteria

[00159] To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

Table 2: Compilation of G + C contents of protein coding regions of maize genes

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

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

[00160] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00161] Table 2 illustrates how high the G+C content is in maize. For the data in Table 2, 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.

[00162] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

Table 3. 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

[00163] In engineering genes encoding a bacterial protein for maize (or other plant, such as

cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 3. 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.

[00164] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00165] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of

the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

[00166] AAD-12 Plant Rebuild Analysis: Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native AAD-12 coding region (SEQ ID NO: 1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO: 1 (AAD-12) is presented as SEQ ID NO: 2. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence AAD-12 (v1) (SEQ ID NO: 3) was developed that encodes a protein (SEQ ID NO: 4) which is the same as the native SEQ ID NO: 2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO: 4). The additional alanine codon (GCT; underlined in SEQ ID NO: 3) encodes part of an NcoI restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical.

[00167] Table 4 shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences (Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

[00168] It is clear from examination of Table 4 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

Table 4. Codon composition comparisons of coding regions of Native AAD-*12*, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

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

[00169] Rebuild for *E. coli* Expression: Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable

to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in Escherichia coli and Salmonella typhimurium cellular and molecular biology, vol. 2, pp. 2047-2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umbarger, H. (eds.) American Society for Microbiology, Washington, D.C.). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions.

[00170] It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO: 4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (L e. Shine Dalgarno sequences). The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO: 5 and encodes the protein disclosed in SEQ ID NO: 4.

[00171] The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 5 presents the codon compositions of the native AAD-12 coding region (Columns A and D), an AAD-12 coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

[00172] It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

Table 5. Codon composition comparisons of coding regions of Native *AAD-12*, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

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

[00173] Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO: 5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino

acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO: 20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO: 21 of PCT/US2005/014737.

[00174] A codon usage table for soybean (Glycine max) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 6. Columns D and H of Table 6 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).

[00175] A biased soybean codon usage table was calculated from the data in Table 6. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

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

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 6 (ignoring the rare codon values in bold font).

[00176] The Weighted % value for each codon is given in Columns C and G of Table 6. 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 6. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

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

[00177] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO: 21 from PCT/US2005/014737 was reverse-

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

[00178] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO: 21 is disclosed as bases 1-1575 of SEQ ID NO: 22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO: 22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston Tex.).

EXAMPLE 3

Cloning of Expression and Transformation Vectors

[00179] Construction of *E. coli*, pET Expression Vector: Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) AAD-12 (v2) was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 µg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

[00180] To differentiate between AAD-12 (v2): pET280 and pCR2.1: pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37 °C. for 4 hours with agitation. Each culture was then spotted onto LB + Kanamycin 50 µg/ml plates, which were incubated at 37 °C. overnight. Colonies that grew on the LB-K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI/XhoI. The final expression construct was given the designation pDAB3222.

[00181] Construction of Pseudomonas Expression Vector: The AAD-12 (v2) open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S,"

as an XbaI-XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD-12 (v2) open reading frame from pDAB725 was transferred into the Pseudomonas expression vector, pMYC1803, as an XbaI-XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 Pseudomonas expression strains.

[00182] Completion of Binary Vectors: The plant optimized gene AAD-12 (v1) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO: 3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO: 6) and M13 Reverse (SEQ ID NO: 7) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-12 (v1) DNA sequence. The AAD-12 (v1) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-12 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: AAD-12 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium.*

[00183] Cloning of Additional Transformation Constructs: All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis et al., 1982).

Example 4

Transformation into Arabidopsis and Selection

[00184] Arabidopsis thaliana Growth Conditions: Wild type 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).

[00185] 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.

[00186] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu mol/m^2$ 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.

[00187] *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.

[00188] 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.

[00189] 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.

[00190] 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 x 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.

[00191] *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 x 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 x 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.

[00192] Selection of Transformed Plants: Freshly harvested T1 seed [AAD-12 (v1) gene] was allowed to dry for 7 days at room temperature. T1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T1 seed (.about.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.

[00193] 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).

[00194] Seven days after planting (DAP) and again 11 DAP, T1 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 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22 ^{o}C. growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22 ± 5 ^{o}C., 50 ± 30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2$ s^1 natural + supplemental light) at least 1 day prior to testing for the ability of AAD-12 (v1) (plant optimized gene) to provide phenoxy auxin herbicide resistance.

[00195] T1 plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis*, 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200 g ae/ha).

[00196] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

[00197] Some T1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluoroxypyr, could be effectively degraded in planta. Herbicides were

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

[00198] Results of Selection of Transformed Plants: The first *Arabidopsis* transformations were conducted using AAD-12 (v1) (plant optimized gene). T1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T1 seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT + Liberty are used for selection. T1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides.

Table 7. *AAD-12 v1* (plant optimized)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence compared to or *AAD-1 v3* (T_4) homozygous resistant population, Pat-Cry1F transformed, auxin-sensitive control.

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

[00199] Table 7 compares the response of AAD-12 (v1) and control genes to impart 2,4-D resistance to *Arabidopsis* T1 transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%),

moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The AAD-12 (v1) gene imparted herbicide resistance to individual T1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event.

Table 8. T_1 *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene Averages	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	6	0	0		0	0
50 g ae/ha R-dichlorprop	0	0	8		63	7
200 g ae/ha R-dichlorprop	0	0	8		85	10
800 g ae/ha R-dichlorprop	0	0	8		96	4
3200 g ae/ha R-dichlorprop	0	0	8		98	2
PAT/Cry1F Averages	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	10	0	0		0	0
50 g ae/ha R-dichlorprop	0	10	0		27	2
200 g ae/ha R-dichlorprop	0	0	10		69	3
800 g ae/ha R-dichlorprop	0	0	10		83	6
3200 g ae/ha R-dichlorprop	0	0	10		90	2
Homozygous AAD-1 (v3) gene T_4 plants	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	10	0	0		0	0
50 g ae/ha R-dichlorprop	10	0	0		0	0
200 g ae/ha R-dichlorprop	10	0	0		0	0
800 g ae/ha R-dichlorprop	10	0	0		0	0
3200 g ae/ha R-dichlorprop	10	0	0		0	0

[00200] 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 7 simply to demonstrate the significant difference between the plants transformed with AAD-12 (v1) versus the wild type or PAT/Cry1F-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or ~6 x field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin

cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00201] Table 8 shows a similarly conducted dose response of T1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 8). AAD-1 and AAD-12 are considered R- and S-specific α-ketoglutarate dioxygenases, respectively.

[00202] AAD-12 (v1) as a Selectable Marker: The ability to use AAD-12 (v1) as a selectable marker using 2,4-D as the selection agent was analyzed initially with Arabidopsis transformed as described above. Approximately 50 T4 generation *Arabidopsis* seed (homozygous for A AD-12 (v1)) were spiked into approximately 5,000 wild type (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 followed by 11 DAP. Since all individuals also contained the PAT gene in the same transformation vector, AAD-12 selected with 2,4-D could be directly compared to PAT selected with glufosinate.

[00203] Treatments were applied with a DeVilbiss spray tip as previously described. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 (v1) can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

[00204] Heritability: A variety of T1 events were self-pollinated to produce T2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T2 siblings. Each individual T2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the T1 families (T2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P>0.05$).

[00205] Seed were collected from 12 to 20 T2 individuals (T3 seed). Twenty-five T3 siblings from each of eight randomly-selected T2 families were progeny tested as previously described. Approximately one-third of the T2 families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show AAD-12 (v1) is stably integrated and inherited in a Mendelian fashion to at least three generations.

Table 9. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Pyridyloxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	Segregating T_2 *AAD-12 (v1)*plants (pDAB724.01.120)	Pat/Cry1f - Control
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
	Inactive DCP metabolite	
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

[00206] Additional Foliar Applications Herbicide Resistance in AAD-12 *Arabidopsis*: The ability of AAD-12 (v1) to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic Arabidopsis was determined by foliar application of various substrates. T2 generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis*. A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluoroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

[00207] There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-AAD-12 control Arabidopsis (Pat/Cry1F). AAD-12-transformed plants were also clearly protected from the triclopyr and fluoroxypyr herbicide injury that was seen in the transformed non-resistant controls (see Table 9). These results confirm that AAD-12 (v1) in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme

with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

[00208] Molecular Analysis of AAD-12 (v1) Arabidopsis: Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-12 (v1) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-12 (v1). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00209] Results showed that all 2,4-D resistant plants assayed, contained PAT (and thus by inference, AAD-12 (v1)). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the AAD-12 (v1) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

[00210] *Arabidopsis* Transformed with Molecular Stack of AAD-12 (v1) and a Glyphosate Resistance Gene: T1 *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (AAD-12 (v1) + EPSPS) which encodes a putative glyphosate resistance trait. T1 transformants were selected using AAD-12 (v1) as the selectable marker as described. T1 plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wild type Columbia-0, AAD-12 (v1) + PAT T4 homozygous lines (pDAB724-transformed), and PAT + Cry1F homozygous line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

Table 10. T_1 *Arabidopsis* response to a range of glyphosate rates applied postemergence (14 DAT).

AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	5	0	0		0	0
26.25 g ae/ha glyphosate	13	2	1		11	16
105 g ae/ha glyphosate	10	1	5		34	38
420 g ae/ha glyphosate	5	6	5		44	37
1680 g ae/ha glyphosate	0	0	16		85	9
PAT/Cry1F Averages	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	5	0	0		0	0
26.25 g ae/ha glyphosate	0	0	5		67	7
105 g ae/ha glyphosate	0	0	5		100	0
420 g ae/ha glyphosate	0	0	5		100	0
1680 g ae/ha glyphosate	0	0	5		100	0
Wild type (Col-0) Averages	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	5	0	0		0	0
26.25 g ae/ha glyphosate	0	0	5		75	13
105 g ae/ha glyphosate	0	0	5		100	0
420 g ae/ha glyphosate	0	0	5		100	0
1680 g ae/ha glyphosate	0	0	5		100	0
pDAB724 T4 (PAT + AAD-12) Averages	% Injury				% Injury	
	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	5	0	0		0	0
26.25 g ae/ha glyphosate	0	0	5		66	8
105 g ae/ha glyphosate	0	0	5		100	0
420 g ae/ha glyphosate	0	0	5		100	0
1680 g ae/ha glyphosate	0	0	5		100	0

[00211] Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differing modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12 + 2,4-D was used effectively as a selectable marker for a true resistance selection.

[00212] AAD-12 *Arabidopsis* Genetically Stacked with AAD-1 to Give Wider Spectrum of Herbicide Tolerance: AAD-12 (v1) (pDAB724) and AAD-1 (v3) (pDAB721) plants were

reciprocally crossed and F1 seed was collected. Eight F1 seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F1 plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

[00213] One hundred F2 seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F2 plants survived glufosinate selection fitting an expected segregation ration for two independently assorting loci for glufosinate resistance (15 R:1 S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop + 560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R: 6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlroprop or triclopyr). The results indicate that AAD-12 (pDAB724) can be successfully stacked with AAD-1 (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D+R-dichlorprop) and (2,4-D+fluoroxypyr+triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F1 generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively).

[00214] Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber et al.), for example, can be used together with the subject AAD-12 genes to impart spectrums of herbicide resistance in transgenic plants of the subject invention.

Example 5

WHISKERS-Mediated Transformation of Corn Using Imazethapyr Selection

[00215] Cloning of AAD-12 (v1): The AAD-12 (v1) gene was cut out of the intermediate vector pDAB3283 as an Nco1/Sac1 fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were

performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1:AAD-12 (v1) cassette. This construct was digested with Not1 and Pvu1 to liberate the gene cassette and digest the unwanted backbone. This was ligated to Not1 cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/AAD-12 (v1)/ZmPer5::OsAct1/AHAS/LZmLip.

[00216] Callus/Suspension Initiation: To obtain immature embryos for callus culture initiation, F1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00217] 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.

[00218] 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.

[00219] 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 (Form a Scientific) filled with liquid nitrogen vapors.

[00220] 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 x 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.

[00221] Stable Transformation: Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28 °C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28 °C., and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume 8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, sterile silicon carbide

whiskers (Advanced Composite Materials, Inc.).

[00222] 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 ~44 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was then added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28 °C. before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00223] Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28 °C. in a dark box.

[00224] After 1 week, filter papers were transferred to 60 x 20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 3 µM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

[00225] Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121 °C.) containing 3 µM imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

[00226] Protocol for Isolate Recovery and Regeneration: Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

Table 11. Characterization of T0 corn plants transformed with AAD-12						
Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD-12 PCR (cloning Region)	AAD-12 PCR (PTU)	AHAS Copy # (Invader)
4101(0)003.001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ae/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	-	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	-	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	Band smaller than expected	15

[00227] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 µM imazethapyr from Pursuit.RTM. DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2} s^{-1}$) for one week, then higher light (40 $\mu Em^{-2} s^{-1}$) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00228] From 4 experiments, full plantlets, comprised of a shoot and root, were formed in vitro on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissues from nine of these "early regenerators" were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the AAD-12 gene and gene cassette, respectively. All had an intact AAD-12 coding region, while 3 did not have a full-length PTU (Table 11). These "early regenerators" were identified as 4101 events to differentiate them

from the traditionally-derived events, which were identified as "1283" events. Plants from 19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T1 seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. T0 plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (AHAS gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the compete coding region for AAD-12; however, further analysis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T1 stage and further characterization utilized the 4101 events.

[00229] Molecular Analysis - Maize Materials and Methods: Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4 °C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00230] Invader assay analysis: The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95 °C. for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63 °C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

[00231] Polymerase chain reaction: A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are Forward-GAACAGTTAG ACATGGTCTA AAGG (SEQ ID NO: 8) and Reverse-GCTGCAACAC TGATAAATGC CAACTGG (SEQ ID NO: 9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 63 °C. for 30 seconds, and 72 °C. for 1 minute and 45 seconds

followed by 72 °C. for 10 minutes.

[00232] Primers for AAD-12 (v1) Coding Region PCR are Forward-ATGGCTCAGA CCACTCTCCA AA (SEQ ID NO: 10) and Reverse-AGCTGCATCC ATGCCAGGGA (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 65 °C. for 30 seconds, and 72 °C. for 1 minute and 45 seconds followed by 72 °C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00233] Southern Blot Analysis: Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2 µg of genomic leaf DNA or 10 µg of genomic callus DNA is subjected to an overnight digestion using BSM I and SWA I restriction enzymes to obtain PTU data.

[00234] After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 x 100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80 °C. and then stored at 4 °C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60 °C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65 °C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80 °C.

[00235] Postemergence Herbicide Tolerance in AAD-12 Transformed T0 Corn: Four T0 events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to

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

[00236] Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The individual clone '001' from event "001" (a.k.a., 4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the AAD-12 coding region (PCR assay) or the presence of the AHAS gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T0 plants events containing an intact coding region. The copy number of the transgene(s) (AHAS, and by inference AAD-12) varied significantly from 1 to 15 copies. Individual T0 plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T1 seed.

[00237] Verification of High 2,4-D Tolerance in T1 Corn: T1 AAD-12 (v1) seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. S urviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751 x Hi II control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event "pDAB4101(0)001.001" was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D + imazethapyr or 2,4-D + glyphosate (see Table 12).

Table 12. Brace Root injury of AAD-12 (v1) transformed T1 plants and untransformed control corn plants: Average Brace Root Injury (0-10 Scale)				
Herbicide	Untransformed Control	AAD-12 (v1) pDAB4101(0) 003.003	AAD-12 (v1) pDAB4101(0) 001.001	AAD-12 (v1) pDAB4101(0) 005.001
0 g ae/ha 2,4-D DMA	0	0	0	0
2240 g ae/ha 2,4-D DMA	9	1	8	0
A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.				

[00238] AAD-12 (v1) Heritability in Corn: A progeny test was also conducted on seven AAD-12 (v1) T1 families that had been crossed with 5XH751. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D (rates $\geq$ 560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

[00239] Stacking of AAD-12 (v1) to Increase Herbicide Spectrum: AAD-12 (v1) (pDAB4101) and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F1 seed was collected. The seed from two F1 lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g ae/ha 2,4-D DMA+1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT. Spray data showed that AAD-12 (v1) can be conventionally stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone + 2,4-D + glyphosate tolerance was observed in F1 plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

Table 13. Data demonstrating increase herbicide tolerance spectrum resulting from an F1 stack of AAD-12 (v1) and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF): Average % Injury 16DAT				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	AAD-12 (v1) pDAB4101(0) 003.R003.AF	AAD-12 (v1) pDAB4101(0) 005.R001.AF
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1
1120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5

[00240] Field Tolerance of pDAB4101 Transformed Corn Plants to 2,4-D, Triclopyr and Fluoroxypyr Herbicides: Field level tolerance trials were conducted on two AAD-12 (v1) pDAB4101 events (4101(0)003.R.003.AF and 4101(0)005.R001.AF) and one Roundup Ready (RR) control hybrid (2P782) at Fowler, Ind. and Wayside, Miss. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxypyr at 280 g ae/ha and an untreated control. The AAD-12 (v1) events contained the AHAS gene as a selectable marker. The F2 corn events were segregating so the AAD-12 (v1) plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

[00241] AAD-12 (v1) event response to 2,4-D, triclopyr, and fluoroxypyr at 14 days after treatment are shown in Table 14. Crop injury was most severe at 14 DAT. The RR control corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at 4480 g ae/ha, which is 8 times (8 x) the normal field use rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4 x rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2 x rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2 x rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluoroxypyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. AAD-12 (v1) events demonstrated increased tolerance with an

average of 8% injury at 5 DAT.

Table 14. Visual injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions: % Visual Injury 14 DAT				
Treatment	Rate (g ae/ha)	AAD-12 4101(0) 003.R.003.AF	AAD-12 4101(0) 005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxypyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

[00242] Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 15 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluoroxypyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn.

Table 15. Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions: Brace toot injury rating (0-10 scale) 28 DAT				
Treatment	Rate (g ae/ha)	AAD-12 4101(0) 003.R.003.AF	AAD-12 4101(0) 005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxypyr	280	0	0	2
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

[00243] Both AAD-12 (v1) corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the AAD-12 events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluoroxypyr caused only moderate brace root swelling and wandering in the wild-type corn with the AAD-12 (v1) events showing no brace root injury.

[00244] This data clearly shows that AAD-12(v1) conveys high level tolerance in corn to

2,4-D, triclopyr and fluoroxypyr at rates far exceeding those commercially used and that cause non-AAD-12 (v1) corn severe visual and brace root injury.

Example 6

Tobacco Transformation

[00245] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (Nicotiana tabacum cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30 °C. and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the Agrobacterium strains (EHA101S containing pDAB3278, aka pDAS1580, AAD-12 (v1)+PAT), grown overnight in a flask on a shaker set at 250 rpm at 28 °C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28 °C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, 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.

[00246] 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 $\mu E/m^2 s^1$).

[00247] Prior to propagation, T0 plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-12 (v1) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4 °C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00248] The DNA samples were diluted to 9 ng/µl and then denatured by incubation in a thermocycler at 95 °C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63 °C. for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

[00249] All events were also assayed for the presence of the AAD-12 (v1) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR AAD-12 were (SdpacodF: ATGGCTCATG CTGCCCTCAG CC) (SEQ ID NO: 12) and (SdpacodR: CGGGCAGGCC TAACTCCACC AA) (SEQ ID NO: 13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 64 °C. for 30 seconds, and 72 °C. for 1 minute and 45 seconds followed by 72 °C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably AAD-12 (v1) since these genes are physically linked) were regenerated and moved to the greenhouse.

Table 16. Tobacco T0 events transformed with pDAS1580 (AAD-12 (v1) + PAT)						
# Tube	Plant ID	Copy # PAT	PTU PCR AAD12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance*
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable

24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High
@Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluroxypyr where tolerance was variable across events.						

[00250] Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T0 Tobacco: T0 plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluoroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluoroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4 x field rate). On the whole, AAD-12 (v1) events were more sensitive to fluoroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T0 plant responses to 560 g ae/ha fluoroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable."

[00251] Verification of High 2,4-D Tolerance in T1 Tobacco: Two to four T0 individuals surviving high rates of 2,4-D and fluoroxypyr were saved from each event and allowed to self

fertilize in the greenhouse to give rise to T1 seed. The T1 seed was stratified, and sown into selection trays much like that of *Arabidopsis*, followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (PAT gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T0 generation. Improved consistency of response is anticipated in T1 plants not having come directly from tissue culture. These plants were compared against wild type KY160 tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140-2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ae/ha triclopyr or 35-560 g ae/ha fluoroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T1 generation is segregating, so some variable response is expected due to difference in zygosity.

Table 17. Segregating *AAD-12* T_1 tobacco plants' response to phenoxy and pyridyloxy auxin herbicides.

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

[00252] No injury was observed at 4 x field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluoroxypyr. The fluoroxypyr injury was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 17). It is important to note that untransformed tobacco is exceedingly sensitive to fluoroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by AAD-12 (v1), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluoroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

[00253] AAD-12 (v1) Heritability in Tobacco: A 100 plant progeny test was also conducted on seven T1 lines of AAD-12 (v1) lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

[00254] Field Tolerance of pDAS1580 Tobacco Plants to 2,4-D, Dichloprop, Triclopyr and Fluoroxypyr Herbicides: Field level tolerance trials were conducted on three AAD-12 (v1) lines

(events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Miss. Tobacco transplants were grown in the greenhouse by planting T1 seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

[00255] The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

Table 18. AAD-12 tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	PDAS1580-[1]-004.001	PDAS1580-[1]-020.016	PDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

[00256] AAD-12 (v1) event response to 2,4-D, triclopyr, and fluoroxypyr are shown in Table 18. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1.times. field application rate. The AAD-12 (v1) lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8.times. rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2 x rate of triclopyr (840 g ae/ha); whereas, AAD-12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three

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

[00257] These results indicate that AAD-12 (v1) transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluoroxypyr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

[00258] AAD-12 (v1) Protection Against Elevated 2,4-D Rates: Results showing AAD-12 (v1) protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 19. T1 AAD-12 (v1) plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T1 AAD-1 (v3) seed was also planted for transformed tobacco controls (see PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

[00259] AAD-12 (v1) and AAD-1 (v3) both effectively protected tobacco against 2,4-D injury at doses up to 4 x commercial use rates. AAD-12 (v1), however, clearly demonstrated a marked advantage over AAD-1 (v3) by protecting up to 64 x the standard field rates.

Table 19. Results demonstrating protection provided by AAD-12 (v1) and AAD-1 (v3) against elevated rates of 2,4-D.

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

[00260] Stacking of AAD-12 to Increase Herbicide Spectrum: Homozygous AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) plants (see PCT/US2005/014737 for the latter) were both reciprocally crossed and F1 seed was collected. The F1 seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regimine as used for the other testing with one of the following treatments: 70, 140, 280 g ae/ha fluoroxypyr (selective for the AAD-12 (v1) gene); 280, 560, 1120 g ae/ha R-dichloroprop (selective for the AAD-1 (v3) gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T2 plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 20.

[00261] The results confirm that AAD-12 (v1) can be successfully stacked with AAD-1 (v3),

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

Table 20. Comparison of auxinic herbicide cross tolerance of AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) T2 plants compared to AAD-12 x AAD-1 F1 cross and to wild type

Treatment	Average % Injury 14 DAT			
	KY160 Wild type control	AAD-12 (v1) (pDAS1580)	AAD-1 (v3) (pDAB721)	AAD-12 (v1) x AAD (v3) F1
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichloprop	25	15	0	0
560 g ae/ha R-dichloprop	60	50	0	0
1120 g ae/ha R-dichloprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

Example 7

Soybean Transformation

[00262] 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.

[00263] Transformants derived from Agrobacterium-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchee et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-12 (v1) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-12 (v1).

[00264] Gateway Cloning of Binary Constructs: The AAD-12 (v1) coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting AAD-12 (v1) plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Calif., Cat #11791-019).

[00265] An NcoI-SacI fragment containing the AAD-12 (v1) coding sequence was digested from DASPICO12 and ligated into corresponding NcoI-SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1//CsVMV promoter//AtuORF23 3'UTR//attL2); pDAB3916 (attL1//AtUbi10 promoter//AtuORF23 3'UTR//attL2); pDAB4458 (attL1//AtUbi3 promoter//AtuORF23 3'UTR//attL2); pDAB4459 (attL1//ZmUbi1 promoter//AtuORF23 3'UTR//attL2); and pDAB4460 (attL1//AtAct2 promoter//AtuORF23 3'UTR//attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4467 (attL1//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4471 (attL1//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4475 (attL1//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); and pDAB4479 (attL1//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2). These

constructs were confirmed via restriction enzyme digestion and sequencing.

[00266] The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3//attR1-ccdB-chloramphenicol resistance-attR2//CsVMV promoter//PATv6//AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #'s 12535-019 and 12535-027, Gateway Technology Version E, Sep. 22, 2003, #25-022. The DNA recombination sequences (attL, and attR,) and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6 AtuORF1 3'UTR); pDAB4468 (RB7 MARv3//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4472 (RB7 MARv3//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4476 (RB7 MARv3//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6 AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR). These constructs were confirmed via restriction enzyme digestion and sequencing.

[00267] Transformation Method 1 - *Agrobacterium*-mediated Transformation: 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. 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.

[00268] Plant transformation production of AAD-12 (v1) tolerant phenotypes. Seed derived explants of "Maverick" and the *Agrobacterium* mediated cot-node transformation protocol was used to produces AAD-12 (v1) transgenic plants.

[00269] *Agrobacterium* Preparation and Inoculation: *Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the AAD-12 (v1) gene and a plant-selectable gene (PAT) cassette within the T-DNA region. Plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the integration of genes before the *Agrobacterium* treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, Mo.

[00270] *Agrobacterium*-mediated transformation of soybean (Glycine max) using the PAT gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out. The seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytagel (Sigma-Aldrich, St. Louis, Mo.). Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3-4 mg/L during shoot elongation in the medium.

[00271] Prior to transferring elongated shoots (3-5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1-3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25 x 100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Convirons. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse

for further acclimation and establishment.

[00272] Assay of Putatively Transformed Plantlets, and Analyses Established T0 Plants in the Greenhouse: The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

[00273] Plants that are transferred to the greenhouse can be assayed for the presence of an active PAT gene further with a non-destructive manner by painting a section of leaf of the T0 primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v),=500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimethylamine salt formulation, preferably 0.5% v/v=2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifolioate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from the plane of the adjacent leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. T0 plants will be allowed to self fertilize in the greenhouse to give rise to T1 seed. T1 plants (and to the extent enough T0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-12 (v1) and PAT genes in transgenic soybean. Rates of 2,4-D used on T0 plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described. T1 plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T0 and T1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be assessed in the T1 generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Individual T0 plants were assessed for the presence of the coding region of the gene of interest (AAD-12 (v1) or PAT v6) and copy number. Determination of the inheritance of AAD-12 (v1) will be made using T1 and T2 progeny segregation with respect to herbicide tolerance as described in previous examples.

[00274] A subset of the initial transformants were assessed in the T0 generation according to

the methods above. Any plant confirmed as having the AAD-12 (v1) coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wild type Maverick soybeans did. PAT-only transformed plants responded the same at wild type plants to leaf paint applications of 2,4-D.

[00275] 2,4-D was applied to a subset of the plants that were of similar size to the wild type control plants with either 560 or 1120 g ae 2,4-D. All AAD-12 (v1)-containing plants were clearly resistant to the herbicide application versus the wild type Maverick soybeans. A slight level of injury (2 DAT) was observed for two AAD-12 (v1) plants, however, injury was temporary and no injury was observed 7 DAT. Wild type control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that AAD-12 (v1) can impart high tolerance (>2.times. field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the AAD12 (v1) genes integration, copy number, and gene expression levels.

[00276] Molecular Analyses - Soybean: Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4 °C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

[00277] Polymerase chain reaction: A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are (Forward-ATAATGCCAG CCTGTTAAAC GCC) (SEQ ID NO: 8) and (Reverse-CTCAAGCATA TGAATGACCT CGA) (SEQ ID NO: 9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 63 °C. for 30 seconds, and 72 °C. for 1 minute and 45 seconds followed by 72 °C. for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward-ATGGCTCATG CTGCCCTCAG CC) (SEQ ID NO: 10) and (Reverse-CGGGCAGGCC TAACTCCACC AA) (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 65 °C. for 30 seconds, and 72 °C. for 1 minute and 45 seconds followed by 72 °C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1%

agarose gel stained with EtBr.

[00278] Southern blot analysis: Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 µg of genomic DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 x 100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80 °C. and then stored at 4 °C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60 °C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65 °C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80 °C.

[00279] Biochemical Analyses - Soybean: Tissue Sampling and Extracting AAD-12 (v1) protein from soybean leaves. Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-hole-punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Protein analysis (ELISA and Western analysis) was completed accordingly.

[00280] T1 Progeny evaluation: T0 plants will be allowed to self fertilize to derive T1 families. Progeny testing (segregation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surviving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

[00281] AAD-12 (v1) transgenic Maverick soybean plants have been generated through

Agrobacterium-mediated transformation system. The T0 plants obtained tolerated up to 2 x levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the AAD1-12 (v1) gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

[00282] Transformation Method 2 - Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue: Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Pat. No. 6,809,232 (Held et al.) to create transformants using constructs provided herein.

[00283] Transformation Method 3 - Biolistic Bombardment of Soybean: This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants.

[00284] Transformation Method 4 - Whiskers Mediated Transformation: Whisker preparation and whisker transformation can be performed according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants.

[00285] Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 minutes and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18-20 hours. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

[00286] Transformation Method 5 - Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006).

Example 8

AAD-12 (v1) in Cotton

[00287] 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 21) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 $\mu E/m^2$, with the photoperiod set at 16 hours of light and 8 hours dark at 28 °C.

[00288] Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 21) 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 21) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 21). 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.

[00289] *Agrobacterium* preparation: Inoculate 35 ml of Y media (Table 21) (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 21) 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.

[00290] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05 mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 21), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 21) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Dessication is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure

(taping versus parafilm).

Table 21. Media for Cotton Transformation								
Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

[00291] Larger, well-developed embryos are isolated and transferred to DK media (Table 21) 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.

[00292] Cell Transformation: Several experiments were initiated in which cotyledon segments were treated with Agrobacterium containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton

callus, either: 0.1 or 0.5 mg/L R-dichlorprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis. Embryogenic cotton callus was stressed using dessication techniques to improve the quality and quantity of the tissue recovered. Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the AAD-12 (v1) gene.

[00293] Plant Regeneration: AAD-12 (v1) cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the AAD-12 (v1) gene provides resistance to 2,4-D in cotton, both the AAD-12 (v1) cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T1 seed or outcrossed with an elite cotton line to produce F1 seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR trants.

Example 9

Agrobacterium Transformation of Other Crops

[00294] 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.

[00295] The Latin names for these and other plants are given below. It should be clear that these and other (non Agrobacterium)transformation techniques can be used to transform AAD-12 (v1), for example, into these and other plants, including but not limited to Maize (Zea mays), Wheat (Triticum spp.), Rice (Oryza spp. and Zizania spp.), Barley (Hordeum spp.), Cotton (Abroma augusta and Gossypium spp.), Soybean (Glycine max), Sugar and table beets (Beta spp.), Sugar cane (Arenga pinnata), Tomato (Lycopersicon esculentum and other spp., Physalis ixocarpa, Solanum incanum and other spp., and Cyphomandra betacea), Potato (Solanum

tubersoum), Sweet potato (Ipomoea betatas), Rye (Secale spp.), Peppers (Capsicum annuum, sinense, and frutescens), Lettuce (Lactuca sativa, perennis, and pulchella), Cabbage (Brassica spp), Celery (Apium graveolens), Eggplant (Solanum melongena), Peanut (Arachis hypogea), Sorghum (all Sorghum species), Alfalfa (Medicago sativua), Carrot (Daucus carota), Beans (Phaseolus spp. and other genera), Oats (Avena sativa and strigosa), Peas (Pisum, Vigna, and Tetragonolobus spp.), Sunflower (Helianthus annuus), Squash (Cucurbita spp.), Cucumber (Cucumis sativa), Tobacco (Nicotiana spp.), Arabidopsis (Arabidopsis thaliana), Turfgrass (Lolium, Agrostis, Poa, Cynadon, and other genera), Clover (Tifolium), Vetch (Vicia). Such plants, with AAD-12 (v1) genes, for example, are included in the subject invention.

[00296] AAD-12 (v1) has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluoroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: Alder (Alnus spp.), ash (Fraxinus spp.), aspen and poplar species (Populus spp.), beech (Fagus spp.), birch (Betula spp.), cherry (Prunus spp.), eucalyptus (Eucalyptus spp.), hickory (Carya spp.), maple (Acer spp.), oak (Quercus spp), and pine (Pinus spp). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose (Rosa spp.), burning bush (Euonymus spp.), petunia (Petunia spp), begonia (Begonia spp.), rhododendron (Rhododendron spp), crabapple or apple (Malus spp.), pear (Pyrus spp.), peach (Prunus spp), and marigolds (Tagetes spp.).

Example 10

Further Evidence of Surprising Results: AAD-12 vs. AAD-2

[00297] 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 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO: 13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00298] The strain of Bradyrhizobium japonicum containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80 °C. in 20%

glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28 °C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28 °C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO: 14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO: 15 of PCT/US2005/014737 (added a Xho I site)].

[00299] 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.sub.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. #F599100). 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.

[00300] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37 °C. with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO: 6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO: 7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

[00301] 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. See PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA

TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR was restriction digested (with Nod, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into Agrobacterium.

[00302] Evaluation of Transformed Arabidopsis: Freshly harvested T1 seed transformed with a plant optimized AAD-12 (v1) or native AAD-2 (v1) gene were planted and selected for resistance to glufosinate as previously described Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

[00303] AAD-12 (v1) and AAD-2 (v1) did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T1 *Arabidopsis* plants. Surprisingly, AAD-2 (v1) and AAD-2 (v2) transformants were far less resistant to 2,4-D than the AAD-12 (v1) gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v1) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (see PCT/US2005/014737). Conversely, AAD-12 (v1) had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D. Tolerance improved slightly for plant-optimized AAD-2 (v2) versus the native gene; however, comparison of both AAD-12 and AAD-2 plant optimized genes indicates a significant advantage for AAD-12 (v1) in planta.

[00304] These results are unexpected given that the *in vitro* comparison of AAD-2 (v1) (see

PCT/US2005/014737) and AAD-12 (v2) indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T1 plants to varying levels; however, little protection from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes (see PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) in planta, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

Example 11

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

[00305] AAD-12 (v1) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluoroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00306] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluoroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

[00307] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluoroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-

inhibiting herbicides.

[00308] Further benefits could include tolerance to 2,4-D, triclopyr or fluoroxypyr required before planting following aryloxyacetic acid auxin herbicide application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluoroxypyr. Dicamba (and many other herbicides) can still be used for the subsequent control of AAD-12 (v1)-transformed dicot crop volunteers.

[00309] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by AAD-12 (v1) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 12

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

[00310] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-12 (v1) would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. AAD-12 (v1)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00311] An advantage to this additional tool is the extremely low cost of the broadleaf

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

[00312] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluoroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

[00313] The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 13

AAD-12 (v1) in Rice

[00314] 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 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-m1 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-hour photoperiod (Zhang et al. 1996).

[00315] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/L glutamine.

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

[00316] 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 hous 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-hour photoperiod. Regenerating shoots were transferred to Magenta boxes containing 1/2 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 1/2 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.

[00317] 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 one with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303 (Hpt-containing vector) to pDAB4101 (AAD-12 (v1)+AHAS), 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8-9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

[00318] Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T0 Rice: Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the presence of the AHAS gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled and molecular characterization was performed that identified seven of these 10 events as containing both the AAD-12 (v1) PTU and the entire AHAS coding region.

[00319] Heritability of AAD-12 (v1) in T1 Rice: A 100-plant progeny test was conducted on five T1 lines of AAD-12 (v1) lines that contained both the AAD-12 (v1) PTU and AHAS coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 coseregated with the AHAS selectable marker as determined by 2,4-D tolerance testing below.

[00320] Verification of High 2,4-D Tolerance in T1 Rice: The following T1 AAD-12 (v1) single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ae/ha 2,4-D DMA (2 x, 4 x, and 8 x typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, `Lamont,` as negative control plants.

Table 22. T1 AAD-12 (v1) and untransformed control response to varying levels of 2,4-D DMA: Average % injury 14 DAT			
Herbicide	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

[00321] Injury data (Table 22) shows that the AAD-12 (v1)-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101(20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

[00322] 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.

[00323] AAD-12 (v1) Expression: All 33 T0 transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

[00324] Field Tolerance of pDAB4101 Rice Plants to 2,4-D and Triclopyr Herbicides: A field level tolerance trial was conducted with AAD-12 (v1) event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Miss. (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2 x rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T1 generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101 [20] rice contained the AHAS gene as a selectable

marker for the AAD-12(v1) gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any AAD-12 (v1) null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

[00325] AAD-12 (v1) event response to 2,4-D and triclopyr are shown in Table 23. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4 x commercial use rate. The AAD-12 (v1) event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2 x rate of triclopyr (560 g ae/ha). The AAD-12 (v1) event demonstrated excellence tolerance to the 2 x rates of triclopyr with no injury observed at either 7 or 15DAT.

[00326] These results indicate that the AAD-12 (v1) transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 I multiple species to provide resistance to a wider spectrum of effective chemistries.

Table 23. AAD-12 T1 generation rice plants response to 2,4-D and triclopyr under field conditions

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

Example 14

AAD-12 (v1) in Canola

[00327] Canola Transformation: The AAD-12 (v1) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera*710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained AAD-12 (v1) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter.

[00328] 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 hours light/8 hours dark.

[00329] Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The Agrobacterium was grown overnight at 28 °C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00330] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/L Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00331] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

[00332] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/L Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00333] Molecular Analysis - Canola Materials and Methods: 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.

[00334] 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-12 (v1) were (SEQ ID NO: 10) (forward) and (SEQ ID NO: 11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94 °C. for 3 minutes and 35 cycles of 94 °C. for 30 seconds, 65 °C. for 30 seconds, and 72 °C. for 2 minutes followed by 72 °C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-12 (v1) events tested positive. Three negative control samples tested negative.

[00335] ELISA: Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 24.

Table 24. Expression of AAD-12 (v1) in Canola plants

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

[00336] Postemergence Herbicide Tolerance in AAD-12(v1) Transformed T0 Canola: Forty-five T0 events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves had emerged (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were then treated with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

[00337] Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T1 (and T2 generation) by selfpollination under controlled, bagged, conditions.

[00338] AAD-12 (v1) Heritability in Canola: A 100 plant progeny test was also conducted on 11 T1 lines of AAD-12 (v1). The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

[00339] AAD-12 (v1) Heritability in Canola: A 100 plant progeny test was also conducted on 11 T1 lines of AAD-12 (v1). The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

[00340] Verification of High 2,4-D Tolerance in T1 Canola : For T1 AAD-12 (v1), 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix #5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

Table 25. T1 AAD-12 (v1) and untransformed control response to varying rates postemergence 2,4-D DMA applications: Average % injury 14 DAT

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

[00341] Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T1 progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T1 event injury data 14DAT may be seen in Table 25. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D.

Surviving plants were transplanted to 51/4" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

[00342] Field Tolerance of pDAB3759 Canola Plants to 2,4-D, Dichloprop, Triclopyr and Fluoroxypyr Herbicides: Field level tolerance trial was conducted on two AAD-12 (v1) events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Ind. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.0011, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

Table 26. AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

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

[00343] Canola response to 2,4-D, triclopyr, and fluoroxypyr are shown in Table 26. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ae/ha which is considered the 4 x rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14DAT with an average injury of 2, 3 and 2% observed at the 1, 2 and 4 x rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2 x rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2 x rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluoroxypyr at 280 g ae/ha caused severe injury (37%) to the non-transformed line at 14DAA. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5DAT.

[00344] These results indicate that AAD-12 (v1) transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluoroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative

efficacy of 2,4-D > triclopyr > fluoroxypyr.

Example 15

Transformation and Selection of the AAD-12 Soybean Event DAS-68416-4

[00345] Transgenic soybean (*Glycine max*) Event DAS-68416-4 was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium* strain EHA101 (Hood et al., 2006), carrying the binary vector pDAB4468 (FIG. 2) with the selectable marker (pat) and the gene of interest (AAD-12) within the T-strand DNA region, was used to initiate transformation.

[00346] *Agrobacterium*-mediated transformation was carried out. Briefly, soybean seeds (cv Maverick) were germinated on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium.* Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium.* Glufosinate selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

[00347] Terminal leaflets of selected plantlets were leaf painted with glufosinate to screen for putative transformants. The screened plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with glufosinate to reconfirm tolerance and deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of the selectable marker gene and/or the gene of interest were carried out. T0 plants were allowed to self fertilize in the greenhouse to give rise to T1 seed.

[00348] The T1 plants were backcrossed and introgressed into elite germplasm (Maverick). This event, soybean Event DAS-68416-4, was generated from an independent transformed isolate. The event was selected based on its unique characteristics such as single insertion site, normal Mendelian segregation and stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance in broad genotype backgrounds and across multiple environmental locations. Additional description of soybean Event DAS-68416-4 has been disclosed in WO 2011/066384, which is incorporated by reference in its entirety.

Example 16

Generation of 2008 Agronomic Data

[00349] An agronomic study with Event DAS-68416-4 soybean and a non-transgenic control (var. Maverick) was conducted in 2008 at six sites located in Iowa, Illinois, Indiana, Nebraska and Ontario, Canada (2 sites). Agronomic determinants, including stand/population count,

seedling/plant vigor, plant height, lodging, disease incidence, insect damage, and days to flowering were evaluated to investigate the equivalency of the soybean Event DAS-68416-4 (with and without herbicide treatments) as compared to the control line Maverick. This study is referred to as Agronomic Experiment S1.

Table 27. Agronomic parameters evaluated in Agnomic Experiment S1.

Trait	Evaluation Timing	Description of Data	Scale
Early population	VC-V2	Number of plants emerged in rows of each plot	Actual count per plot
Seedling vigor	VC-V2	Visual estimate of average vigor of emerged plants per plot	1-10 scaled based on growth of the non-transformed soybeans 10 = Growth equivalence to non-transformed 9 = Plant health is 90% as compared to non-transformed, etc.
Plant vigor / injury	After post-emergent herbicide applications	Injury from herbicide applications	1-10 scale based on growth of the non-transformed soybeans 10 = Growth equivalence to non-transformed 9 = Plant health is 90% as compared to non-transformed, etc.
Plant height	Approximately R6	Height from soil surface to the tip of the highest leaf when extended by hand	Height in cm (average of 10 plants per plot)
Lodging	Approximately R8	Visual estimate of lodging severity	Visual estimate on 0-100% scale based on the number of plants lodged
Final population	Approximately R8	The number of plants remaining in rows of each plot	Actual count per plot, including plants removed during previous sampling

[00350] The test and control soybean seed were planted at a seeding rate of approximately 112 seeds per 25 ft row with a row spacing of approximately 30 inches (75 cm). At each site, three replicate plots of each treatment were established, with each plot consisting of 2-25 ft rows. Plots were arranged in a randomized complete block (RCB) design, with a unique randomization at each site. Each soybean plot was bordered by two rows of a non-transgenic soybean of similar maturity. The entire trial site was surrounded by a minimum of 10 ft of a non-transgenic soybean of similar relative maturity.

[00351] Herbicide treatments were applied with a spray volume of approximately 20 gallons per acre (187 L/ha). These applications were designed to replicate maximum label rate commercial practices. 2,4-D was applied as three broadcast over-the-top applications for a seasonal total of 3 lb ae/A. Individual applications of 1.0 lb ae A (1,120 g/ha) were made at pre-emergence and approximately V4 and R2 growth stages. Glufosinate was applied as two broadcast over-the-top applications for a seasonal total of 0.74 lb ai/A (828 g ai/ha). Individual applications of 0.33 lb ai/A and 0.41 lb ai/A (374 and 454 g ai/ha) were made at approximately V6 and R1 growth stages.

[00352] Analysis of variance was conducted across the field sites for the agronomic data using a mixed model (SAS Version 8; SAS Institute 1999). Entry was considered a fixed effect, and location, block within location, location-by-entry, and entry-by-block within location were designated as random effects. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between the control and unsprayed soybean Event DAS-68416-4 (unsprayed), soybean Event DAS-68416-4 sprayed with glufosinate (soybean Event DAS-68416-4 + glufosinate), soybean Event DAS-68416-4 sprayed with 2,4-D (soybean Event DAS-68416-4 + 2,4-D) and soybean Event DAS-68416-4 sprayed with both glufosinate and 2,4-D (soybean Event DAS-68416-4 + both) transgenic entries using *t*-tests. Adjusted P-values were also calculated using the False Discovery Rate (FDR) to control for multiplicity (Benjamini and Hochberg, 1995).

[00353] An analysis of the agronomic data collected from the control, soybean Event DAS-68416-4 unsprayed, soybean Event DAS-68416-4 + 2,4-D, soybean Event DAS-68416-4 + glufosinate, and soybean Event DAS-68416-4 + both herbicides was conducted. No statistically significant differences were observed for stand count, early population, seedling vigor, injury after application, lodging, final stand count or days to flowering (Table 28). For height, a significant paired t-test was observed between the control and the soybean Event DAS-68416-4 + 2,4-D spray. However, no significant overall treatment effect was observed, differences were very small between the soybean Event DAS-68416-4 treatment and the control, and differences were not shared among the different soybean Event DAS-68416-4 treatments. Based on these results, soybean Event DAS-68416-4 was agronomically equivalent to the near-isogenic non-transgenic control.

Table 28. Analysis of agronomic characteristics from Agronomic Experiment S1.

Analyte	Overall Treatment Effect (Pr>F)[a]	Control	Unsprayed (P-value,[b] Adj. P)[c]	Sprayed Glufosinate (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Stand Count (no. of plants)	0.774	170	172 (0.709,0.824)	175 (0.311,0.575)	173 (0.476,0.672)	175 (0.269,0.575)
Early Population (% emergence)[d]	0.714	76.7	77.4 (0.738,0.824)	79.1 (0.301,0.575)	79.0 (0.327,0.575)	79.4 (0.256,0.575)
Seedling Vigor[e]	0.547	9.72	9.39 (0.146,0.575)	9.50 (0.326,0.575)	9.44 (0.222,0.575)	9.39 (0.146,0.575)
Vigor/Injury App. 2[e]	0.511	10.0	9.86 (0.461,0.671)	9.89 (0.555,0.718)	9.83 (0.378,0.611)	9.67 (0.087,0.575)
Vigor/Injury App. 3[e]	0.462	10.0	10.0 (1.000,1.000)	9.89 (0.320,0.575)	9.83 (0.141,0.575)	9.89 (0.320,0.575)
Vigor/Injury App. 5[e]	0.431	9.94	9.89 (0.721,0.824)	9.78 (0.289,0.575)	9.67 (0.085,0.575)	9.78 (0.289,0.575)
Height (cm)	0.144	101	98.1 (0.145,0.575)	99.2 (0.390,0.611)	96.1 (**0.020**,0.575)	97.2 (0.062,0.575)
Lodging (%)	0.948	17.2	18.2 (0.885,0.904)	21.3 (0.551,0.718)	20.7 (0.606,0.746)	21.7 (0.511,0.700)
Final Stand Count (no. of plants)	0.268	156	154 (0.770,0.840)	161 (0.335,0.575)	155 (0.817,0.853)	163 (0.127,0.575)
Flowering Days[f]	0.452	49.0	49.5 (0.261,0.575)	49.4 (0.395,0.611)	48.7 (0.568,0.718)	49.2 (0.668,0.801)

[a] Overall treatment effect estimated using an F-test.

[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.

[c] P-values adjusted using a False Discovery Rate (FDR) procedure.

[d] 0-100% scale; (Stand count divided by the no. of seeds planted) * 100.

[e] Visual estimate on 1-10 scale; 10 = growth equivalent to non-transformed plants.

[f] Visual estimate on 0-100% scale; 0% = no damage.

[f] The number of days from the time of planting until flowering.

Bolded P-values are significant (<0.05).

Example 17

Generation of 2009 Agronomic Data

[00354] An agronomic study with soybean Event DAS-68416-4 and a non-transgenic control (var. Maverick) was conducted in 2009 at 8 sites located in Arkansas, Iowa, Illinois, Indiana, Missouri, and Nebraska. Agronomic determinants, including stand/population count, seedling/plant vigor, plant height, disease incidence, insect damage, and days to flowering were evaluated to investigate the equivalency of the soybean Event DAS-68416-4 soybeans (with

and without herbicide treatments) to the control (Table 29).

Table 29. Data collected in agronomic and yield trials, 2009.

Characteristic	Evaluation Timing	Description	Units reported	Test *
Emergence	VC - V2	Stand count in 1 meter section of row divided by number of seeds planted per meter	%	B
Seedling vigor	V1 - V3	General seedling vigor	1 (low) to 10 (high)	B
Visual injury	Post V3 application	Visual injury 1 day post herbicide application at V3 stage	%	S
Visual injury	Post V3 application	Visual injury 7 days post herbicide application at V3 stage	%	S
Visual injury	Post V3 application	Visual injury 14 days post herbicide application at V3 stage	%	S
Days to Flower		Number of days from planting to when 50% of plants are at R1	days	B
Stand count	R2	Number of plants in one meter section of row		B
Visual injury	Post R2 application	Visual injury 1 day post herbicide application at R2 stage	%	S
Visual injury	Post R2 application	Visual injury 7 days post herbicide application at R2 stage	%	S
Visual injury	Post R2 application	Visual injury 14 days post herbicide application at R2 stage	%	S
Disease incidence	~R6	Opportunistic note on any disease that occured at a location	%	B
Insect damage	~R6	Opportunistic note on any insect damage that occured at a location	%	B
Plant Height	R8	Final height of plot at R8	cm	B
Maturity	R8	Number of days from planting to when 95% of plants in plot have reached their mature color	days	B
Lodging	R8	Degree of lodging in a plot	1 (none) - 5 (flat)	B
Yield	R8	Weight of seed produced by the plot	bu/acre	B
100 seed weight	R8	Weight of 100 random seeds from the harvested plot	g	B

* B – Sprayed and Unsprayed tests, S – Sprayed tests only

[00355] A randomized-complete-block design was used for trials. Entries were soybean Event DAS-68416-4, a Maverick control line, and commercially available non-transgenic soybean lines. The test, control and reference soybean seed were planted at a seeding rate of approximately 112 seeds per row with row spacing of approximately 30 inches (75 cm). At each site, 4 replicate plots of each treatment were established, with each plot consisting of 2-25

ft rows. Each soybean plot was bordered by 2 rows of a non-transgenic soybean (Maverick). The entire trial site was surrounded by a minimum of 4 rows (or 10 ft) of non-transgenic soybean (Maverick). Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

[00356] Herbicide treatments were applied to replicate maximum label rate commercial practices. Treatments consisted of a non-sprayed control and herbicide applications of 2,4-D, glufosinate, 2,4-D/glufosinate applied at the specified growth stages. For the 2,4-D applications, the herbicide was applied at a rate of 1.0 lb ae /A (1,120 g ae/ha) at the V4 and R2 growth stages. For the glufosinate treatments, applications were made to plants at the V4 and V6-R2 growth stages. For both applications, glufosinate was applied at a rate of 0.33 lb ai/A (374 g ai/ha) and 0.41 lb ai/A (454 g ai/ha) for the V4 and V6-R2 applications, respectively. Entries for both herbicide applications were soybean Event DAS-68416-4 and the controls including non-transgenic Maverick. Maverick plots were expected to die after herbicide application.

[00357] Analysis of variance was conducted across the field sites for the agronomic data using a mixed model (SAS Version 8; SAS Institute 1999). Entry was considered a fixed effect, and location, block within location, location-by-entry, and entry-by-block within location were designated as random effects. Analysis at individual locations was done in an analogous manner with entry as a fixed effect, and block and entry-by-block as random effects. Data were not rounded for statistical analysis. Significant differences were declared at the 95% confidence level, and the significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between unsprayed AAD-12 (unsprayed), AAD-12 sprayed with glufosinate (AAD- 12 + glufosinate), AAD- 12 sprayed with 2,4-D (AAD- 12 + 2,4-D) and AAD- 12 sprayed with both glufosinate and 2,4-D (AAD-12 + 2,4-D + glufosinate) transgenic entries and the control entry using T-tests.

[00358] Due to the large number of contrasts made in this study, multiplicity was an issue. Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise p-values is very high ($1-0.95^{\text{nuber of comparisoils}}$). In this study there were four comparisons per analyte (16 analyzed observation types for agronomics), resulting in 64 comparisons for agronomics. Therefore, the probability of declaring one or more false differences based on unadjusted p-values was 99% for agronomics ($1-0.95^{64}$.)

[00359] An analysis of the agronomic data collected from the control, AAD-12 unsprayed, AAD- 12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + 2,4-D + glufosinate entries was

conducted. For the across-site analysis, no statistically significant differences were observed for seedling vigor, final population, plant vigor/injury (V4, Rl), lodging, disease incidence, insect damage, days to flowering, days to maturity, number of pods, number of seeds, yield, and plant height. For stand count and early population, a significant paired t-test was observed between the control and the AAD-12 + glufosinate entry, but was not accompanied by a significant overall treatment effect or FDR adjusted p-value. For plant vigor/injury (R2), significant paired t-tests and a significant overall treatment effect were observed between the control and both the AAD-12 + glufosinate and AAD-12 + 2,4-D + glufosinate entries, but were not accompanied by a significant FDR adjusted p-value. The mean results for all of these variables were also within the range found for the reference lines tested in this study.

Example 18

Transformation and Selection of the AADl Event pDAS 1740-278

[00360] The AADl event, pDAS 1740-278, was produced by WHISKER - mediated transformation of maize line Hi-II. The transformation method used is described in US Patent Application # 20090093366. An Fspl fragment of plasmid pDAS1740 (FIG. 3), also referred to as pDAB3812, was transformed into the maize line. This plasmid construct contains the plant expression cassette containing the RB7 MARv3 : : Zea mays Ubiquitin 1 promoter v2 // AADl v3 // Zea mays PER5 3'UTR :: RB 7 MARv4 plant transcription unit (PTU).

[00361] Numerous events were produced. Those events that survived and produced healthy, haloxyfop-resistant callus tissue were assigned unique identification codes representing putative transformation events, and continually transferred to fresh selection medium. Plants were regenerated from tissue derived from each unique event and transferred to the greenhouse.

[00362] Leaf samples were taken for molecular analysis to verify the presence of the AAD-I transgene by Southern Blot, DNA border confirmation, and genomic marker assisted confirmation. Positive TO plants were pollinated with inbred lines to obtain Tl seed. Tl plants of Event pDAS 1470-278-9 (DAS-40278-9) was selected, self-pollinated and characterized for five generations. Meanwhile, the Tl plants were backcrossed and introgressed into elite germplasm (XHH 13) through marker-assisted selection for several generations. This event was generated from an independent transformed isolate. The event was selected based on its unique characteristics such as single insertion site, normal Mendelian segregation and stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance in broad genotype backgrounds and across multiple environmental locations. Additional description regarding the corn Event pDAS-1740-278-9 has been disclosed in WO 2011/022469, which is incorporated by reference in its entirety.

Example 19

Herbicide Application and Agronomic Data

[00363] Herbicide treatments were applied with a spray volume of approximately 20 gallons per acre (187 L/ha).

[00364] These applications were designed to replicate maximum label rate commercial practices. Weedar 64 (026491-0006) at concentration 39%, 3,76 lb ae/gal, 451 g ae/l and Assure II (106155) at concentration 10.2%, 0.87 lb ai/gal, 104 g ai/g were used.

[00365] 2,4-D (Weedar 64) was applied as 3 broadcast over-the-top applications to Test Entries 4 and 5 (seasonal total of 3 Ib ae/A). Individual applications were at pre-emergence and approximately V4 and V8 -V8.5 stages. Individual target application rates were 1.0 lb ae/A for Weedar 64, or 1120 g ae/ha. Actual application rates ranged from 1096 - 1231 g ae/A.

[00366] Quizalofop (Assure II) was applied as a single broadcast over-the-top application to Test Entries 3 and 5. Application timing was at approximately V6 growth stage. The target application rate was 0.0825 lb ai/A for Assure II, or 92 g ai/ha. Actual application rates ranged from 90.8 - 103 g ai/ha. Agronomic characteristics were recorded for all test entries within Blocks 2, 3, and 4 at each location. Table 30 lists characteristics that were measured.

Table 30. Agronomic data for corn Event pDAS-1740-278-9		
Trait	Evaluation Timing	Description of Data
Early Population	Vl and V4	Number of plants emerged per plot
Seedling Vigor	V4	Visual estimate of average vigor of emerged plants per plot
Plant Vigor/Injury	Approximately 1-2 weeks after applications	Injury from herbicide applications
Time to Silking	Approximately 50% Silking	The number of accumulated heat units from the time of planting until approximately 50% of the plants have emerged silks.
Time to Pollen Shed	Approximately 50% Pollen Shed	The number of accumulated heat units from the time of planting until approximately 50% of the plants are shedding pollen
Pollen Viability	Approximately 50%	Evaluation of pollen color and shape over time
Plant Height	Approximately R6	Height to the tip of the tassel
Ear Height	Approximately R6	Height to the base of the primary ear
Stalk Lodging	Approximately R6	Visual estimate of percent of plants in the plot with stalks broken below the primary ear
Root Lodging	Approximately R6	Visual estimate of percent of plants in the plot leaning approximately 30° or more in the first ~1/2 meter above the soil surface
Final Population	Approximately R6	The number of plants remaining per plot
Days to Maturity	Approximately R6	The number of accumulated heat units from the time of planting until approximately 50% of the plants have reached physiological maturity
Stay Green	Approximately R6	Overall plant health
Disease Incidence	Approximately R6	Visual estimate of foliar disease incidence
Insect Damage	Approximately R6	Visual estimate of insect damage
Note: Heat Unit = ((MAX temp + MIN temp) / 2) – 50 °F		

[00367] An analysis of the agronomic data collected from the control, aad-l unsprayed, aad-l + 2,4-D, aad-\ + quizalofop, and aad-\ + both entries was conducted. For the across-site analysis, no statistically significant differences were observed for early population (Vl and V4), vigor, final population, crop injury, time to silking, time to pollen shed, stalk lodging, root lodging, disease incidence, insect damage, days to maturity, plant height, and pollen viability (shape and color) values in the across location summary analysis. For stay green and ear height, significant paired t-tests were observed between the control and the aad-l + quizalofop entries, but were not accompanied by significant overall treatment effects or False Discovery Rates (FDR) adjusted p-values (Table 31).

Table 31. Summary analysis of agronomic characteristics results across locations for the DAS-40278-9 aad-1 corn (sprayed and unsprayed) and control.

Analyte	Overall Trt. Effect (pr>F)[a]	Control	Unsprayed (P-value,[b] Adj. P)[c]	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Early population V1 (no. of plants)	(0.351)	42.8	41.3 (0.303, 0.819)	41.7 (0.443, 0.819)	41.9 (0.556, 0.819)	44.1 (0.393, 0.819)
Early population V4 (no. of plants)	(0.768)	43.1	43.3 (0.883, 0.984)	43.7 (0.687, 0.863)	44.3 (0.423, 0.819)	44.8 (0.263, 0.819)
Seedling Vigor[d]	(0.308)	7.69	7.39 (0.197, 0.819)	7.36 (0.161, 0.819)	7.58 (0.633, 0.819)	7.78 (0.729, 0.889)
Final population (no. of plants)	(0.873)	40.1	39.6 (0.747, 0.889)	39.7 (0.802, 0.924)	39.9 (0.943, 1.00)	41.1 (0.521, 0.819)
Crop Injury - 1st app.[e]	NA[l]	0	0	0	0	0
Crop Injury - 2nd app.[e]	(0.431)	0	0 (1.00, 1.00)	0 (1.00, 1.00)	0 (1.00, 1.00)	0.28 (0.130, 0.819)
Crop Injury - 3rd app.[e]	NA	0	0	0	0	0
Crop Injury - 4th app.[e]	NA	0	0	0	0	0
Time to Silking (heat units)[f]	(0.294)	1291	1291 (0.996, 1.00)	1293 (0.781, 0.917)	1304 (0.088, 0.819)	1300 (0.224, 0.819)
Time to Pollen Shed (heat units)[f]	(0.331)	1336	1331 (0.564, 0.819)	1342 (0.480, 0.819)	1347 (0.245, 0.819)	1347 (0.245, 0.819)
Pollen Shape 0 minutes (%)[g]	(0.872)	10.9	10.9 (0.931, 1.00)	11.3 (0.546, 0.819)	11.4 (0.439, 0.819)	11.3 (0.605, 0.819)
Pollen Shape 30 minutes (%)	(0.486)	49.2	50.8 (0.618, 0.819)	46.4 (0.409, 0.819)	48.1 (0.739, 0.889)	51.9 (0.409, 0.819)
Pollen Shape 60 minutes (%)	(0.724)	74.4	74.7 (0.809, 0.924)	73.6 (0.470, 0.819)	73.9 (0.629, 0.819)	75.0 (0.629, 0.819)
Pollen Shape 120 minutes (%)	(0.816)	82.6	82.6 (1.00, 1.00)	82.6 (1.00, 1.00)	82.6 (1.00, 1.00)	82.5 (0.337, 0.819)
Pollen Color 30 minutes (%)[h]	(0.524)	51.9	52.5 (0.850, 0.960)	48.9 (0.306, 0.819)	50.3 (0.573, 0.819)	53.6 (0.573, 0.819)
Pollen Color	(0.332)	75.3	75.9	74.2	74.2	75.9

60 minutes (%)			(0.612, 0.819)	(0.315, 0.819)	(0.315, 0.819)	(0.612, 0.819)
Pollen Color 120 minutes (%)	NA	84.0	84.0	84.0	84.0	84.0
Stalk Lodging (%)	(0.261)	5.11	5.22 (0.356, 0.819)	5.00 (0.356, 0.819)	5.00 (0.356, 0.819)	5.00 (0.356, 0.819)
Root Lodging (%)	(0.431)	0.44	0.17 (0.457, 0.819)	0.72 (0.457, 0.819)	0.17 (0.457, 0.819)	0.11 (0.373, 0.819)
Stay Green[i]	(0.260)	4.67	4.28 (0.250, 0.819)	3.92 (0.034[m], 0.819)	4.17 (0.144, 0.819)	4.11 (0.106, 0.819)
Disease Incidence[j]	(0.741)	6.42	6.22 (0.383, 0.819)	6.17 (0.265, 0.819)	6.17 (0.265, 0.819)	6.17 (0.265, 0.819)
Insect Damage[k]	(0.627)	7.67	7.78 (0.500, 0.819)	7.78 (0.500, 0.819)	7.72 (0.736, 0.889)	7.56 (0.500, 0.819)
Days to Maturity (heat units)[f]	(0.487)	2411	2413 (0.558, 0.819)	2415 (0.302, 0.819)	2416 (0.185, 0.819)	2417 (0.104, 0.819)
Plant Height (cm)	(0.676)	294	290 (0.206, 0.819)	290 (0.109, 0.819)	291 (0.350, 0.819)	291 (0.286, 0.819)
Ear Height (cm)	(0.089)	124	120 (0.089, 0.819)	118 (0.018[m], 0.786)	121 (0.214, 0.819)	118 (0.016[m], 0.186)

[a] Overall treatment effect estimated using an F-test.

[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.

[c] P-values adjusted using a False Discovery Rate (FDR) procedure.

[d] Visual estimate on 1-9 scale; 9 = tall plants with large robust leaves.

[e] 0-100% scale; with 0 = no injury and 100 = dead plant.

[f] The number of heat units that have accumulated from the time of planting.

[g] 0-100% scale; with % pollen grains with collapsed walls.

[h] 0-100% scale; with % pollen grains with intense yellow color.

[i] Visual estimate on 1-9 scale with 1 no visible green tissue.

[J] Visual estimate on 1-9 scale with 1 being poor disease resistance.

[k] Visual estimate on 1-9 scale with 1 being poor insect resistance.

[l] NA = statistical analysis not performed since no variability across replicates or treatment.

[m] Statistical difference indicated by P- Value <0.05.

Example 20

Additional Argonomic Trials

[00368] Agronomic characteristics of corn line 40278 compared to a near-isoline corn line were evaluated across diverse environments. Treatments included 4 genetically distinct hybrids

and their appropriate near-isoline control hybrids tested across a total of 21 locations.

[00369] The four test hybrids were medium to late maturity hybrids ranging from 99 to 113 day relative maturity. Experiment A tested event DAS-40278-9 in the genetic background Inbred C x BC3S1 conversion. This hybrid has a relative maturity of 109 days and was tested at 16 locations (Table 32). Experiment B tested the hybrid background Inbred E x BC3S1 conversion, a 113 day relative maturity hybrid. This hybrid was tested at 14 locations, using a slightly different set of locations than Experiment A. Experiments C and D tested hybrid backgrounds BC2S1 conversion x Inbred D and BC2S1 conversion x Inbred F, respectively. Both of these hybrids have a 99 day relative maturity and were tested at the same 10 locations.

[00370] For each trial, a randomized complete block design was used with two replications per location and two row plots. Row length was 20 feet and each row was seeded at 34 seeds per row. Standard regional agronomic practices were used in the management of the trials.

[00371] Data were collected and analyzed for eight agronomic characteristics; plant height, ear height, stalk lodging, root lodging, final population, grain moisture, test weight, and yield. The parameters plant height and ear height provide information about the appearance of the hybrids. The agronomic characteristics of percent stalk lodging and root lodging determine the harvestability of a hybrid. Final population count measures seed quality and seasonal growing conditions that affect yield. Percent grain moisture at harvest defines the maturity of the hybrid, and yield (bushels/acre adjusted for moisture) and test weight (weight in pounds of a bushel of corn adjusted to 15.5% moisture) describe the reproductive capability of the hybrid.

[00372] Analysis of variance was conducted across the field sites using a linear model. Entry and location were included in the model as fixed effects. Mixed models including location and location by entry as random effects were explored, but location by entry explained only a small portion of variance and its variance component was often not significantly different from zero. For stock and root lodging a logarithmic transformation was used to stabilize the variance, however means and ranges are reported on the original scale. Significant differences were declared at the 95% confidence level. The significance of an overall treatment effect was estimated using a t-test.

[00373] Results from these agronomic characterization trials can be found in Table 32. No statistically significant differences were found for any of the four 40278 hybrids compared to the isoline controls (at $p<0.05$) for the parameters of ear height, stalk lodging, root lodging, grain moisture, test weight, and yield. Final population count and plant height were statistically different in Experiments A and B, respectively, but similar differences were not seen in comparisons with the other 40278 hybrids tested. Some of the variation seen may be due to low

levels of genetic variability remaining from the backcrossing of the DAS-40278-9 event into the elite inbred lines. The overall range of values for the measured parameters are all within the range of values obtained for traditional corn hybrids and would not lead to a conclusion of increased weediness. In summary, agronomic characterization data indicate that 40278 corn is biologically equivalent to conventional corn.

Table 32. Analysis of agronomic characteristics.

Experiment A

Parameter (units)	**Treatment**	**Mean**	**Range**		**P-value**
			Min	**Max**	
Plant Height (inches)	AAD-1	96.31	94.00	99.00	0.6174
	Control	95.41	95.00	98.00	
Ear Height (inches)	AAD-1	41.08	30.00	48.00	0.4538
	Control	44.42	40.00	47.00	
Stalk Lodging (%)	AAD-1	3.64	0.00	27.70	0.2020
	Control	2.49	0.00	28.57	
Root Lodging (%)	AAD-1	1.00	0.00	7.81	0.7658
	Control	0.89	0.00	28.33	
Final Population (plants/acre in 1000's)	AAD-1	31.06	27.00	36.00	0.0230
	Control	32.17	27.00	36.00	
Grain Moisture (%)	AAD-1	22.10	14.32	27.80	0.5132
	Control	21.84	14.52	31.00	
Test Weight (lb/bushel)	AAD-1	54.94	51.10	56.80	0.4123
	Control	54.66	51.00	56.80	
Yield (bushels/acre)	AAD-1	193.50	138.85	229.38	0.9712
	Control	187.05	99.87	256.72	

Experiment B

Parameter (units)	**Treatment**	**Mean**	**Range**		**P-value**
			Min	**Max**	
Plant Height (inches)	AAD-1	106.92	104.00	108.00	0.0178
	Control	100.79	95.00	104.00	
Ear Height (inches)	AAD-1	51.75	49.00	50.00	0.1552
	Control	45.63	38.00	50.00	
Stalk Lodging (%)	AAD-1	1.24	0.00	15.07	0.1513
	Control	0.72	0.00	22.22	
Root Lodging (%)	AAD-1	0.64	0.00	6.15	0.2498
	Control	0.40	0.00	9.09	
Final Population (plants/acre in 1000's)	AAD-1	31.30	26.00	37.00	0.4001
	Control	30.98	25.00	35.00	

Grain Moisture (%)	AAD-1	23.71	14.34	28.70	0.9869
	Control	23.72	13.39	31.10	
Test Weight (lb/bushel)	AAD-1	56.96	50.90	59.50	0.2796
	Control	56.67	52.00	60.10	
Yield (bushels/acre)	AAD-1	200.08	102.32	258.36	0.2031
	Control	205.41	95.35	259.03	
Experiment C					
Parameter (units)	**Treatment**	**Mean**	**Range**		**P-value**
			Min	**Max**	
Plant Height (inches)	AAD-1	95.92	94.00	96.00	0.1262
	Control	90.92	90.00	90.00	
Ear Height (inches)	AAD-1	47.75	41.00	50.00	0.4630
	Control	43.75	37.00	46.00	
Stalk Lodging (%)	AAD-1	6.74	0.00	27.47	0.4964
	Control	5.46	0.00	28.12	
Root Lodging (%)	AAD-1	0.3512	0.00	7.58	0.8783
	Control	0.3077	0.00	33.33	
Final Population (plants/acre in 1000's)	AAD-1	32.78	29.00	36.00	0.0543
	Control	31.68	24.00	35.00	
Grain Moisture (%)	AAD-1	19.09	13.33	25.90	0.5706
	Control	19.36	13.66	26.50	
Test Weight (lb/bushel)	AAD-1	54.62	42.10	58.80	0.1715
	Control	55.14	52.80	58.40	
Yield (bushels/acre)	AAD-1	192.48	135.96	243.89	0.2218
	Control	200.35	129.02	285.58	
Experiment D					
Parameter (units)	**Treatment**	**Mean**	**Range**		**P-value**
			Min	**Max**	
Stalk Lodging (%)	AAD-1	7.29	0.00	9.26	0.4364
	Control	4.17	0.00	39.06	
Final Population (plants/acre in 1000's)	AAD-1	29.93	27.00	34.00	0.0571
	Control	31.86	29.00	35.00	
Grain Moisture (%)	AAD-1	18.74	19.40	24.40	0.4716
	Control	19.32	13.35	25.70	
Test Weight (lb/bushel)	AAD-1	56.59	54.80	58.30	0.0992
	Control	55.50	52.70	57.40	
Yield (bushels/acre)	AAD-1	203.55	196.51	240.17	0.7370
	Control	199.82	118.56	264.11	

[00374] Agronomic characteristics of hybrid corn containing event DAS-40278-9 compared

to near- isoline corn were collected from multiple field trials across diverse geographic environments for a growing season. The results for hybrid corn lines containing event DAS-40278-9 as compared to null plants are listed in Table 33.

Table 33. Yield, percent moisture, and final population results for hybrid corn containing evnt DAS-40278-9 as compared to the near-isoline control.

Name	Yield	Grain Moisture (%)	Final Population (Plants/acre reported in 1000's)
Hybrid Corn Contianing DAS-40278-9	218.1	21.59	31.69
Control Hybrid Corn	217.4	21.91	30.42

[00375] Agronomic characteristics for the hybrid corn lines containing event DAS-40278-9 and null plants sprayed with the herbicides quizalofop (280 g ae/ha) at the V3 stage of development and 2,4-D (2,240 g ae/ha) sprayed at the V6 stage of development are in Table 34.

Table 34. Agronomic data for event DAS 40278-9 as compared to the near-isoline control.

Trial	Yield	Grain Moisture (%)	Stock Lodge (%)	Root Lodge (%)	Final Population (plants/acre reported in 1000's)
Spray Trial					
Hybrid Corn #1 containing DAS-40278-9	214.9	23.4	0.61	2.19	30
Control Hybrid Corn #1	177.9	23.46	0.97	36.32	28.36
LSD (0.5)	13.3	1.107	0.89	10.7	1.1
Non Spray					
Hybrid Corn #1 containing DAS-40278-9	219.6	22.3	0.95	1.78	30.8
Control Hybrid Corn #1	220.3	22.51	0.54	1.52	30.55
LSD (0.5)	6.9	0.358	0.98	1.65	0.7
Spray Trial					
Hybrid Corn #2 containing DAS-40278-9	198.6	26.76	0.38	2.08	29.29
Control Hybrid Corn #2	172.3	23.76	1.5	39.16	28.86
LSD (0.5)	13.3	1.107	0.89	10.7	1.1
Non Spray					
Hybrid Corn #2 containing DAS-40278-9	207.8	24.34	0.22	0.59	31
Control Hybrid Corn #2	206.2	24.88	0.35	0.12	30.94
LSD (0.5)	8.0	0.645	0.55	1.79	0.9

Example 21

2,4-D Increases Growth of 2,4-D Resistant Soybean

[00376] Transgenic soybean with AAD-12 transgene provides protection to the soybean plant while weeds are destroyed by application of 2,4-D. It has been unexpectedly observed that 2,4-D also increase growth in 2,4-D tolerant soybean. This increased growth has resulted in increases in plant height and/or yield of sprayed plots compared to non-sprayed plots.

[00377] Increase in plant growth and/or yield resulting from the application of 2,4-D is described for soybean plants genetically engineered to the tolerant to 2,4-D. Trials were grown across multiple locations covering the North American soybean growing region. Entries included elite lines into which event DAS-68416-4 (which conditions tolerance to 2,4-D) had been introgressed. Treatments consisted of non-sprayed and 2,4-D sprayed treatment applied at both the V3 and R2 growth stages. Plots were measured for various agronomic characteristics throughout the season including plant height and grain yield. Weeds were controlled throughout the season in both sprayed and non-sprayed plots to eliminate any competition effect. At the conclusion of the trial, data analysis measured a significant increase in both height and yield for those entries which had been sprayed with 2,4-D compared with those which received no treatment. An increase in yield is an additional benefits to the weed control delivered by 2,4-D on 2,4-D resistant soybeans.

[00378] Field trials were run in 2011 to compare the agronomic characteristics of soybean event DAS-68416-4 (International Patent Application No. 2011/066384) that had been sprayed with 2,4-D, with the agronomic characteristics of unsprayed soybean event DAS-68416-4. The field trials contained entries of 4 elite soybean lines into which soybean event DAS-68416-4 had been introgressed, and the respective null isolines of the 4 elite soybean lines which did not contain soybean event DAS-68416-4. The trials were planted across differing geographical locations (ten locations in total). The experiment was set up as a modified split plot with two replications per location. Whole plots were treatments and subplots were entries. Each plot consisted of two rows, 12.5 feet long, planted 30 inches apart. The sprayed plots were treated with 2,4-D (1120 g ae/ha) sprayed at the V3 and R2 growth stages. Throughout the season, field plots were maintained under normal agronomic practices and kept free from weeds. Various agronomic characteristics were measured for the soybean plants to determine how the application of 2,4-D affected the performance of the soybean agronomic characteristics. The tested agronomic characteristics and the growth stage when the data were collected are listed in Table 35.

Table 35. List of agronomic characteristics measured in 2011 yield trials to compare 2,4-D sprayed and unsprayed soybean event DAS-68416-4.	
Characteristic Measured	Growth Stage of Measurement
1. Emergence: Stand count (above) divided by the number of seeds planted in a one meter section multiplied by 100.	Calculated based on early stand count
2. Seedling vigor: Percent vigor with 0% representing a plot with all dead plants and 100% representing plots that look very healthy.	V1 – V3
3. Days to Flowering: Days from planting when 50% of the plants in the plot began to flower.	R1
4. Stand count at R2: Number of plants in a representative one meter section of row at the R2 growth stage.	R2
5. Disease incidence: Severity of disease in the plot rated on a scale of 0-100%.	R6
6. Insect damage: Percentage of plant tissue in the plot damaged by insects.	R6
7. Plant height: Average height in centimeters of the plants in each plot measured from the soil surface to the tip after leaves have fallen.	R8
8. Lodging: Percent lodging at harvest time with 0% = no lodging and 100% = all plants in a plot flat on the ground.	R8
9. Days to maturity. Days from planting when 95% of the pods in a plot reached their dry down color.	R8
10. Shattering: Percentage of pods shattered per plot.	R8
11. Yield: Bushels per acre adjusting to 13% moisture.	R8
12. 100 seed weight: For each plot count out 100 seeds and record the weight in grams.	R8

[00379] At the end of the soybean growing season, data from all locations were combined and an across location analysis was performed. Data analysis was carried out using JMP® Pro 9.0.3 (SAS, Cary, NC). Least square means from the analysis are reported in Table 28. The application of 2,4-D on soybean event DAS-68416-4 containing the AAD-12 transgene resulted in a conditioning effect of increased growth. The increased growth culminated in significantly greater yield and plant height measurements in field plots sprayed with 2,4-D as compared to field plots not sprayed with 2,4-D. These increases were ascertainable when the data was analyzed cumulatively across all locations. In contrast, the increased yield for soybean event DAS-68416-4 sprayed with 2,4-D was diminished by a location by treatment interaction. Both average height and yield were increased about 5% by applications of 2,4-D in Table 36.

Table 36. Least square means from the across location analysis comparing soybean event DAS-68416-4 that was sprayed with 2,4-D to unsprayed plants. Levels not connected by the same letter are significantly different.		
Treatments Applied	2,4-D at 1,120 g ae/ha (at V3 and R2 stages)	Unsprayed
Emergence (%)	77 (A)	74 (A)
Vigor V1-V3 (%)	87 (A)	87 (A)
Days to Flowering (days from planting)	44 (A)	44 (A)
Stand Count at R2 (plants/ m)	21 (A)	22 (A)
Disease Incidence R6 (%)	1 (A)	1 (A)
Insect Damage R6 (%)	2 (A)	2 (A)
Height (cm)	81 (A)	77 (A)
Maturity (days from planting)	109 (A)	109 (A)
Lodging (%)	10 (A)	8 (B)
Shattering (%)	0 (A)	1 (A)
Yield (bu/acre)	56.4 (A)	53.7 (B)
100 Seed Weight (g)	14.8 (A)	14.8 (A)

[00380] As shown in Table 37, at least one of the ten locations (Location #a3) reported significantly higher yield harvests for the unsprayed soybean event DAS-68416-4 plants as compared to the 2,4-D sprayed soybean event DAS-68416-4 plants. When the results for all of the locations were accumulated the application of 2,4-D on soybean event DAS-68416-4 containing the AAD-12 transgene indicated a conditioning effect resulting in increased growth. For instance, the yield of soybean event DAS-68416-4 plants sprayed with 2,4-D was 56.4 bu/acre which is considerably greater than the yield of unsprayed soybean event DAS-68416-4 plants which was 53.7 bu/acre. Likewise, the height of soybean event DAS-68416-4 plants sprayed with 2,4-D was 81 cm which is considerably greater than the height of unsprayed soybean event DAS-68416-4 plants which was 77 cm.

Table 37. Least square means for yield from specific locations comparing soybean event DAS-68416-4 that was sprayed with 2,4-D to unsprayed plants. Levels not connected by the same letter are significantly different.

Location Number	Treatment	Yield (bu/acre)		Yield %
Location #a1	Sprayed	51	A	121.5
	Unsprayed	42	B	100
Location #a2	Sprayed	67	A	115.6
	Unsprayed	58	B	100
Location #a3	Sprayed	44	B	88
	Unsprayed	50	A	100
Location #a4	Sprayed	68	A	97
	Unsprayed	70	A	100
Location #a5	Sprayed	75	A	102.8
	Unsprayed	73	A	100
Location #a6	Sprayed	57	A	132.6
	Unsprayed	43	B	100
Location #a7	Sprayed	48	A	102.2
	Unsprayed	47	A	100
Location #a8	Sprayed	39	A	91
	Unsprayed	43	A	100
Location #a9	Sprayed	57	A	101.8
	Unsprayed	56	A	100
Location #a10	Sprayed	59	A	107.3
	Unsprayed	55	A	100
Average	Sprayed	-	-	106

Example 22

2,4-D Increases Growth of 2,4-D Resistant Soybean in 2,4-D/Glyphosate Combination

[00381] Similar field trials as in the previous Example were run in 2010 but with two applications of 2,4-D in combination with glyphosate. Results show that increased growth of 2,4-D resistant soybean, in plant height and/or yield of sprayed plots compared to non-sprayed plots, is due to application of 2,4-D.

[00382] Significant treatment effects were observed for a number of parameters measured. Both 2,4-D and glyphosate were sprayed at the V3 and R2 growth stages. The trials were planted across differing geographical locations (six locations in total). The tested agronomic characteristics and the growth stage when the data were collected are listed in Table 30. The average height was increased 6% and average yield was increased 17% for sprayed soybean in Table 38. In addition, average seed weight was increased 6% for sprayed soybean.

Table 38. Least square means from the across location analysis comparing 2,4-D tolerant soybean that was sprayed with 2,4-D plus glyphosate to unsprayed plants. Levels not connected by the same letter are significantly different.		
Treatments Applied	2,4-D plus glyphosate Both at 1,120 g ae/ha (at V3 and R2 stages)	Unsprayed
Emergence (%)	54 (A)	54 (A)
Vigor V1-V3 (%)	7 (A)	7 (A)
Days to Flowering (days from planting)	41 (A)	41 (A)
Stand Count at R2 (plants/ m)	15 (A)	15 (A)
Disease Incidence R6 (%)	4 (A)	4 (A)
Insect Damage R6 (%)	17 (A)	14 (B)
Height (cm)	109 (A)	103 (B)
Maturity (days from planting)	117 (A)	116 (B)
Lodging (%)	17 (A)	9 (B)
Shattering (%)	0 (A)	0 (A)
Yield (bu/acre)	43.4 (A)	37.0 (B)
100 Seed Weight (g)	12.2 (A)	11.5 (B)

[00383] As shown in Table 39, certain geographical variations were also observed in this Example. The average yield was increased 21.6% for sprayed soybean in Table 39.

Table 39. Least square means for yield from specific locations 2,4-D tolerant soybean that was sprayed with 2,4-D plus glyphosate to unsprayed plants.				
Location Number	Treatment	Yield (bu/acre)		Yield %
Location #b1	Sprayed	39	A	162.5
	Unsprayed	24	B	100
Location #b2	Sprayed	51	A	104.1
	Unsprayed	49	A	100
Location #b3	Sprayed	56	A	155.5
	Unsprayed	36	B	100
Location #b4	Sprayed	35	A	106.1
	Unsprayed	33	A	100
Location #b5	Sprayed	48	A	104.3
	Unsprayed	46	A	100
Location #b6	Sprayed	32	A	97.0
	Unsprayed	33	A	100
Average	Sprayed	-	-	121.6

Example 23

Yield Trial Results Comparing Sprayed and Non-sprayed Treatments

[00384] 2,4-D resistant transgenic crop plants transformed with an aryloxyalkanoate dioxygenase (AAD) resulted in increased yield when treated with a stimulating amount of

herbicide comprising an aryloxyalkanoate moiety. Soybean events comprising an AAD-12 gene expression cassette were tested in replicated yield trials under sprayed and non-sprayed conditions. There was one series of experiments which contained early soybeans adapted to northern latitudes and another series of experiments which contained late soybeans adapted to more southern latitudes. In previous experiments there were instances where soybean entries comprising an AAD-12 gene expression cassette were treated with 2,4-D during the growing season exhibited and increased yield relative to the unsprayed checks.

[00385] A modified split plot design with 2 replications was used for the trials. Each plot was 2 rows wide with 30 inch row spacing and 12.5 feet long. There was a 2.5 to 3 foot alleyway between plots planted end to end to allow for movement within the trial during the season. The sprayed blocks were sprayed sequentially (twice) during the growing season with 2,4-D choline + glyphosate (premix) at 2185 g ae/ha + AMS at 2% weight per weight.

Table 40. List of analysis locations for yield trials comparing sprayed verses non-sprayed treatments.

Location	Trial
Atlantic, IA	early
Brookings, SD	early
Cherry Grove, MN	early
Deerfield, MI	early
Kirklin, IN	early
Otterbein, IN	early
Richland, IA	early
Wyoming, IL	early
Atlantic, IA	late
Carlyle, IL	late
Fisk, MO	late
Otterbein, IN	late
Seymour, IL	late
Stewardson, IL	late
Sycamore, GA	late
Tallassee, AL	late

[00386] The first application was at the V3 growth stage and the second application at R2 growth stage. Both the experimentatal and control field trials were kept weed free throughout

the season by use of conventional herbicides or hand weeding. Data were collected on emergence, seedling vigor, crop injury, flowering date, stand count at R2, disease incidence, insect damage plant height, maturity date, lodging, shattering 100 seed weight and yield. Data were analyzed using JMP® Pro 9.0.3. Table 40 lists the locations that were used in the final analysis. Some locations which were planted were not included in the analysis due to within plot variability.

[00387] Across location analysis were performed for both the early and late trials. Tables 41 and 42 show the yield analysis of variance for the early and late trials respectively.

Table 41. Across location (8 locations) analysis of variance for yield in the early variety sprayed vs non-sprayed trials.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
NAME	8	8	57.030	3.780	0.001
TRT	1	1	5.989	12.409	0.013
NAME*TRT	8	8	183.000	0.530	0.833

[00388] For both the early and late trials there was a significant (P=0.05) name effect. This was expected since each elite soybean line into which an event had been introgressed was from a different genetic background.

Table 42. Across location (8 locations) analysis of variance for yield in the late variety sprayed vs non-sprayed trials.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
NAME	11	11	76.020	3.096	0.002
TRT	1	1	7.039	3.050	0.124
NAME*TRT	11	11	257.700	0.499	0.903

[00389] A significant treatment effect was measured for the early trial indicating that the sprayed and non-sprayed treatments differed for yield. For the late trial there was not a significant treatment effect which indicates that sprayed and non-sprayed plots did not differ for yield.

Table 43. Table of least squares yield means from early yield trial.		
Treatment number	Yield (bu/acre)	
289-1(HOMO),Non-sprayed	42.0	A
289-1(HOMO),Sprayed	46.0	A
289-2(HOMO),Non-sprayed	41.8	A
289-2(HOMO),Sprayed	45.7	A
7471638-26(HOMO),Non-sprayed	38.2	B
7471638-26(HOMO),Sprayed	42.9	A
76983-1(HOMO),Non-sprayed	38.4	B
76983-1(HOMO),Sprayed	42.5	A
76983-2(HOMO),Non-sprayed	39.6	A
76983-2(HOMO),Sprayed	42.9	A
75209(HOMO),Non-sprayed	46.4	A
75209(HOMO),Sprayed	47.6	A
75209[1](HOMO),Non-sprayed	48.1	B
75209[1](HOMO),Sprayed	52.7	A
75357-71(HOMO),Non-sprayed	46.2	A
75357-71(HOMO),Sprayed	49.5	A
99345-31[4](HOMO),Non-sprayed	40.1	B
99345-31[4](HOMO),Sprayed	46.0	A

[00390] For both the early and late trials the name by treatment interaction effect was not significant indicating that the effect of the treatment (or lack of an effect) was the same for each entry in a particular trial.

[00391] Table 43 shows average yield for each entry by treatment combination in the early trial, where HOMO stands for homozygous. Values followed by the same letter (within a given variety) are not different according to Student's t at P=0.05. There were four entries which exhibited higher yield when sequentially sprayed at V3 and R3 with 2,4-D choline + glyphosate (premix) at 2185 g ae/ha + AMS.

[00392] Table 44 shows average yield for each entry by treatment combination. Values followed by the same letter (within a given variety) are not different according to Student's t at P=0.05. As reported above there was not a significant treatment effect or treatment by entry effect for the late trial so mean separation was not carried out. Letters in the table indicate that

there was no difference between sprayed and non-sprayed treatments in the late test.

Table 44. Table of least squares yield means from the 2012 late yield trial.		
Treatment number	Yield (bu/acre)	
348-1(HOMO),Non-sprayed	54.5	A
348-1(HOMO),Sprayed	54.7	A
348[3](HOMO),Non-sprayed	51.1	A
348[3](HOMO),Sprayed	54.5	A
4075433-15(HOMO),Non-sprayed	59.6	A
4075433-15(HOMO),Sprayed	60.4	A
75226-1(HOMO),Non-sprayed	52.1	A
75226-1(HOMO),Sprayed	55.2	A
75226-2(HOMO),Non-sprayed	51.1	A
75226-2(HOMO),Sprayed	52.2	A
75505(HOMO),Non-sprayed	50.1	A
75505(HOMO),Sprayed	54.6	A
99753-81(HOMO),Non-sprayed	56.1	A
99753-81(HOMO),Sprayed	55.4	A
75358-72(HOMO),Non-sprayed	50.7	A
75358-72(HOMO),Sprayed	53.8	A
75358-72[1](HOMO),Non-sprayed	48.4	A
75358-72[1](HOMO),Sprayed	50.1	A
99753-75[4](HOMO),Non-sprayed	52.1	A
99753-75[4](HOMO),Sprayed	53.4	A
Control-1,Non-sprayed	49.2	A
Control-1,Sprayed	51.4	A
Control-2,Non-sprayed	49.6	A
Control-2,Sprayed	52.0	A

[00393] Results from yield trials in this example once again show that in some environments for some soybean genotypes there may be an increase in yield following application of 2,4-D. In the past two years such yield increase has been observed in yield trials that have been run in MG 2 growing region.

Example 24

Comparison Between Soybean and Corn

[00394] The yield results from the field trials in soybean comprising an AAD-12 transgene indicate that an application of 2,4-D may increase the yield of soybeans in certain environments for certain soybean genotypes. These results are surprising when compared to the transgenic corn events which comprise an AAD-1 transgene. The yield of AAD-1 transgenic corn plants did not consistently show a statistically singnificant increase in yield after sprayed with 2,4-D. These AAD-1 transgenic corn plants are biologically equivalent to conventional corn. Additional field studies in diverse geographical locales were completed from 2010 through 2012 on hybrid corn lines. Throughout these field studies the yield of the corn lines sprayed with 2,4-D (2,185 g ae/ha and 4,370 g ae/ha) were compared to untreated control corn lines (e.g., not sprayed with 2,4-D). The results of these experiments further substantiate that corn plants containing the AAD-1 transgene do not result in a significant increase in yield as a result of treatment with a 2,4-D spray. Comparatively, a yield increase has been shown in some soybean genotypes following an application of 2,4-D. The observed yield increase in soybean genotypes which is shown following an application of 2,4-D is an unexpected improvement that is applicable for increasing the yield of crop plants. The disclosed method can be deployed for using a 2,4-D treatment to increase the yield of transgenic crop plants, for example expressing an AAD-12 gene.

[00395] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of improving yield of 2,4-D resistant crop plants, comprising treating the plants with a stimulating amount of a herbicide comprising an aryloxyalkanoate moiety.

2. The method of claim 1, wherein the 2,4-D resistant crop plants are transgenic plants transformed with an aryloxyalkanoate dioxygenase (AAD).

3. The method of claim 2, wherein the aryloxyalkanoate dioxygenase (AAD) is AAD-12.

4. The method of claim 1, wherein the herbicide comprising an aryloxyalkanoate moiety is a phenoxy herbicide or phenoxyacetic herbicide.

5. The method of claim 1, wherein the herbicide comprising an aryloxyalkanoate moiety is 2,4-D.

6. The method of claim 5, wherein the 2,4-D comprises 2,4-D choline or 2,4-D dimethylamine (DMA).

7. The method of claim 1, wherein the treating is performed at least once at an application rate of 2,4-D as employed also for weed control.

8. The method of claim 1, wherein the treating is performed twice at an application rate of 2,4-D as employed also for weed control.

9. The method of claim 8, wherein 2,4-D is applied at the V3 and R2 growth stages of soybean with 2,4-D tolerance.

10. The method of claim 1, wherein the treating is performed at least three times at an application rate of 2,4-D as employed also for weed control.

11. The method of claim 1, wherein the 2,4-D resistant crop plants are under stress.

12. The method of claim 1, wherein the 2,4-D resistant crop plants are also treated with a herbicide different than 2,4-D for weed control.

13. The method of claim 12, wherein the herbicide different than 2,4-D is a phosphor-herbicide or aryloxyphenoxypropionic herbicide.

14. The method of claim 13, wherein the phosphor-herbicide comprises glyphosate, glufosinate, their derivatives, or combinations thereof.

15. The method of claim 13, wherein the phosphor-herbicide is in form of ammonium salt, isopropylammonium salt, isopropylamine salt, or potassium salt.

16. The method of claim 13, wherein the aryloxyphenoxypropionic herbicide comprises chlorazifop, fenoxaprop, fluazifop, haloxyfop, quizalofop, their derivatives, or combinations thereof.

17. The method of claim 1, wherein the 2,4-D resistant crop plants are treated at least once with 25 g ae/ha to 5000 g ae/ha 2,4-D.

18. The method of claim 1, wherein the 2,4-D resistant crop plants are treated at least once with 100 g ae/ha to 2500 g ae/ha 2,4-D.

19. The method of claim 1, wherein the herbicide comprising an aryloxyalkanoate moiety reaches the 2,4-D resistant crop plants via root absorption.

20. The method of claim 13, wherein the phosphor-herbicide reaches the 2,4-D resistant crop plants via root absorption.

21. The method of claim 13, wherein the aryloxyphenoxypropionic herbicide reaches the 2,4-D resistant crop plants via root absorption.

22. The method of claim 2, wherein the transgenic plants transformed with an aryloxyalkanoate dioxygenase (AAD) are selected from cotton, soybean, and canola.

23. A method of improving yield of 2,4-D resistant crop plants comprising

(a) transforming plant cells with a nucleic acid molecule comprising a nucleotide sequence encoding an aryloxyalkanoate dioxygenase (AAD);

(b) selecting transformed cells;

(c) regenerating the plants from the transformed cells; and

(d) treating the plants with a stimulating amount of a herbicide comprising an aryloxyalkanoate moiety.

24. The method of claim 23, wherein the aryloxyalkanoate dioxygenase (AAD) is AAD-12.

25. The method of claim 23, wherein the nucleic acid molecule comprises a selectable marker which is not an aryloxyalkanoate dioxygenase (AAD).

26. The method of claim 25, wherein the selectable marker is phosphinothricin acetyltransferase gene (pat) or bialaphos resistance gene (bar).

27. The method of claim 23, wherein the nucleic acid molecule is plant-optimized.

28. The use of 2,4-D in the manufacture of transgenic plants with 2,4-D resistance with increased yield as compared to its non-transgenic parent plants.

29. The use of claim 28, wherein the 2,4-D is applied at least once with 25 g ae/ha to 5000 g/ha 2,4-D.

30. The use of claim 28, wherein the 2,4-D is applied at least once with 100 g ae/ha to 2500 g ae/ha 2,4-D.

31. The use of claim 28, wherein the 2,4-D comprises 2,4-D choline or 2,4-D dimethylamine (DMA).

32. The use of claim 28, wherein the 2,4-D resistant crop plants are treated with 2,4-D at least two times before flowering.

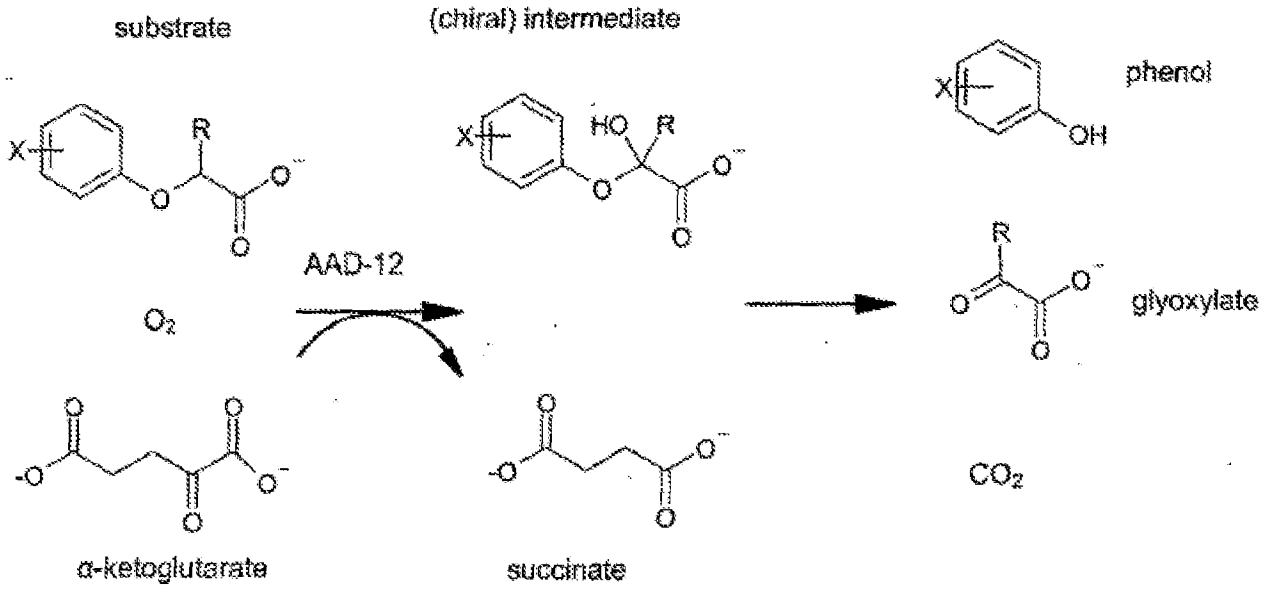
substrate
(chiral) intermediate
phenol
AAD-12
O_2
glyoxylate
CO_2
α-ketoglutarate
succinate

FIG. 1

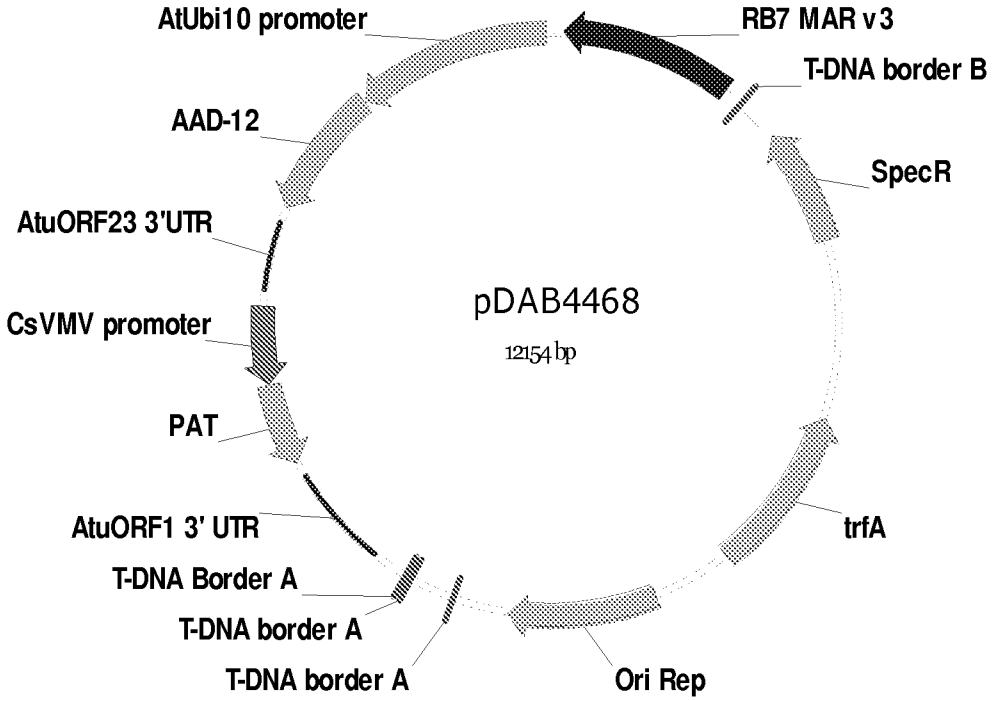
AtUbi10 promoter
RB7 MAR v3
T-DNA border B
AAD-12
SpecR
AtuORF23 3'UTR
pDAB4468
12154 bp
CsVMV promoter
PAT
AtuORF1 3' UTR
trfA
T-DNA Border A
T-DNA border A
T-DNA border A
Ori Rep

FIG. 2

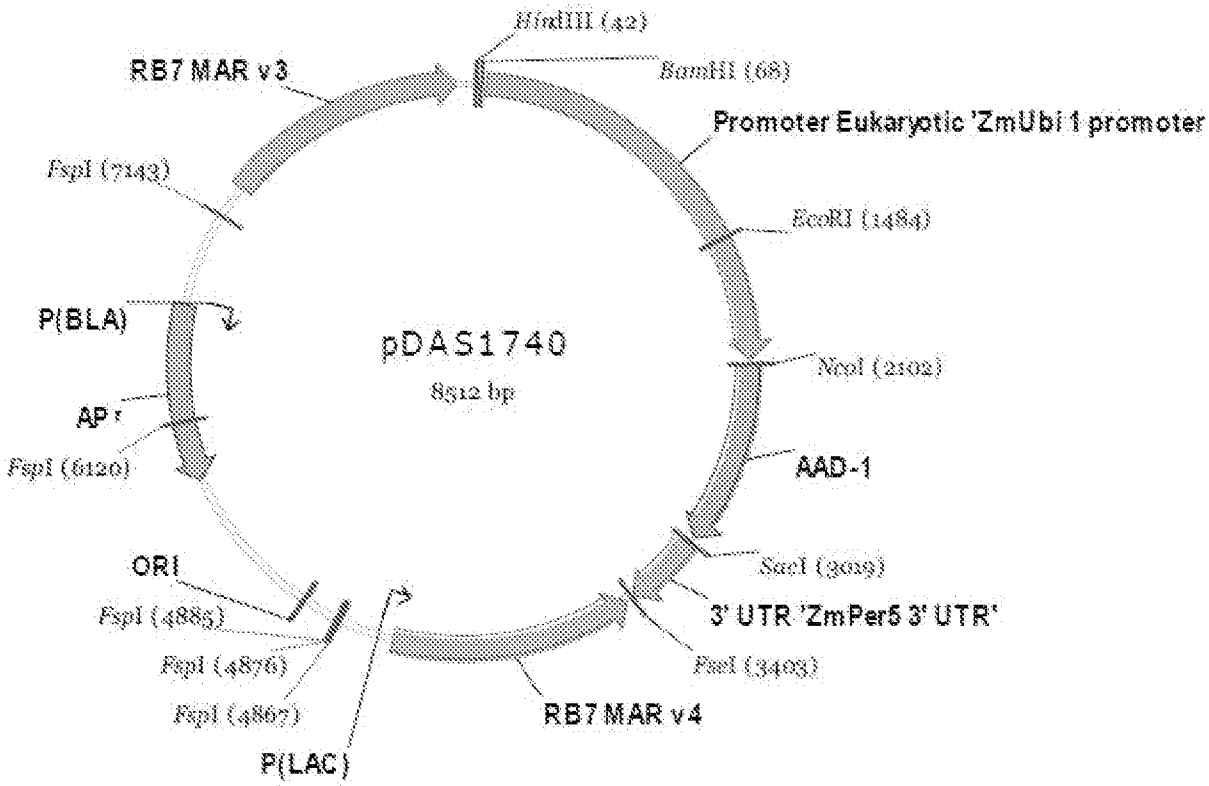
HindIII (42)
RB7 MAR v3
BamHI (68)
Promoter Eukaryotic 'ZmUbi 1 promoter
FspI (7143)
EcoRI (1484)
P(BLA)
pDAS1740
8512 bp
NcoI (2102)
AP r
FspI (6120)
AAD-1
SacI (3039)
ORI
FspI (4885)
3' UTR 'ZmPer5 3' UTR'
FspI (4876)
FseI (3403)
FspI (4867)
RB7 MAR v4
P(LAC)

FIG. 3