EXHIBIT 11

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(54) **HERBICIDE TOLERANCE GENES AND METHODS OF USE THEREOF**

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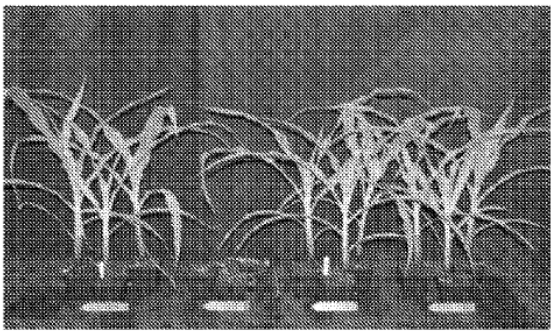
C12N 9/02	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/8274*** (2013.01); ***C12N 9/0071*** (2013.01); ***A01N 57/00*** (2013.01); *C12Y 114/11033* (2013.01)

(57) **ABSTRACT**

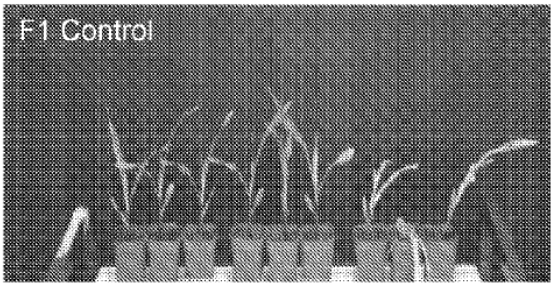
Polypeptides and recombinant DNA molecules useful for conferring tolerance to AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides are provided in the present invention, as well as herbicide tolerant transgenic plants, seeds, cells, and plant parts containing the recombinant DNA molecules, as well as methods of using the same.



Untreated Quizalofop Untreated Quizalofop

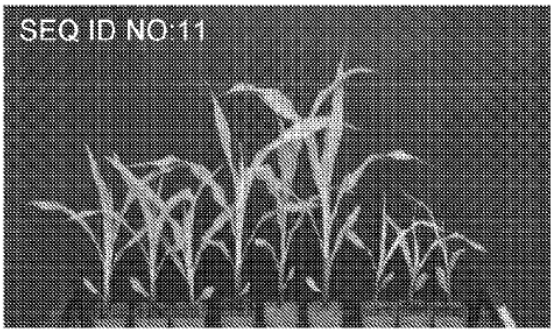
LH244 SEQ ID NO:11

FIG. 1A



20C/20C 28C/20C 38C/30C

FIG. 1B



20C/20C 28C/20C 38C/30C

FIG. 1C

[redacted]

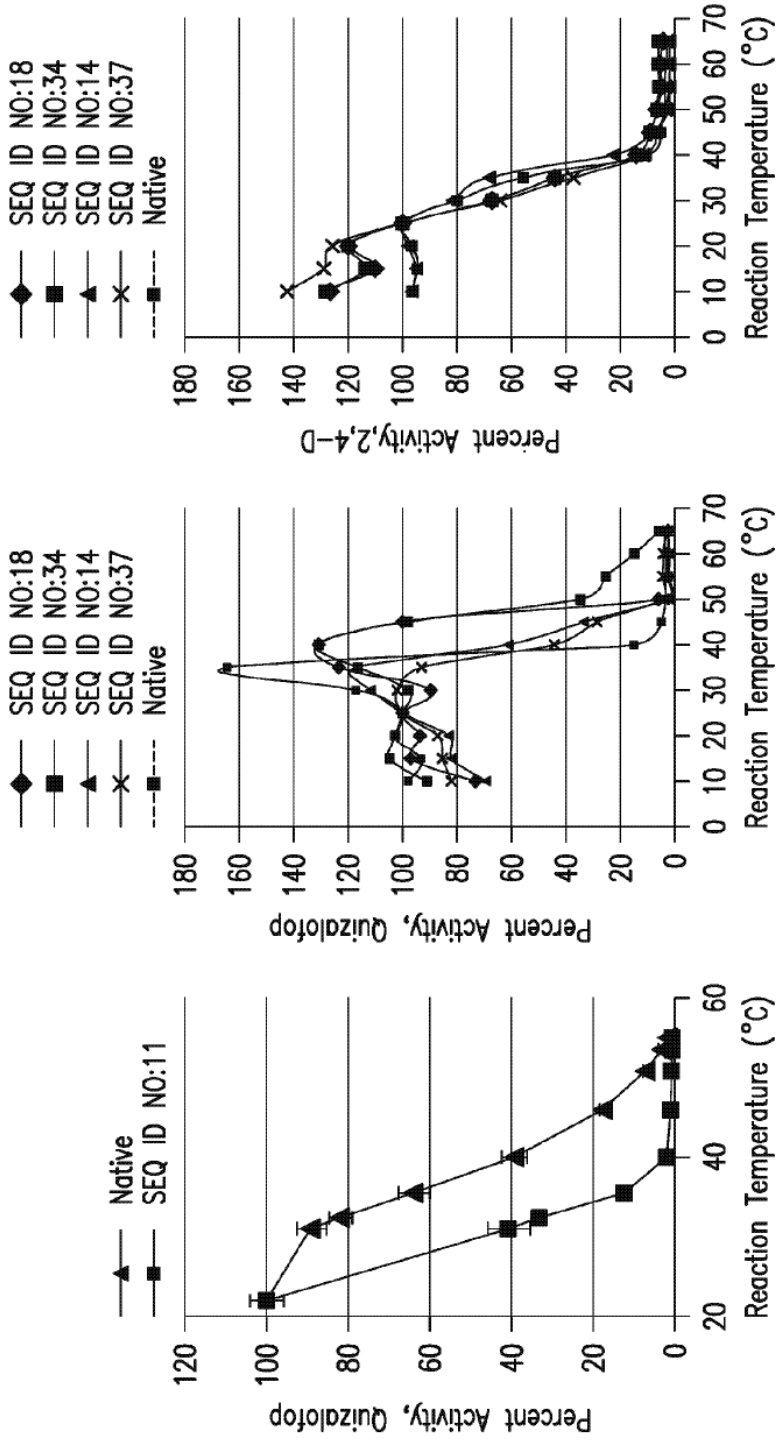


FIG.2A

FIG.2B

FIG.2C

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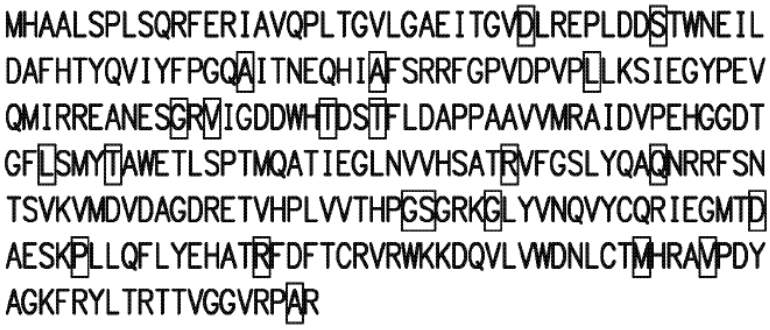

FIG.3

[redacted]

COR-AAD1-00385706

COR-AAD1-00385707

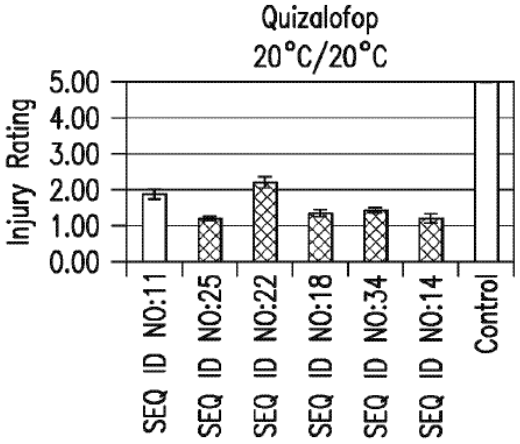

FIG.4A

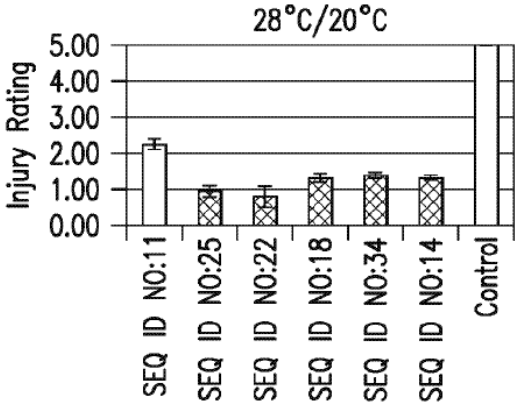

FIG.4B

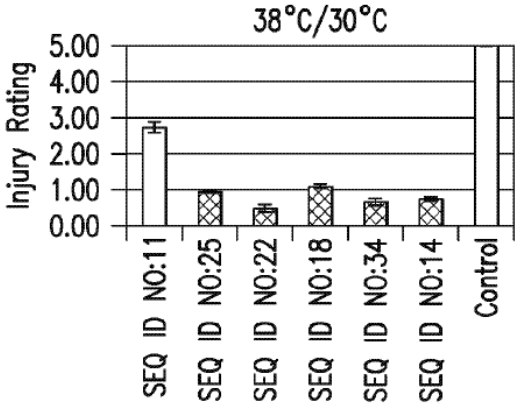

FIG.4C

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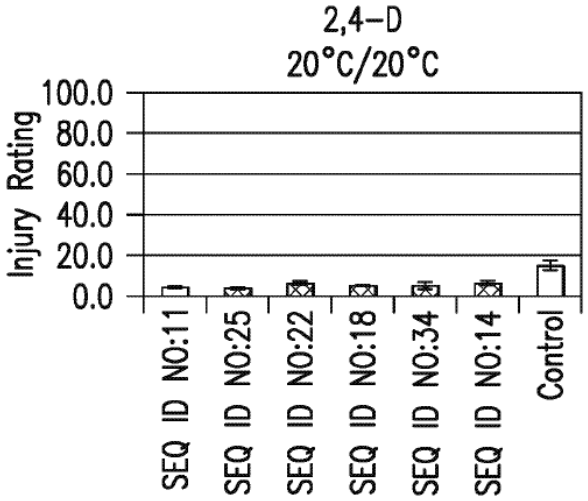

FIG.4D

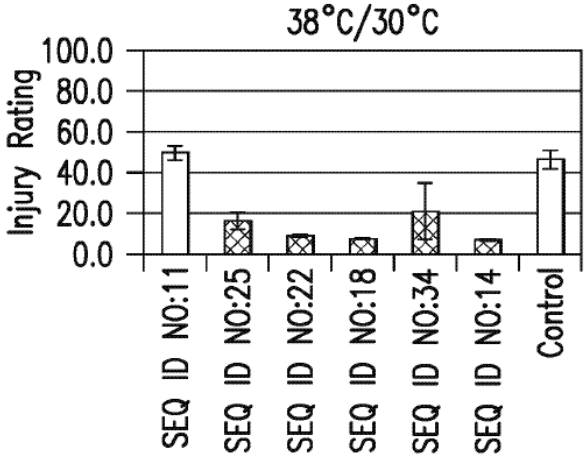

FIG.4E

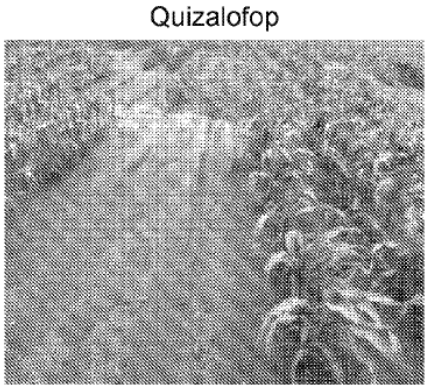
Quizalofop
F1 Control
MON-HT2
+CTP

FIG.5A

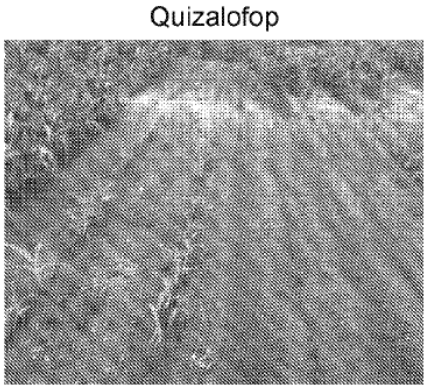
Quizalofop
MON-HT2
no CTP
F1 Control

FIG.5B

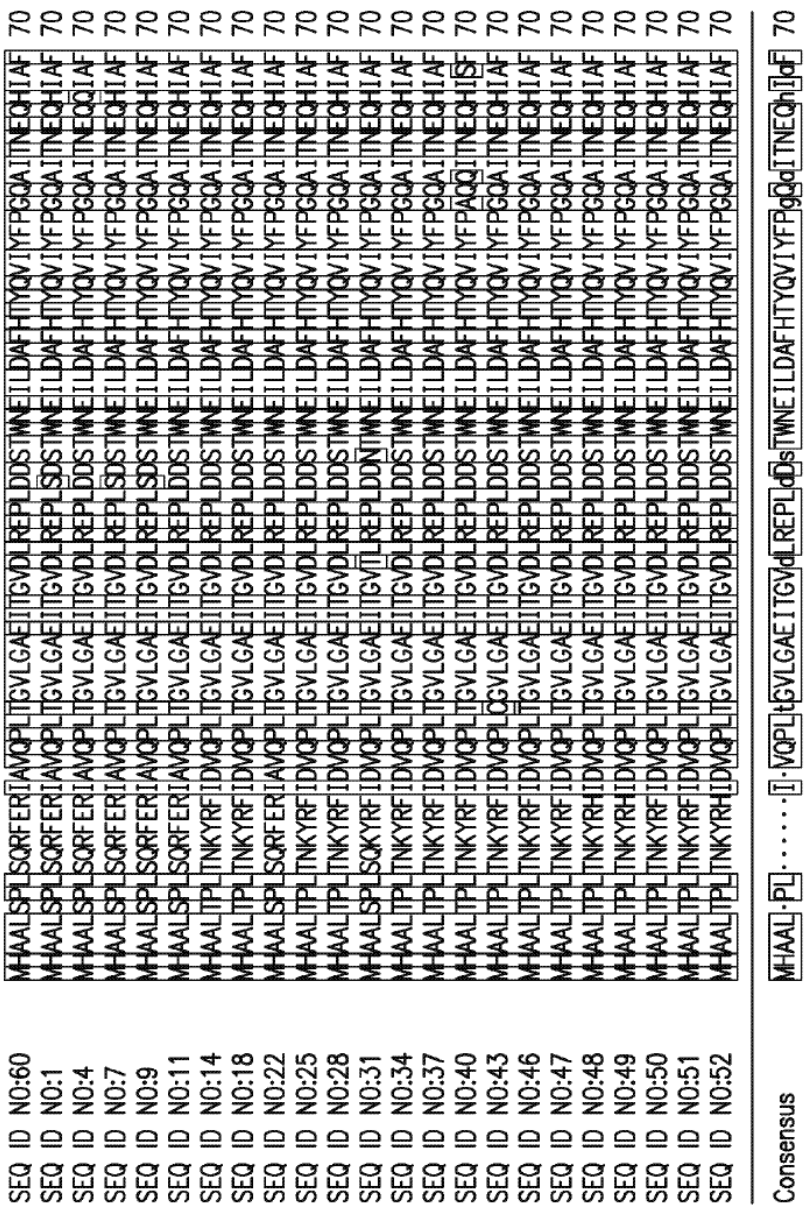

FIG.6A

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COR-AAD1-00385711

FIG.6B

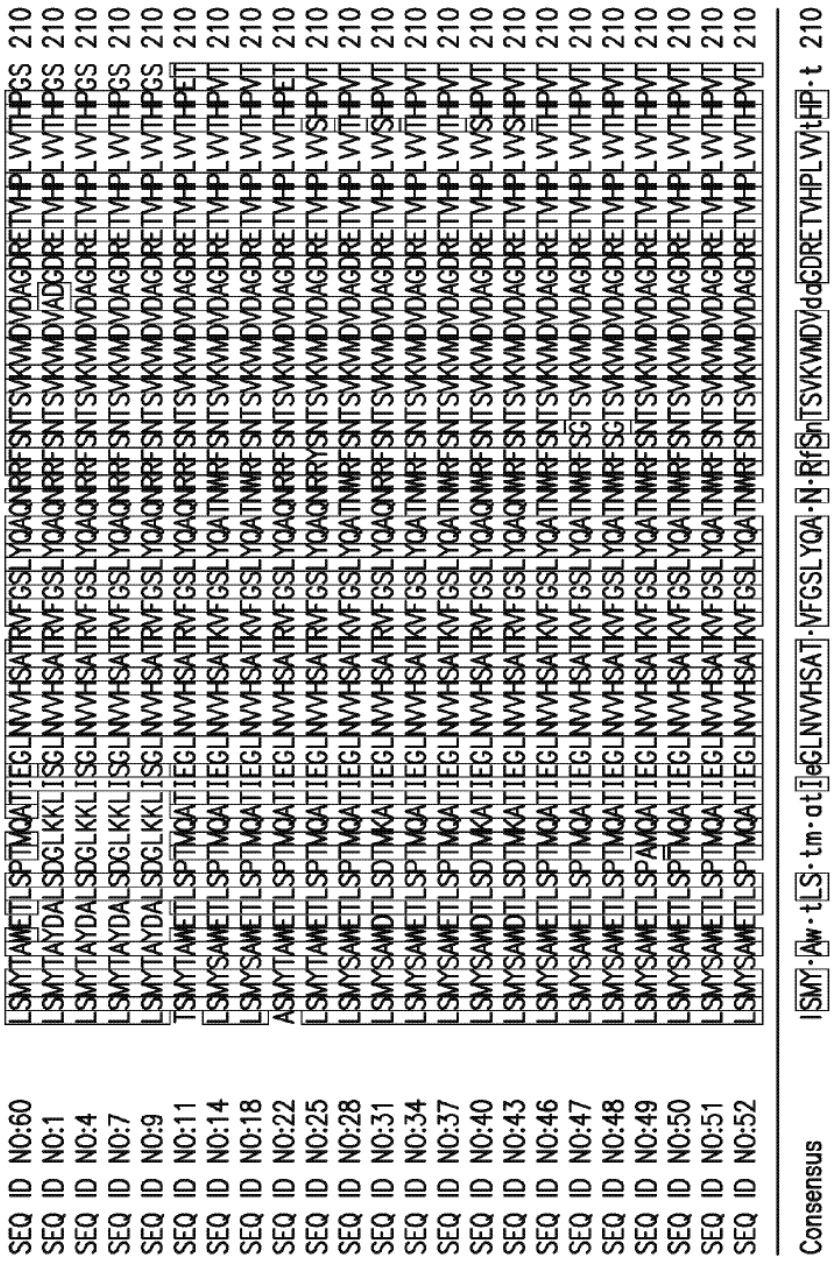

FIG.6C

COR-AAD1-00385712

COR-AAD1-00385713

FIG.6D

COR-AAD1-00385714

FIG.6E

HERBICIDE TOLERANCE GENES AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/064,343, filed on Oct. 15, 2014, herein incorporated by reference in its entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] The sequence listing that is contained in the file named "MONS378US_ST25", which is 118,584 bytes (measured in MS-WINDOWS) and created on Sep. 11, 2015, is filed herewith by electronic submission and incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0003] 1. Field of the Invention

[0004] The present invention generally relates to the field of biotechnology. More specifically, the invention relates to recombinant DNA molecules encoding enzymes that degrade herbicides. The invention also relates to transgenic plants, parts, seeds, cells, and plant parts containing the recombinant DNA molecules, as well as methods of using the same.

[0005] 2. Description of Related Art

[0006] Agricultural crop production often utilizes transgenic traits created using the methods of biotechnology. A heterologous gene, also known as a transgene, is introduced into a plant to produce a transgenic trait. Expression of the transgene in the plant confers a desirable trait, such as herbicide tolerance, on the plant. Examples of transgenic herbicide tolerance traits include glyphosate tolerance, glufosinate tolerance, and dicamba tolerance. With the increase of weed species resistant to the most commonly used herbicides, new herbicide tolerance traits are needed in the field. Herbicides of particular interest are the aryloxyphenoxypropionate (AOPP) herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides. AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides provide control of a spectrum of glyphosate-resistant weeds, thus making a trait conferring tolerance these herbicides particularly useful in a cropping system combined with other herbicide tolerance trait(s).

[0007] The *Sphingobium herbicidovorans* strain MH isolated from a dichloroprop-degrading soil sample was identified as being capable of cleaving the ether bond of various phyenoxyalkanoic acid herbicides, utilizing this as its sole carbon and energy source for growth (HPE Kohler, Journal of Industrial Microbiology & Biotechnology (1999) 23:336-340). Catabolism of the herbicides is carried out by two different enantioselective alpha-ketoglutarate-dependent dioxygenases, RdpA (R-dichloroprop dioxygenase) and SdpA (S-dichloroprop dioxygenase). (A Westendorf, et al., Microbiological Research (2002) 157:317-322; Westendorf, et al., Acta Biotechnologica (2003) 23(1):3-17). RdpA has been isolated from *Sphingobium herbicidovorans* (GenBank Accessions AF516752 (DNA) and AAM90965 (protein)) and *Delftia acidovorans* (GenBank Accessions NG_036924 (DNA) and YP_009083283 (protein)) (TA Mueller, et al., Applied and Environmental Microbiology (2004) 70 (10): 6066-6075.) The RdpA and SdpA genes have been used for plant transformation to confer herbicide tolerance to crops (TR Wright, et al., Proceedings of the National Academy of Sciences USA, (2010) 107(47):20240-5). Improving the activity of the RdpA enzyme using protein engineering techniques to create a protein for use in transgenic plants would permit higher rates of herbicide application, thus improving transgenic crop safety and weed control measures.

BRIEF SUMMARY OF THE INVENTION

[0008] The invention provides a polypeptide that has at least about 92% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52. In one embodiment, the polypeptide has oxygenase activity against at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

[0009] The invention provides a recombinant DNA molecule comprising a nucleic acid sequence encoding a polypeptide that has at least about 92% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52. In one embodiment, the recombinant DNA molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2, 3, 5, 6, 8, 10, 12, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41, 42, 44, 45, and 53-59. In another embodiment, the recombinant DNA molecule encodes a polypeptide with oxygenase activity against at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides. In another embodiment, the recombinant DNA molecule is operably linked to a heterologous promoter functional in a plant cell. In another embodiment, the recombinant DNA molecule is operably linked to a DNA molecule encoding a chloroplast transit peptide that functions to localize an operably linked polypeptide within a cell.

[0010] The invention provides a DNA construct comprising a heterologous promoter functional in a plant cell operably linked to a recombinant DNA molecule comprising a nucleic acid sequence encoding a polypeptide that has at least about 92% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52. In one embodiment, the recombinant DNA molecule is operably linked to a DNA molecule encoding a chloroplast transit peptide that functions to localize an operably linked polypeptide within a cell. In another embodiment, the recombinant DNA molecule encodes a polypeptide that has an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52 and the expression of the polypeptide in a transgenic plant confers herbicide tolerance to the plant. In another embodiment, the DNA construct is present in the genome of a transgenic plant.

[0011] The invention provides a transgenic plant, seed, cell, or plant part comprising a recombinant DNA molecule comprising a nucleic acid sequence encoding a polypeptide that has at least about 92% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52. In one embodiment, the transgenic plant, seed, cell, or plant part comprises a transgenic trait for tolerance to at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides. In another embodiment, the transgenic plant, seed, cell, or plant part comprises a DNA construct of the

invention. In another embodiment, the transgenic plant, seed, cell, or plant part comprises a polypeptide of the invention.

[0012] The invention provides a method for conferring herbicide tolerance to a plant, seed, cell, or plant part comprising expressing in the plant, seed, cell, or plant part a polypeptide of the invention. In one embodiment, the method for conferring herbicide tolerance is used with a transgenic plant, seed, cell, or plant part that comprises a transgenic trait comprising a recombinant DNA molecule of the invention. In one embodiment, the method for conferring herbicide tolerance is used with an herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

[0013] The invention provides a method of plant transformation, comprising introducing a DNA construct of the invention into a plant cell and regenerating a plant therefrom that comprises the DNA construct and is tolerant to at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides. In one embodiment, the method of plant transformation includes crossing the regenerated plant with itself or with a second plant and collecting seed from the cross.

[0014] The invention provides a method for controlling weeds in a plant growth area by contacting a plant growth area comprising a transgenic plant or seed of the invention with at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides, where the transgenic plant or seed is tolerant to the herbicide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIGS. **1**A-**1**C. Control and MON-HT55 (SEQ ID NO:11) transgenic maize plants following treatment with quizalofop-P. FIG. **1**A shows control and transgenic maize plants either untreated or treated with 1× quizalofop-P (0.08 lb ai/acre). FIG. **1**B shows F1 hybrid control maize plants and FIG. **1**C shows F1 hybrid MON-HT55 transgenic maize plants. Plants were grown at daytime/night-time temperatures of (1) 20° C./20° C., (2) 28° C./20° C., or (3) 38° C./30° C. prior to being sprayed with 2× quizalofop-P.

[0016] FIGS. **2**A-**2**C. Graphs showing temperature-dependent activity of engineered proteins. FIG. **2**A shows activity of MON-HT55 (SEQ ID NO:11) and the wild-type RdpA enzyme when tested with quizalofop-P as the substrate. FIG. **2**B shows activity of MON-HT1 (SEQ ID NO:14), MON-HT2 (SEQ ID NO:18), MON-HT7 (SEQ ID NO:34), MON-HT8 (SEQ ID NO:37) and the wild-type RdpA when tested with quizalofop-P as the substrate. FIG. **2**C shows activity of MON-HT1, MON-HT2, MON-HT7, MON-HT8 and the wild-type RdpA when tested with 2,4-D as the substrate. Data are normalized to the activity of each protein at 25° C.

[0017] FIG. **3**. The protein sequence of wild-type RdpA (SEQ ID NO:60) with exemplary amino acid positions useful for protein engineering boxed.

[0018] FIGS. **4**A-**4**E. The average injury rating after 2× quizalofop-P (0.16 lb ai/acre) (**4**A, **4**B, and **4**C) or 4× 2,4-D (4 lb ae/acre) (**4**D and **4**E) application to F1 hybrid maize plants (homozygous R1 expressing MON-HT×MON89034 inbred) expressing MON-HT55 (SEQ ID NO:11), MON-HT1 (SEQ ID NO:14), MON-HT2 (SEQ ID NO:18), MON-HT3 (SEQ ID NO:22), MON-HT4 (SEQ ID NO:25), MON-HT7 (SEQ ID NO:34), or F1 hybrid control (NK603×MON89034). Data from plants acclimated at daytime and night time temperatures set at 20° C. (20° C./20° C.) prior to application of 2× quizalofop-P (FIG. **4**A) or 4× 2,4-D (FIG. **4**D); FIG. **4**B shows data from plants acclimated at daytime temperature of 28° C. and night time temperature of 20° C. (28° C./20° C.) prior to application of 2× quizalofop-P; FIG. **4**C shows data from plants acclimated at daytime temperature of 38° C. and night time temperature of 30° C. (38° C./30° C.) prior to application of 2× quizalofop-P (FIG. **4**C) or 4× 2,4-D (FIG. **4**E).

[0019] FIGS. **5**A-**5**B. FIG. **5**A and FIG. **5**B: Control and transgenic maize plants comprising MON-HT2 (SEQ ID NO:20, encoding SEQ ID NO:18) with a CTP or without a CTP where the plants received quizalofop-P at 16× rates (1.28 lb ai/acre) applied at V2 followed by V4 and the photos taken 10 to 14 days after quizalofop-P application.

[0020] FIGS. **6**A-**6**E. Multi-sequence alignment of protein sequences for wild-type RdpA from *S.herbicidovorans* (SEQ ID NO:60) and SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52 with the consensus sequence (provided as SEQ ID NO:61) provided at the bottom of each of FIGS. **6**A, **6**B, **6**C, **6**D, **6**E.

BRIEF DESCRIPTION OF THE SEQUENCES

[0021] SEQ ID NO:1-3 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT51.

[0022] SEQ ID NO:4-6 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT52.

[0023] SEQ ID NO:7-8 are the amino acid sequence and bacterial codon polynucleotide sequence of MON-HT53.

[0024] SEQ ID NO:9-10 are the amino acid sequence and bacterial codon polynucleotide sequence of MON-HT54.

[0025] SEQ ID NO:11-13 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT55.

[0026] SEQ ID NO:14-17 are the amino acid sequence, bacterial codon polynucleotide sequence, monocot codon optimized polynucleotide sequence, and dicot codon optimized polynucleotide sequence of MON-HT1.

[0027] SEQ ID NO:18-21 are the amino acid sequence, bacterial codon polynucleotide sequence, monocot codon optimized polynucleotide sequence, and dicot codon optimized polynucleotide sequence of MON-HT2.

[0028] SEQ ID NO:22-24 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT3.

[0029] SEQ ID NO:25-27 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT4.

[0030] SEQ ID NO:28-30 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT5.

[0031] SEQ ID NO:31-33 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT6.

[0032] SEQ ID NO:34-36 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT7.

[0033] SEQ ID NO:37-39 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT8.

[0034] SEQ ID NO:40-42 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT9.

[0035] SEQ ID NO:43-45 are the amino acid sequence, bacterial codon polynucleotide sequence, and monocot codon optimized polynucleotide sequence of MON-HT10.

[0036] SEQ ID NO:46-52 are the amino acid sequences of MON-HT11, MON-HT13, MON-HT14, MON-HT15, MON-HT16, MON-HT17, and MON-HT18.

[0037] SEQ ID NO:53-59 are the dicot codon optimized polynucleotide sequences of MON-HT11, MON-HT13, MON-HT14, MON-HT15, MON-HT16, MON-HT17, and MON-HT18.

[0038] SEQ ID NO:60 is the amino acid sequence for wild-type RdpA from *Sphingobium herbicidovorans*.

[0039] SEQ ID NO:61 is the consensus sequence of FIG. **6**A-FIG. **6**E.

DETAILED DESCRIPTION OF THE INVENTION

[0040] The following definitions and methods are provided to better define the invention and to guide those of ordinary skill in the art in the practice of the invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

[0041] The invention overcomes the limitations of the prior art by providing novel, engineered proteins, referred to herein as MON-HT proteins, and the recombinant DNA molecules that encode them as well as compositions and methods using these. The MON-HT proteins are oxygenases that can inactivate aryloxyphenoxypropionate (AOPP) herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides. As used herein, inactivating an herbicide means making the herbicide no longer have its herbicidal activity against a plant. The MON-HT proteins exhibit novel substrate selectivity, useful enzyme kinetics, and increased enzyme stability at elevated temperature. Transgenic plants expressing a MON-HT protein demonstrate improved tolerance to application of AOPP, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

Engineered Proteins and Recombinant DNA Molecules

[0042] The invention provides novel, engineered proteins and the recombinant DNA molecules that encode them. As used herein, the term "engineered" refers to a non-natural DNA, protein, or organism that would not normally be found in nature and was created by human intervention. An "engineered protein" is a protein whose polypeptide sequence was conceived of and created in the laboratory using one or more of the techniques of protein engineering, such as protein design using site-directed mutagenesis and directed evolution using random mutagenesis and DNA shuffling. For example, an engineered protein may have one or more deletions, insertions, or substitutions relative to the coding sequence of the wild-type protein and each deletion, insertion, or substitution may consist of one or more amino acids. Examples of engineered proteins are provided herein as SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52.

[0043] Engineered proteins provided by the invention are enzymes that have oxygenase activity. As used herein, the term "oxygenase activity" means the ability to oxidize a substrate by transferring the oxygen from molecular oxygen to the substrate, co-product, or an intermediary. The oxygenase activity of the engineered proteins provided by the invention can inactivate one or more of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

[0044] As used herein, "wild-type" means naturally-occurring. As used herein, a "wild-type DNA molecule", "wild-type polypeptide", or a "wild-type protein" is a naturally-occurring DNA molecule, polypeptide, or protein, that is, a DNA molecule, polypeptide, or protein pre-existing in nature. A wild-type version of a polypeptide, protein, or DNA molecule may be useful for comparison with an engineered protein or gene. An example of a wild-type protein useful for comparison with the engineered proteins provided by the invention is the RdpA enzyme from *Sphingobium herbicidovorans* strain MH. An example of a wild-type DNA molecule useful for comparison with the recombinant DNA molecules provided by the invention is the RdpA gene from *Sphingobium herbicidovorans* strain MH. A wild-type version of a protein or DNA molecule may be useful as a control in an experiment.

[0045] As used herein, "control" means an experimental control designed for comparison purposes. For example, a control plant in a transgenic plant analysis is a plant of the same type as the experimental plant (that its, the plant to be tested) but does not contain the transgenic insert, recombinant DNA molecule, or DNA construct of the experimental plant. An example of a control plant useful for comparison with transgenic maize plants is non-transgenic LH244 maize (U.S. Pat. No. 6,252,148) and with transgenic soy plants is non-transgenic A3555 soybean (U.S. Pat. No. 7,700,846).

[0046] As used herein, the term "recombinant" refers to a non-natural DNA, polypeptide, or protein that is the result of genetic engineering and as such would not normally be found in nature and was created by human intervention. A "recombinant DNA molecule" is a DNA molecule comprising a DNA sequence that does not naturally occur and as such is the result of human intervention, for example, a DNA molecule that encodes an engineered protein. Another example is a DNA molecule comprised of a combination of at least two DNA molecules heterologous to each other, such as a protein-coding DNA molecule and an operably linked heterologous promoter. An example of a recombinant DNA molecule is a DNA molecule comprising at least one sequence selected from SEQ ID NO:2, 3, 5, 6, 8, 10, 12, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41, 42, 44, 45 and 53-59. A "recombinant polypeptide" or "recombinant protein" is a polypeptide or protein comprising an amino acid sequence that does not naturally occur and as such is the result of human intervention, for example, an engineered protein.

[0047] The term "transgene" refers to a DNA molecule artificially incorporated into the genome of an organism as a result of human intervention, such as by plant transformation methods. As used herein, the term "transgenic" means comprising a transgene, for example a "transgenic plant" refers to a plant comprising a transgene in its genome and a "transgenic trait" refers to a characteristic or phenotype conveyed or conferred by the presence of a transgene incorporated into the plant genome. As a result of such genomic alteration, the transgenic plant is something distinctly different from the related wild-type plant and the transgenic trait is a trait not naturally found in the wild-type plant. Transgenic plants of the invention comprise the recombinant DNA molecules and engineered proteins provided by the invention.

[0048] As used herein, the term "heterologous" refers to the relationship between two or more things derived from different sources and thus not normally associated in nature. For example, a protein-coding recombinant DNA molecule is heterologous with respect to an operably linked promoter if

such a combination is not normally found in nature. In addition, a particular recombinant DNA molecule may be heterologous with respect to a cell or organism into which it is inserted when it would not naturally occur in that particular cell or organism.

[0049] As used herein, the term "protein-coding DNA molecule" or "polypeptide-coding DNA molecule" refers to a DNA molecule comprising a nucleotide sequence that encodes a protein or polypeptide. A "protein-coding sequence" or "polypeptide-coding sequence" means a DNA sequence that encodes a protein or polypeptide. A "sequence" means a sequential arrangement of nucleotides or amino acids. The boundaries of a protein-coding sequence or polypeptide-coding sequence are usually determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus A protein-coding molecule or polypeptide-coding molecule may comprise a DNA sequence encoding a protein or polypeptide sequence. As used herein, "transgene expression", "expressing a transgene", "protein expression", "polypeptide expression", "expressing a protein", and "expressing a polypeptide" mean the production of a protein or polypeptide through the process of transcribing a DNA molecule into messenger RNA (mRNA) and translating the mRNA into polypeptide chains, which may be ultimately folded into proteins. A protein-coding DNA molecule or polypeptide-coding DNA molecule may be operably linked to a heterologous promoter in a DNA construct for use in expressing the protein or polypeptide in a cell transformed with the recombinant DNA molecule. As used herein, "operably linked" means two DNA molecules linked in manner so that one may affect the function of the other. Operably-linked DNA molecules may be part of a single contiguous molecule and may or may not be adjacent. For example, a promoter is operably linked with a protein-coding DNA molecule or polypeptide-coding DNA molecule in a DNA construct where the two DNA molecules are so arranged that the promoter may affect the expression of the transgene.

[0050] As used herein, a "DNA construct" is a recombinant DNA molecule comprising two or more heterologous DNA sequences. DNA constructs are useful for transgene expression and may be comprised in vectors and plasmids. DNA constructs may be used in vectors for the purpose of transformation, that is the introduction of heterologous DNA into a host cell, in order to produce transgenic plants and cells, and as such may also be contained in the plastid DNA or genomic DNA of a transgenic plant, seed, cell, or plant part. As used herein, a "vector" means any recombinant DNA molecule that may be used for the purpose of plant transformation. Recombinant DNA molecules as set forth in the sequence listing, can, for example, be inserted into a vector as part of a construct having the recombinant DNA molecule operably linked to a promoter that functions in a plant to drive expression of the engineered protein encoded by the recombinant DNA molecule. Methods for constructing DNA constructs and vectors are well known in the art. The components for a DNA construct, or a vector comprising a DNA construct, generally include, but are not limited to, one or more of the following: a suitable promoter for the expression of an operably linked DNA, an operably linked protein-coding DNA molecule, and a 3' untranslated region (3'-UTR). Promoters useful in practicing the present invention include those that function in a plant for expression of an operably linked polynucleotide. Such promoters are varied and well known in the art and include those that are inducible, viral, synthetic, constitutive, temporally regulated, spatially regulated, and/or spatio-temporally regulated. Additional optional components include, but are not limited to, one or more of the following elements: 5'-UTR, enhancer, leader, cis-acting element, intron, chloroplast transit peptides (CTP), and one or more selectable marker transgenes.

[0051] The DNA constructs of the invention may include a CTP molecule operably linked to the protein-coding DNA molecules provided by the invention. A CTP useful in practicing the present invention includes those that function to facilitate localization of the engineered protein molecule within the cell. By facilitating protein localization within the cell, the CTP may increase the accumulation of engineered protein, protect it from proteolytic degradation, enhance the level of herbicide tolerance, and thereby reduce levels of injury after herbicide application. CTP molecules for use in the present invention are known in the art and include, but are not limited to the *Arabidopsis thaliana* EPSPS CTP (Klee et al., 1987), the *Petunia hybrida* EPSPS CTP (della-Cioppa et al., 1986), the maize cab-m7 signal sequence (Becker et al., 1992; PCT WO 97/41228) and the pea glutathione reductase signal sequence (Creissen et al., 1991; PCT WO 97/41228).

[0052] Recombinant DNA molecules of the present invention may be synthesized and modified by methods known in the art, either completely or in part, especially where it is desirable to provide sequences useful for DNA manipulation (such as restriction enzyme recognition sites or recombination-based cloning sites), plant-preferred sequences (such as plant-codon usage or Kozak consensus sequences), or sequences useful for DNA construct design (such as spacer or linker sequences). The present invention includes recombinant DNA molecules and engineered proteins having at least about 80% (percent) sequence identity, about 85% sequence identity, about 90% sequence identity, about 91% sequence identity, about 92% sequence identity, about 93% sequence identity, about 94% sequence identity, about 95% sequence identity, about 96% sequence identity, about 97% sequence identity, about 98% sequence identity, and about 99% sequence identity to any of the recombinant DNA molecule or engineered protein sequences provided herein, for instance, to a recombinant DNA molecule comprising a sequence selected from the group consisting of SEQ ID NO:2, 3, 5, 6, 8, 10, 12, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41, 42, 44, 45, and 53-59. As used herein, the term "percent sequence identity" or "% sequence identity" refers to the percentage of identical nucleotides or amino acids in a linear polynucleotide or polypeptide sequence of a reference ("query") sequence (or its complementary strand) as compared to a test ("subject") sequence (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide or amino acid insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the Sequence Analysis software package of the GCG® Wisconsin Package® (Accelrys Inc., San Diego, Calif.), MEGA1ign (DNAStar, Inc., 1228 S. Park St., Madison, Wis. 53715), and MUSCLE (version 3.6) (RC

Edgar, Nucleic Acids Research (2004) 32(5):1792-1797) with default parameters. An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, that is, the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more sequences may be to a full-length sequence or a portion thereof, or to a longer sequence.

[0053] Engineered proteins may be produced by changing (that is, modifying) a wild-type protein to produce a new protein with a novel combination of useful protein characteristics, such as altered Vmax, Km, substrate specificity, substrate selectivity, and protein stability. Modifications may be made at specific amino acid positions in a protein and may be a substitution of the amino acid found at that position in nature (that is, in the wild-type protein) with a different amino acid. Exemplary amino acid positions relative to the protein sequence of wild-type protein RdpA (SEQ ID NO:60) useful for protein engineering are depicted in FIG. **3**. FIGS. **6**A, **6**B, **6**C, **6**D, **6**E provide a multi-sequence alignment of the wild-type RdpA protein sequence and engineered protein sequences SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52. An engineered protein can be designed that has at least about 92% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52 and comprises at least one of these amino acid mutations. Engineered proteins provided by the invention thus provide a new protein with one or more altered protein characteristics relative to the wild-type protein found in nature. In one embodiment of the invention, an engineered protein has altered protein characteristics such as improved or decreased activity against one or more herbicides or improved protein stability as compared to a similar wild-type protein, or any combination of such characteristics. In one embodiment, the invention provides an engineered protein, and the recombinant DNA molecule encoding it, having at least about 80% sequence identity, about 85% sequence identity, about 90% sequence identity, about 91% sequence identity, about 92% sequence identity, about 93% sequence identity, about 94% sequence identity, about 95% sequence identity, about 96% sequence identity, about 97% sequence identity, about 98% sequence identity, and about 99% sequence identity to an engineered protein sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52 Amino acid mutations may be made as a single amino acid substitution in the protein or in combination with one or more other mutation(s), such as one or more other amino acid substitution(s), deletions, or additions. Mutations may be made as described herein or by any other method known to those of skill in the art.

Transgenic Plants

[0054] An aspect of the invention includes transgenic plant cells, transgenic plant tissues, transgenic plants, and transgenic seeds that comprise the recombinant DNA molecules and engineered proteins provided by the invention. These cells, tissues, plants, and seeds comprising the recombinant DNA molecules and engineered proteins exhibit herbicide tolerance to one or more of aryloxyphenoxypropionate (AOPP) herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

[0055] Suitable methods for transformation of host plant cells for use with the current invention include virtually any method by which DNA can be introduced into a cell (for example, where a recombinant DNA construct is stably integrated into a plant chromosome) and are well known in the art. An exemplary and widely utilized method for introducing a recombinant DNA construct into plants is the *Agrobacterium* transformation system, which is well known to those of skill in the art. Transgenic plants can be regenerated from a transformed plant cell by the methods of plant cell culture. A transgenic plant homozygous with respect to a transgene (that is, two allelic copies of the transgene) can be obtained by self-pollinating (selfing) a transgenic plant that contains a single transgene allele with itself, for example an R0 plant, to produce R1 seed. One fourth of the R1 seed produced will be homozygous with respect to the transgene. Plants grown from germinating R1 seed can be tested for zygosity, typically using a SNP assay, DNA sequencing, or a thermal amplification assay that allows for the distinction between heterozygotes and homozygotes, referred to as a zygosity assay.

[0056] Plants, seeds, plant parts, plant tissues, and cells provided by the invention exhibit herbicide tolerance to one or more of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxyl acid herbicides. AOPP herbicides target the plant's acetyl-coenzyme A carboxylase (ACCase), which is part of the fatty acid biosynthetic pathway. Grass plants are sensitive to these herbicides because they contain herbicide-sensitive ACCase in their plastids and cytosol. AOPP herbicides are well-known in the art and commercially available. Examples of AOPP herbicides include, but are not limited to, clodinafop, cyhalofop, diclofop, fenoxaprop, fenoxaprop-P, fenthiaprop, fluazifop, fluazifop-P, haloxyfop, isoxapyrifop, metamifop, propaquizafop, quizalofop, quizalofop-P, and trifop. The phenoxy acid and pyridinyloxy acid herbicides are synthetic auxins similar to the plant growth hormone indoleacetic acid (IAA). Broad-leaf plants are sensitive to these herbicides, which induce rapid, uncontrolled growth, eventually killing the plant. Examples of phenoxy acid herbicides include, but are not limited to, 2,4-D; 2,4-DB; clomeprop; dichlorprop; fenoprop; MCPA; MCPB, and mecoprop. Examples of pyridinyloxy acid herbicides include, but are not limited to, triclopyr; fluroxypyr; aminopyralid, clopyralid, and picloram.

[0057] Herbicides may be applied to a plant growth area comprising the plants and seeds provided by the invention as a method for controlling weeds. Plants and seeds provided by the invention comprise an herbicide tolerance trait and as such are tolerant to the application of one or more AOPP herbicides, or phenoxy acid herbicides, or pyridinyloxyl acid herbicides. The herbicide application may be the recommended commercial rate (1×) or any fraction or multiple thereof, such as twice the recommended commercial rate (2×). Herbicide rates may be expressed as acid equivalent per pound per acre (lb ae/acre) or pounds active ingredient per acre (lb ai/acre). The herbicide application comprises at least one herbicide selected from the group consisting of AOPP herbicides, and phenoxy acid herbicides, and pyridinyloxy acid herbicides. The plant growth area may or may not comprise weed plants at the time of herbicide application. An herbicidally effective dose of AOPP herbicides for use in the area for controlling weeds should consist of a range from

[redacted] COR-AAD1-00385719

about 0.01 lb ai/acre to about 16 lb ai/acre over a growing season. For example, a 1× rate of quizalofop-P would be a rate of 0.08 lb ai/acre. An herbicidally effective dose of phenoxy acid herbicides for use in the area for controlling weeds should consist of a range from about 0.01 lb ae/acre to about 16 lb ae/acre over a growing season. For example, a 1× rate of 2,4-D would be a rate of about 0.75 lb ae/acre to 1.0 lb ae/acre. An herbicidally effective dose of pyridinyloxy acid herbicides for use in the area for controlling weeds should consist of a range from about 0.01 lb ae/acre to about 16 lb ae/acre over a growing season. For example, a 1× rate of fluroxypyr would be a rate of about 0.13 to 0.48 lb ae/acre.

[0058] The herbicide application may be sequentially or tank mixed with one, two, or a combination of several AOPP herbicides, phenoxy acid herbicides, pyridinyloxy acid herbicides, or any other compatible herbicide. Multiple applications of one herbicide or of two or more herbicides, in combination or alone, may be used over a growing season to areas comprising transgenic plants of the invention for the control of a broad spectrum of dicot weeds, monocot weeds, or both, for example, two applications (such as a pre-planting application and a post-emergence application or a pre-emergence application and a post-emergence application) or three applications (such as a pre-planting application, a pre-emergence application, and a post-emergence application or a pre-emergence application and two post-emergence applications).

[0059] As used herein, "tolerance" or "herbicide tolerance" means a plant, seed, plant tissue, plant part, or cell's ability to resist the toxic effects of one or more herbicide(s). The herbicide tolerance of a plant, seed, plant tissue, plant part, or cell may be measured by comparing the plant, seed, plant tissue, plant part, or cell to a suitable control. For example, the herbicide tolerance may be measured or assessed by applying an herbicide to a plant comprising a recombinant DNA molecule encoding a protein capable of conferring herbicide tolerance (the test plant) and a plant not comprising the recombinant DNA molecule encoding the protein capable of conferring herbicide tolerance (the control plant) and then comparing the plant injury of the two plants, where herbicide tolerance of the test plant is indicated by a decreased injury rate as compared to the injury rate of the control plant. An herbicide tolerant plant, seed, plant tissue, plant part, or cells exhibits a decreased response to the toxic effects of an herbicide when compared to a control plant, seed, plant tissue, plant part, or cell. As used herein, an "herbicide tolerance trait" is a transgenic trait imparting improved herbicide tolerance to a plant as compared to a wild-type plant or control plant.

[0060] The transgenic plants, progeny, seeds, plant cells, and plant parts of the invention may also contain one or more additional transgenic traits. Additional transgenic traits may be introduced by crossing a plant containing a transgene comprising the recombinant DNA molecules provided by the invention with another plant containing an additional transgenic trait(s). As used herein, "crossing" means breeding two individual plants to produce a progeny plant. Two transgenic plants may thus be crossed to produce progeny that contain the transgenic traits. As used herein "progeny" means the offspring of any generation of a parent plant, and transgenic progeny comprise a DNA construct provided by the invention and inherited from at least one parent plant. Alternatively, additional transgenic trait(s) may be introduced by co-transforming a DNA construct for that additional transgenic trait (s) with a DNA construct comprising the recombinant DNA molecules provided by the invention (for example, with all the DNA constructs present as part of the same vector used for plant transformation) or by inserting the additional trait(s) into a transgenic plant comprising a DNA construct provided by the invention or vice versa (for example, by using any of the methods of plant transformation on a transgenic plant or plant cell). Such additional transgenic traits include, but are not limited to, increased insect resistance, increased water use efficiency, increased yield performance, increased drought resistance, increased seed quality, improved nutritional quality, hybrid seed production, and herbicide tolerance, in which the trait is measured with respect to a wild-type plant or control plant. Such additional transgenic traits are known to one of skill in the art; for example, a list of such traits is provided the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) and can be found on their website at www. aphis. usda.gov.

[0061] Transgenic plants and progeny that contain a transgenic trait provided by the invention may be used with any breeding methods that are commonly known in the art. In plant lines comprising two or more transgenic traits, the transgenic traits may be independently segregating, linked, or a combination of both in plant lines comprising three or more transgenic traits. Back-crossing to a parental plant and outcrossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of breeding methods that are commonly used for different traits and crops are well known to those of skill in the art. To confirm the presence of the transgene(s) in a particular plant or seed, a variety of assays may be performed. Such assays include, for example, molecular biology assays, such as Southern and northern blotting, PCR, and DNA sequencing; biochemical assays, such as detecting the presence of a protein product, for example, by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole plant.

[0062] Introgression of a transgenic trait into a plant genotype is achieved as the result of the process of backcross conversion. A plant genotype into which a transgenic trait has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired transgenic trait may be referred to as an unconverted genotype, line, inbred, or hybrid.

[0063] As used herein, the term "comprising" means "including but not limited to".

EXAMPLES

[0064] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit, and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein with the same or similar result achieved. All such similar substitutes and modi-

fications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

Example 1

Initial Protein Engineering and Enzyme Analysis

[0065] Novel, engineered proteins and recombinant DNA molecules encoding these proteins were conceived of and created in the laboratory using the techniques of protein engineering. The engineered proteins are enzymes that have oxygenase activity and were engineered to have altered abilities to inactivate AOPP herbicides, phenoxy acid herbicides, or both relative to the wild-type protein.

[0066] Sixteen known proteins with oxygenase activity were selected and used to create a consensus homology sequence alignment. This was used in combination with structure-guided analyses to inform rational design strategies. From these analyses, five regions each from 13 to 21 amino acids in length, referred to herein as "islands", were selected for mutatagenesis. Mutations in each of these regions were created using techniques known to those of skill in the art, such as Alanine-Scanning Mutations; Homology-Scanning Mutations; Pro/Gly Scanning Mutations; Region Swaps or Mutations; and combinations of these various techniques (see, M Lehmann and M Wyss, Current Opinion in Biotechnology (2001) 12(4):371-375; B Van den Burg and VGH Eijsink, Current Opinion in Biotechnology (2002) 13(4):333-337; and Weiss et al., Proc Natl Acad Sci U S A (2000) 97(16):8950-8954). Using these methods, more than 1,200 unique engineered proteins and the recombinant DNA molecules that encode them were generated for further analysis and characterization. Because of the large number of engineered proteins produced for testing, and the need to test and compare the enzymatic activity of each protein, a high-throughput bacterial protein expression and enzyme assay system was developed for rapid analysis using crude bacterial extracts.

[0067] The high-throughput bacterial protein expression was achieved by synthesizing a recombinant DNA molecule encoding each engineered protein and cloning this into a bacterial expression vector with a C-terminal histidine tag (His-tag) operably linked to the recombinant DNA molecule. The vectors were used to transform *Escherichia coli* (*E. coli*), and bacterial expression of the engineered proteins was induced. Overnight *E. coli* cultures were grown in 96-well plates and the cultures were centrifuged to pellet the bacteria. The bacterial pellets were lysed by adding 100 ul lysis master mix (10 ml of Bacteria Protein Extraction Reagent (B-PER®) II (Pierce Biotechnology, Rockford, Ill.; cat. no. 78260); 10 ul Lysozyme (10 ug/ml final; Lysozyme American Bioanalytical, Natick, Mass.; cat. no. AB011780-00005); and 40 ul Benzonase® Nuclease (100 Units/ml final, Novagen, Darmstadt, Germany; cat no 71206-3)) to each well. The plates were vortexed then incubated for 30 minutes at 4° C. 400 ul of MOPS buffer (pH 6.57) was added to each well and the debris pelleted by centrifugation. The lysate supernatant was carefully removed and used as the crude bacterial extract for subsequent enzymatic analysis.

[0068] The high-throughput herbicide degradation enzyme assay was designed to assay the enzymatic activity of the engineered proteins towards various herbicides using crude bacterial extract. The engineered protein's oxygenase activity (that is, its enzymatic activity) was measured by an end point colorimetric assay utilizing detection of phenol products through measurement of absorbance at 510 nm from 4-aminoantipyrine and potassium ferricyanide. This assay was based on the assay described in Fukomori and Hausinger, Journal of Biological Chemistry (1993) 268(32):24311-24317. Enzyme reactions were assayed in 96-well plates in a total volume of 150 ul that contained: 20 mM MOPS pH 6.75, 50-200 uM NH4FeSO4, 50-200 uM sodium ascorbate, 1 mM alpha-ketoglutarate (aKG), 10 ul of *E. coli* cell lysate containing expressed engineered protein, and substrate (either an AOPP herbicide or phenoxy acid herbicide). Upon initiation of the reaction with substrate, the plate was incubated at various temperatures for various times and quenched (terminated) by the addition of EDTA to a final concentration of 6.25 mM or by the addition of 15 ul of pH 10 buffer (50 mM boric acid, 50 mM KC1) followed by 15 ul of 0.2% 4-aminoantipyrine and 15 ul of 0.8% potassium ferricyanide. Absorbance measurements were made on a standard laboratory spectrometer. Assays were scaled as needed to enhance throughput. Standard curves were generated using purified protein or product standards.

[0069] Using this high-throughput bacterial protein expression and enzyme assay system, activities of approximately 1200 engineered proteins were measured relative to the activity of a selected wild-type protein, RdpA. In a 96 well plate assay, there were 3 controls (crude bacterial extract without an engineered protein) and 3 positive controls (crude bacterial extract with wild-type protein). The absorbance of the wells was measured and the protein activity was calculated using the following formula:

$$Activity_i = \left(\frac{\left(Absorbance_i - Absorbance_{pET}\right)}{\left(Absorbance_{WT} - Absorbance_{pET}\right)} \right) \times 100$$

where, Activity, is the activity of the sample, Absorbance, is the absorbance of the sample, $Absorbance_{WT}$ is the absorbance of the wells containing extract from *E. coli* expressing the wild-type enzyme, and $Absorbance_{pET}$ is the absorbance of the wells containing extract from *E. coli* without an engineered protein. Activity for each unique engineered protein was measured in duplicate and was reported as an average of the two measurements.

[0070] Based on the results from the high-throughput enzyme assay system, approximately 545 unique engineered proteins were selected for further analysis using purified engineered protein. In the purified engineered protein prep assay, crude bacterial expression lysates were prepared using QUIAGEN® Ni-NTA Agarose (Qiagen, Valencia, Calif., cat. no. 30230) following manufacturer's protocol.

[0071] The purified engineered proteins were assayed using the herbicide degradation enzyme assay described in this Example 1 with the AOPP herbicide quizalofop as the substrate. The results of the purified engineered protein assays largely confirmed the results from the high-throughput enzyme assay. Assay results for seven of the approximately 545 engineered proteins are shown in Table 1, where the enzymatic activity is expressed as the activity of the sample relative to the activity of the wild-type RdpA enzyme (calculated as described in this Example 1). These data from these assays yielded the surprising result that combinations of specific mutations performed significantly better than others and demonstrated that the enzymatic activity of the engineered proteins could be altered significantly.

TABLE 1

Replicate Number	Island 1-2-3	Island 1-2-5	Island 1-3-4	Island 2-3-4	Island 2-3-5	Island 2-4-5	Island 3-4-5
1	−5.94	−4.14	−4.86	−2.84	138.99	48.12	4.06
2	−3.54	−2.64	−3.9	−0.05	117.67	48.84	5.56
3		−3.85			140.75		
4		−0.65			113.77		
5		−0.05			150.58		
6		2.14			141.27		

[0072] Using the information learned from the first assays, protein engineering was then done as described previously to produce additional engineered proteins, which were tested as described in this Example 1. The results of the high-throughput enzyme assay with quizalofop-P as the substrate for five of these additional engineered proteins are provided in Table 2.

TABLE 2

SEQ ID NO	MON-HT	Replicate 1	Replicate 2	Replicate 3	Average quizalofop-P activity
1	MON-HT51	117.02	124.87	110.17	117.35
4	MON-HT52	92.17	54.46	89.75	78.79
7	MON-HT53	105.34	105.31	106.13	105.60
9	MON-HT54	131.85	149.77	130.56	137.39
11	MON-HT55	118.48	99.79		109.13

[0073] Further protein characterization, such as Km, Vmax, and crystal structure analysis, was performed using the five engineered proteins from Table 2. For this detailed analysis, purified protein was prepared as follows: 2 ml overnight cultures of *E. coli* expressing a transgene encoding a given MON-HT protein were used to inoculate 500 ml of broth and grown at 37° C. for 4 hours followed by culture at 15° C. for approximately 36 hours. Then 250 ml of the 500 ml bacterial culture was pelleted by centrifugation and resuspended in 25 ml of extraction buffer (20 mM Tris, pH 7.8, 300 mM NaCl, 5 mM beta-mercaptoethanol (BME), 20 mM imidazole (Fluka/Sigma-Aldrich, St. Louis, Mo.), 125 units/ml of benzonase and 10K units/ml of lysozyme (Novagen, Darmstadt, Germany) The cell slurry was passed through a cell disruptor once at 20000 psi and then this cell lysate was clarified by centrifugation at 35,000×g for 20 min at 4° C. The supernatant containing the soluble His-tagged proteins was used for protein purification. For this purification, the supernatant was applied to a 1 ml HisTrapTM FF column (Nickel Sepharose) (GE Healthcare, Piscataway, N.J.) using an AKTaxpress™ system (GE Healthcare, Piscataway, N.J.) following the standard manufacturer's protocol. The wash buffer consisted of: 20 mM Tris pH 7.8, 300 mM NaCl, 20 mM imidazole and 5 mM BME. The composition of elution buffer was the same as the wash buffer except with 500 mM imidazole. The eluate from the nickel column was desalted on a Quick Spin Protein Sephadex G-25 fine column (Roche Applied Science, Indianapolis, Ind.) following the manufacturer's protocol. The eluted protein was in buffer consisting of: 20 mM Tris pH 7.8, 50 mM NaCl and 5 mM BME. Protein extract purity was assessed by SDS-PAGE analysis. Protein concentration was determined by Bradford assay using Bio-Rad Protein Assay dye reagent (Biorad, Hercules, Calif., cat no 500-0006).

[0074] Purified protein for the five engineered proteins was analyzed using the enzyme assay described in this Example 1, but with four different AOPP herbicides as substrates: quizalofop-P, haloxyfop, fenoxaprop, and fluazifop. Standard curves were generated using 2,4-dichlorophenol (2,4-DCP), which was used to produce a general phenol standard curve. The amount of phenol generated in the assay by the engineered proteins was calculated based on this standard curve. The controls were purified wild-type enzyme, no enzyme, and no substrate. Enzyme kinetic measurements of the five engineered proteins was done using 0, 20, 40, 80, 160, 320, 640, or 1280 μM of quizalofop-P, haloxyfop, fenoxaprop, or fluazifop herbicides. Table 3 shows the Km and Vmax (expressed as relative values) measured for the five proteins with the four AOPP herbicide substrates. The protein characteristics of these five engineered proteins with each of the four AOPP herbicides as substrates demonstrated that the enzymatic activity, namely Km and Vmax, of the engineered proteins could be altered significantly through protein engineering.

TABLE 3

	Quizalofop-P		Haloxyfop		Fenoxaprop		Fluazifop	
	Km (uM)	Vmax	Km (uM)	Vmax	Km (uM)	Vmax	Km (uM)	Vmax
MON-HT51	570	120	250	48	870	180	4.0	12
MON-HT52	420	39	280	45	490	420	14	43
MON-HT53	690	95	290	64	1100	640	1.0	40
MON-HT54	560	32	380	40	2100	630	ND	ND
MON-HT55	250	320	450	190	200	450	50	320

Example 2

Expression of Engineered Proteins in Maize

[0075] Plant transformation vectors were constructed each comprising a recombinant DNA molecule encoding one of three engineered proteins with the protein-coding sequence optimized for monocot expression, MON-HT51 (SEQ ID NO:3), MON-HT52 (SEQ ID NO:6), and MON-HT55 (SEQ

[redacted] COR-AAD1-00385722

ID NO:13). The vectors were created using different combinations of promoter, leader, intron, and 3'UTR and with and without a CTP operably linked to the protein-coding sequence. Also included in the vectors was a second DNA cassette comprising a cp4-EPSPS coding sequence to be used in transgenic plants for glyphosate tolerance. Immature maize (LH244) embryos were transformed with these vectors using *Agrobacterium tumifaciens* and standard methods known in the art. Regenerated R0 transgenic plantlets were grown in the green house and sprayed at approximately V2-V4 growth stage with 0.04 or 0.08 lb ai/acre quizalofop-P (Assure™ II, E.I. DuPont), representing 0.5× and 1× rates, respectively. Leaf samples were used to identify transgenic plants with a single copy of the transgenic DNA insert (that is, single event plants). R0 plants that contained only a single copy and passed either 0.5× or 1× quizalofop-P spray testing were selfed to produce R1 seed. No events were obtained with the constructs containing MON-HT52. Only one event was regenerated from the construct containing MON-HT51 with a CTP and from the construct containing MON-HT51 without a CTP. Two pairs of vectors containing MON-HT55 were transformed, with each pair differing only in containing a CTP or not containing a CTP.

[0076] R1 plants expressing MON-HT55 with and without an operably linked CTP were grown in the green house and quizalofop-P herbicide was applied at the V2 growth stage at a rate of 0.08 lb ae/acre (1×). Plants were evaluated for injury eleven days after treatment. The R1 plants were segregating for the trait in typical Mendelian ratio, and the expected numbers (~25%) of null segregants (progeny plants not containing the transgenic trait) were seen that did not survive the herbicide treatment. All R1 transgenic plants expressing MON-HT55 with an operably linked CTP, with the exception of those representing one event, showed only minor chlorotic speckling on the youngest exposed leaves following application of quizalofop-P. No injury scores over 5% were recorded for these plants after herbicide application. The unsprayed transgenic plants also did not differ phenotypically from the unsprayed control plants. FIG. **1**A shows control LH244 plants and transgenic plants comprising MON-HT55 (SEQ ID NO:13) 18 days after application of quizalofop-P.

[0077] To assess the effect of using a CTP to target the engineered protein to the plant cell chloroplast, transgenic plants comprising a transgene insert with and without a CTP operably linked to the protein-coding sequence were compared. The plants comprising a CTP operably linked to the protein-coding sequence showed better tolerance to quizalofop-P compared to those without a CTP. In the R1 green house testing described in this Example 2, most of the transgenic plants comprising a CTP operably linked to the protein-coding sequence showed complete quizalofop-P tolerance. The plants not comprising a CTP operably linked to the protein-coding sequence showed quizalofop-P tolerance but with some moderate injury phenotypes. These results demonstrated that the use of a CTP to target the engineered protein to the plant cell chloroplast enhanced the transgenic plant's quizalofop-P tolerance. This unexpected finding was tested again in trait efficacy field trials with R1 plants comprising either MON-HT51 with or without a CTP operably linked to the protein-coding sequence or MON-HT55 with or without a CTP operably linked to the protein-coding sequence. These R1 plants were single-copy, but were still segregating. In this field trial, seed was planted in the field and treated as follows: 2× (0.16 lb ai/acre) quizalofop-P at pre-plant, 2× (0.14 lb ai/acre) haloxyfop at V4 growth stage, then 2× quizalofop-P at V8 growth stage. A higher percentage of plants comprising a CTP operably linked to the protein-coding sequence survived quizalofop-P and haloxyfop applications and had lower injury scores compared to plants not comprising a CTP operably linked to the protein-coding sequence. Data are provided in Table 4. This confirmed the unexpected finding that a CTP operably linked to the protein-coding sequence confers higher plant tolerance to herbicide application for the engineered proteins.

TABLE 4

Protein	CTP	Unique events	Average % Herbicide Injury
MON-HT51	No	1	95%
MON-HT51	Yes	1	35%
MON-HT55	No	12	49%
MON-HT55	Yes	3	25%
MON-HT55	No	2	90%
MON-HT55	Yes	7	24%

[0078] Inbred trait efficacy field trials were conducted to assess tolerance to the AOPP herbicides and sensitivity to the cyclohexanediones (CHD) herbicides in an inbred background. R2 inbred plants were generated by selfing a homozygous transgenic R1 plant and collecting seed. R2 inbred plants containing MON-HT55 with or without a CTP or MON-HT51 with a CTP and were evaluated at two field locations. Herbicide treatment was 2× quizalofop-P at 0.16 lb ai/acre applied PRE (after planting but before emergence) followed by quizalofop-P at 0.16 lb ai/acre applied at V4 growth stage followed by quizalofop-P at 0.16 lb ai/acre applied at V8 growth stage. Plots were rated for crop injury 7-10 days after herbicide application on a scale of 0-100 with zero being no injury and 100 being complete crop death. All data were subjected to analysis of variance and means separated at LSD (0.05). Most of the inbred R1 plants showed no injury, confirming that both MON-HT55 and MON-HT51, with or without a CTP, confer quizalofop-P tolerance to maize To test for sensitivity to CHD herbicides, which is desirable for use in volunteer control, plants were treated with a 1× rate of clethodim (0.25 lb ai/acre) at V8 growth stage. Volunteer control using a 1× rate of clethodim was 100% effective for all transgenic plants tested. Hybrid trait efficacy field trials were conducted to assess tolerance to the AOPP herbicides and sensitivity to the cyclohexanediones (CHD) herbicides in a hybrid background. F1 hybrid plants were produced by crossing an R1 inbred plant with a non-transgenic plant and collecting seed. The resulting F1 plants containing MON-HT55 (SEQ ID NO:13) with or without a CTP or MON-HT51 (SEQ ID NO:3) without a CTP were evaluated at six field locations. The hybrid trait efficacy field trials were conducted at six locations under a range of environmental conditions, including high heat and drought conditions during the field season. This permitted the engineered protein to be evaluated in maize under high temperature and water stress conditions. Data are provided in Table 5. Initial injury from 2× applications of quizalofop-P were higher than desired (>10% injury) at 7-10 days after application. Plants eventually grew out of most of the injury, with generally less injury from the V8 growth stage application as compared to the V4 growth stage application. Excessive injury was also noted when quizalofop-P was applied very early (for example, at VE-V2 growth stage).

TABLE 5

Protein	CTP	Unique events evaluated	% injury after V4 spray	% injury after V8 spray
MON-HT51	Yes	4	51.8	33.3
MON-HT55	No	9	46.8	78.8
MON-HT55	Yes	17	42.6-48.3	14.2-38.8

[0079] The finding that hybrid plants expressing the engineered proteins MON-HT55 or MON-HT51 were sensitive to quizalofop-P application when grown in field conditions of high temperature was confirmed using a plant-based assay. The plant-based assay was designed to test tolerance to quizalofop-P of F1 hybrids in the growth chamber prior to field testing. The assay was developed using F1 hybrids of plants containing maize events NK603 (U.S. Pat. No. 8,273,959)× MON89034 (U.S. Pat. No. 8,581,047) and F1 hybrids of plants containing MON-HT55×MON89034. F1 hybrid seed was germinated in a growth chamber for 1 week and then moved to one of three different growth chambers to acclimate for two days at day/night temperatures of 20° C./20° C., 28° C/20° C., and 38° C./30° C. prior to application of 2× (0.16 lb ai/acre) quizalofop-P. As expected, the plants not containing MON-HT55 at all temperature regimens were severely injured by 2× quizalofop-P application (FIG. **1**B). The transgenic plants containing MON-HT55 showed good tolerance to the 2× quizalofop-P application when acclimated to day/night temperatures of 20° C./20° C. or 28° C./20° C., but showed significant sensitivity when acclimated to day/night temperatures of 38° C./30° C. (FIG. **1**C). This confirmed that the plant-based assay could be used to screen proteins in plants in the growth chamber for temperature-sensitive activity.

[0080] The data demonstrated that the engineered proteins could be expressed in transgenic plants to confer herbicide tolerance and that unsprayed transgenic plants did not differ phenotypically from the unsprayed control plants. The data also confirmed that expression of the engineered proteins in plants permitted use of CHD herbicides for volunteer control. Unexpectedly, the data showed that use of a CTP for chloroplast targeting of the engineered protein enhanced the herbicide tolerance trait and that the herbicide tolerance provided by the engineered proteins was temperature-sensitive, decreasing under high temperature conditions.

Example 3

Optimizing Engineered Proteins

[0081] The finding that hybrid events expressing the engineered proteins MON-HT55 or MON-HT51 were sensitive to quizalofop-P application when grown in field conditions of high temperature was surprising and provided an additional protein characteristic capable of being altered through protein engineering. A new series of in vitro enzyme assays and a plant-based enzyme activity assay were developed to test proteins for sensitivity to high temperatures.

[0082] To create engineered proteins optimized for activity in higher temperatures, the protein motif analysis used in the first two rounds of protein engineering was combined with crystal structure data for several of the engineered proteins. This was used to inform additional rounds of mutagenesis, performed as described previously, and approximately 1400 additional engineered proteins were thus generated. These were combined with the approximately 1200 engineered proteins described in Example 1 for a total of approximately 2600 engineered proteins for screening with the new temperature sensitivity assay to identify proteins optimized for activity in higher temperatures.

[0083] To analyze these engineered proteins for activity in higher temperatures, the in vitro enzyme assay in Example 1 was modified to specify pre-heating all of the assay components to the desired temperature for 5 minutes before combining the components and then maintaining the reaction at the desired temperature for the duration of the reaction. To normalize the assay measurements, quizalofop-P was used as the substrate for the reaction and enzyme activity was normalized based on the 25° C. readings. Using these parameters, the temperature at which the enzyme activity was half of maximum ($T_{1/2}$) was calculated. The $T_{1/2}$ for MON-HT55 was calculated to be 29° C., and the $T_{1/2}$ for wild-type RdpA was calculated to be 38° C. (FIG. **2**A).

[0084] Because of the large number of variants to test, a five-level screening process was used. Table 6 shows the approximate number of variants tested in the different screenings.

TABLE 6

	Engineered proteins tested
First screen	~2600
Second screen	~1250
Third screen	94
Fourth screen	47
Fifth screen	11

[0085] The first screen was performed with approximately 2600 engineered proteins and used the high-throughput bacterial protein expression and enzyme assay system with crude bacterial lysates as described in Example 1 but modified to be conducted at the desired temperatures of 25° C. and 40° C. with the post-quenching color development done at 25° C. From this screen, approximately 1250 engineered proteins were selected and advanced. The second screen was similar, but included protein normalization across samples. From this screen, 94 engineered proteins were selected and advanced. The third screen used purified protein with the herbicide degradation enzyme assay as described in Example 1, but modified to be conducted at the desired temperatures of 25° C. and 40° C. with the post-quenching color development done at 25° C. From this screen, 47 engineered proteins were selected and advanced. The fourth screen was done using purified protein, the protein concentrations were normalized, and the screening included quizalofop-P and (for a subset of the proteins variants) 2,4-D as substrates with end-point assays done at 23° C. and 40° C. From this screen, thirteen engineered proteins were selected and advanced.

[0086] For the fifth screen, recombinant protein for each of the eleven engineered proteins was produced and purified for an in-depth biochemical analysis. This biochemical analysis included: (1) kinetic analysis (Vmax and Km), (2) activity assays over a range of temperatures, (3) protein melting analysis, (4) activity on additional AOPP herbicides substrates, and (5) mass spectrometry analysis on peptides to confirm identity. The biochemical analyses were also done with purified recombinant wild-type protein and MON-HT55 protein for comparison. For the kinetic analysis, a non-end-point assay was conducted at 23° C. with either quizalofop-P or 2,4-D as substrate. The recombinant proteins for these

assays were produced in bacteria and purified using a 6-His tag fused at the C-terminal end of the protein. Results of the kinetic analysis with either quizalofop-P or 2,4-D as substrate are presented for ten of the engineered proteins, wild-type protein, and MON-HT55 protein in Table 7 (standard error is shown in parenthetical). Vmax is expressed as specific activity, umol herbicide product mg enzyme-1 min-1; Km expressed as mM herbicide substrate. NDB indicates that enzyme activity may be evident at higher concentrations of herbicide, but activity under the concentrations tested was not robust enough to provide proper kinetic characterization. For MON-HT55, low activity levels (Vmax) with 2,4-D as the substrate resulted in low confidence in the reported value. MON-HT7 had a Vmax with quizalofop-P that is approximately 40% greater than the wild-type enzyme and a Vmax for 2,4-D that is only about half of that of the wild-type enzyme. MON-HT1 had a Vmax for quizalofop-P that is about half that of the wild-type enzyme and a Vmax for 2,4-D that is 9.5-fold higher than that of the wild-type enzyme. The differentiation of the protein kinetics for MON-HT1 and MON-HT7 were surprising because there are only four amino acid differences between MON-HT1 and MON-HT7. Specifically, MON-HT1 has the following amino acids at the indicated position: I82; F105; T112; and V273, and MON-HT7 has the following amino acids at the indicated position: L82; V105; S112; and A273.

TABLE 7

Protein	Vmax Quizalofop	Km Quizalofop	Vmax 2,4-D	Km 2,4-D
MON-HT55 (SEQ ID NO: 11)	0.44 (−0.04)	0.32 (−0.07)	0.03 (−0.02)	0.69 (−0.66)
MON-HT1 (SEQ ID NO: 14)	0.76 (−0.04)	0.17 (−0.03)	0.95 (−0.03)	0.03 (0.00)
MON-HT2 (SEQ ID NO: 18)	1.41 (−0.11)	0.41 (−0.06)	0.23 (−0.01)	0.04 (−0.01)
MON-HT3 (SEQ ID NO: 22)	0.77 (−0.04)	0.38 (−0.05)	0.10 (−0.01)	0.27 (−0.06)
MON-HT4 (SEQ ID NO: 25)	0.30 (−0.02)	0.24 (−0.05)	NDB	NDB
MON-HT5 (SEQ ID NO: 28)	0.57 (−0.03)	0.17 (−0.03)	NDB	NDB
MON-HT6 (SEQ ID NO: 31)	0.39 (−0.03)	0.10 (−0.03)	NDB	NDB
MON-HT7 (SEQ ID NO: 34)	1.94 (−0.12)	0.33 (−0.05)	0.04 (−0.01)	0.06 (−0.04)
MON-HT8 (SEQ ID NO: 37)	0.46 (−0.02)	0.11 (−0.02)	0.11 (−0.01)	0.14 (−0.05)
MON-HT9 (SEQ ID NO: 40)	0.53 (−0.02)	0.10 (−0.01)	NDB	NDB
MON-HT10 (SEQ ID NO: 43)	0.78 (−0.05)	0.13 (−0.03)	NDB	NDB
Wild-type RdpA	1.38 (−0.06)	0.27 (−0.03)	0.10 (−0.01)	0.03 (−0.01)

[0087] Enzyme activity over a range of temperatures was analyzed in-depth for MON-HT1, MON-HT2, MON-HT7, and MON-HT8. These assays were conducted as described above. Quizalofop-P or 2,4-D was used as the substrate for these reactions and activity was normalized based on the activity of the wild-type enzyme at 25° C. The activity curves obtained are presented in FIG. **2**B (with quizalofop-P as the substrate) and FIG. **2**C (with 2,4-D as the substrate). Using quizalofop-P as the substrate, MON-HT55 was the most temperature sensitive, with a $T_{1/2}$ of 29° C. The wild-type enzyme had a $T_{1/2}$ of 38° C. MON-HT1 and MON-HT8 were less temperature sensitive than the wild-type enzyme with a $T_{1/2}$ of 42° C. and 41° C., respectively, at which temperatures the wild-type enzyme is 90% inactive. MON-HT2 and MON-HT7 were much less temperature sensitive with a $T_{1/2}$ of 46° C. and 47° C., respectively, at which temperatures the wild-type enzyme is completely inactive. When 2,4-D was used as the substrate, the wild-type enzyme had a $T_{1/2}$ of 36° C. MON-HT2, MON-HT7, and MON-HT8 were all slightly more temperature sensitive with a lower $T_{1/2}$ than the wild-type enzyme. MON-HT1 was slightly less temperature sensitive, with a $T_{1/2}$ about 1° C. higher than the wild-type enzyme.

[0088] A protein melting analysis was also conducted. For the protein melting determinations, purified enzyme was added to 96-well microtiter plates in standard storage buffer (30 mM Tris pH7.5, 150 mM NaCl) with or without 50 μM Fe2+ and 1.0 mM aKG. Protein unfolding was then detected with SYPRO® orange protein gel stain (Invitrogen™ catalog #S6651, Life Technologies, Grand Island, N.Y.) in a BioRad CFX96™ Real time PCR machine (BioRad, Hercules, Calif.) with readings taken between 10° C. to 95° C. in 0.5° C. steps. The $T_{1/2}$ (here, the temperature where 50% of the protein was unfolded) is shown in Table 8. The wild-type enzyme showed stabilization with 50 uM Fe2+ and 1.0 mM aKG. In contrast, 50 uM Fe2+ and 1.0 mM aKG had little effect on the stability of any of the engineered proteins. MON-HT55, MON-HT3, MON-HT4, MON-HT6, and MON-HT10 had melting temperatures in the range of 41° C. to 48° C., which is below the melting temperature of the wild-type enzyme. MON-HT1, MON-HT2, MON-HT5, MON-HT7, MON-HT8, and MON-HT9 had melting temperatures between 58° C. and 67° C., which is 8° C. to 17° C. higher than the wild-type enzyme. For MON-HT7 and MON-HT1, the difference in melting point was 11° C. in buffer without Fe2+ and aKG and 8° C. in buffer with Fe2+ and aKG. This was surprising since there are only four amino acid differences between the two enzymes. This data on the melting point of the enzymes confirms that the engineered proteins have been optimized for protein stability at higher temperatures. This data also matches the enzyme activity assay results for the proteins conducted at different temperatures.

TABLE 8

Protein	Buffer	Buffer plus Fe2+ and aKG
MON-HT55 (SEQ ID NO: 11)	41° C.	41° C.
MON-HT1 (SEQ ID NO: 14)	56° C.	59° C.
MON-HT2 (SEQ ID NO: 18)	55° C.	58° C.
MON-HT3 (SEQ ID NO: 22)	46° C.	48° C.
MON-HT4 (SEQ ID NO: 25)	43° C.	44° C.
MON-HT5 (SEQ ID NO: 28)	60° C.	61° C.
MON-HT6 (SEQ ID NO: 31)	44° C.	44° C.
MON-HT7 (SEQ ID NO: 34)	67° C.	67° C.
MON-HT8 (SEQ ID NO: 37)	61° C.	61° C.
MON-HT9 (SEQ ID NO: 40)	61° C.	60° C.
MON-HT10 (SEQ ID NO: 43)	44° C.	44° C.
Wild-type RdpA	42° C.	50° C.

[0089] The enzyme activity of MON-HT protein variants with haloxyfop, fenoxaprop, fluazifop, and dichlorprop as

substrates was determined using the enzyme activity assay conducted at 23° C. with purified enzyme. The activity was recorded as the maximum activity as a percentage of the wild-type enzyme's activity, which was set at 100%. Data are provided in Table 9. MON-HT55, MON-HT3, MON-HT4, MON-HT5, and MON-HT9 had maximum activities for all four substrates lower than, or equal to, the maximum activity of the wild-type enzyme with the same substrate. With haloxyfop as the substrate MON-HT1, MON-HT2, MON-HT7, and MON-HT10 had a maximum activity that was greater than that of the wild-type enzyme. With fenoxaprop as the substrate MON-HT1, MON-HT2, MON-HT6, MON-HT7, MON-HT8, and MON-HT10 had a maximum activity that was greater than that of the wild-type enzyme. With fluazifop as the substrate MON-HT2 and MON-HT7 had a maximum activity that was greater than that of the wild-type enzyme. With dichlorprop as the substrate MON-HT1, MON-HT7, and MON-HT8 had a maximum activity that was greater than that of the wild-type enzyme.

TABLE 9

Protein	Haloxyfop	Fenoxaprop	Fluazifop	Dichlorprop
MON-HT55 (SEQ ID NO: 11)	58	34	38	31
MON-HT1 (SEQ ID NO: 14)	134	175	67	175
MON-HT2 (SEQ ID NO: 18)	142	121	124	92
MON-HT3 (SEQ ID NO: 22)	67	83	60	100
MON-HT4 (SEQ ID NO: 25)	49	53	52	39
MON-HT5 (SEQ ID NO: 28)	100	92	46	36
MON-HT6 (SEQ ID NO: 31)	75	333	47	37
MON-HT7 (SEQ ID NO: 34)	193	210	161	210
MON-HT8 (SEQ ID NO: 37)	99	106	50	106
MON-HT9 (SEQ ID NO: 40)	91	67	41	19
MON-HT10 (SEQ ID NO: 43)	124	233	48	29
Wild-type RdpA	100	100	100	100

[0090] The enzyme identities were confirmed using mass spectroscopy. For this analysis, purified protein was separated on a PAGE gel and stained. The stained bands were then cut out, destained, and trypsin digested using standard protocols. Trypsin digested protein preparations were separated on a Dionox UltiMat® 3000 RSLCnano LC System (Thermo Scientific, Sunnyvale, Calif.) using a Thermo Scientific™ AQUASIL™ C-18 Javelin™ Guard column under standard conditions and injected for MS-MS analysis using a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Sunnyvale, Calif.).

[0091] To optimize proteins for increased activity in the presence of phenoxy acid herbicides, computational protein engineering was performed on the crystal structure for several of the engineered proteins. This was used to inform additional rounds of mutagenesis, performed as described previously but using the bacterial sequence, SEQ ID NO:15, encoding the protein sequence MON-HT1 (SEQ ID NO:14) as the starting sequence. Approximately 472 additional engineered proteins were generated. These were combined with the approximately 2600 engineered proteins described in Example 1 and Table 6 for a total of approximately 3072 engineered proteins to identify proteins optimized for activity in the presence of 2,4-D. The first screen of the new variants was the high-throughput (HTP) bacterial protein expression and enzyme assay system with crude bacterial lysates as described in Example 1, but modified to be conducted at the desired temperatures of 25° C. and 40° C. with the post-quenching color development done at 25° C. Following this HTP screen, approximately 34 engineered proteins were selected and advanced into screening with protein normalization across all of the samples. From this screen, 12 engineered proteins were selected and advanced to screening with purified protein assayed with the herbicide degradation enzyme assay as described in Example 1. Enzyme heat stability was assayed with limited protein melting assays. From this screen, 7 engineered proteins were selected and advanced for in plant testing. Three enzyme variants were selected for detailed characterization using purified protein, where the protein concentrations were normalized, and the screening included quizalofop-P and 2,4-D as substrates, as well as additional herbicides shown in Table 10 and Table 12 and protein melting characterization.

[0092] Kinetic analysis using a non-endpoint assay was conducted at 23° C. with either quizalofop-P or 2,4-D as substrate, as detailed above for purified protein of the wild-type enzyme, MON-HT1, MON-HT13, MON-HT15, and MON-HT17. The data demonstrate a significant and unexpected enhancement of enzymatic activity of MON-HT13, MON-HT15, and MON-HT17 relative to both the wild-type RdpA enzyme and the MON-HT1 enzyme when tested with 2,4-D as a substrate. Specifically, all three variants showed a roughly 2.5 to 3-fold increase in activity (Vmax) relative to MON-HT1. Enzymatic activity of MON-HT13, MON-HT15, and MON-HT17 variants with quizalofop as the substrate was roughly similar to the activity of MON-HT1. See Table 10.

TABLE 10

	Quizalofop		2,4-D	
Protein	Vmax	Km	Vmax	Km
Wild-type RdpA	2.76 (0.11)	0.09 (0.013)	0.25 (0.01)	0.13 (0.017)
MON-HT1 (SEQ ID NO: 14)	1.62 (0.05)	0.12 (0.012)	1.17 (0.01)	0.03 (0.002)
MON-HT13 (SEQ ID NO: 47)	1.51 (0.05)	0.12 (0.013)	3.38 (0.05)	0.08 (0.004)

TABLE 10-continued

	Quizalofop		2,4-D	
Protein	Vmax	Km	Vmax	Km
MON-HT15 (SEQ ID NO: 49)	1.52 (0.06)	0.12 (0.017)	3.53 (0.04)	0.07 (0.004)
MON-HT17 (SEQ ID NO: 51)	1.53 (0.05)	0.14 (0.014)	2.91 (0.04)	0.07 (0.004)

[0093] A protein melting analysis was conducted as detailed above. The melting temperatures of MON-HT13, MON-HT15, and MON-HT17 was similar to the melting temperature of MON-HT1 with $T_{1/2}$ in buffer in the range of 55-58° C., and with $T_{1/2}$ in buffer plus Fe2+ and aKG in the range of 60-62° C. These data indicate that the MON-HT13, MON-HT15, and MON-HT17 variants have a similar enzyme heat stability compared to MON-HT 1. This data on the melting point of the enzyme variants confirms that the engineered proteins have been optimized for protein stability at higher temperatures. See Table 11.

TABLE 11

	Melting temp ° C.	
Protein	Buffer	Buffer plus Fe2+ and aKG
Wild-type RdpA	43	53
MON-HT1 (SEQ ID NO: 14)	58	62
MON-HT13 (SEQ ID NO: 47)	57	62
MON-HT15 (SEQ ID NO: 49)	55	60
MON-HT17 (SEQ ID NO: 51)	57	61

[0094] The enzyme activity of MON-HT protein variants with triclopyr, fluroxypyr, MCPA, MCPB, mecoprop, as substrates was determined using the enzyme activity assay conducted at 23° C. with purified enzyme. The activity was recorded as the maximum activity as a percentage of the wild-type RdpA enzyme's activity, which was set at 100%. Data are provided in Table 12. For each of the proteins assayed (MON-HT1, MON-HT13, MON-HT15, and MON-HT17) with the herbicides triclopyr and fluroxypyr as the substrate, there was detectable activity, especially in the engineered variants, but activity was not robust enough to quantify. There was no detectable activity for each of the proteins assayed (MON-HT1, MON-HT13, MON-HT15, and MON-HT17) with the herbicide MCPB as the substrate. The enzymatic activity with mecoprop as a substrate was reduced for each of the MON-HT1, MON-HT13, MON-HT15, and MON-HT17 variants compared to the wild-type RdpA enzyme. An unexpected result was that enzymatic activity with MCPA as a substrate was roughly 6-fold greater for MON-HT1, and approximately 10-fold greater for MON-HT13, MON-HT15, and MON-HT17 compared to the wild-type RdpA enzyme. See Table 12.

TABLE 12

	Mecoprop		MCPA	
Protein	Vmax	Km	Vmax	Km
Wild-type RdpA	100	100	100	100
MON-HT1 (SEQ ID NO: 14)	63	142	600	21
MON-HT13 (SEQ ID NO: 47)	37	92	1800	68

TABLE 12-continued

	Mecoprop		MCPA	
Protein	Vmax	Km	Vmax	Km
MON-HT15 (SEQ ID NO: 49)	25	57	1300	59
MON-HT17 (SEQ ID NO: 51)	27	65	1400	62

Example 4

Expression of Optimized Engineered Proteins in Maize

[0095] Ten unique engineered proteins optimized for activity at higher temperatures were selected for maize transformation and analysis in plants. DNA constructs were produced for expressing these engineered proteins with codon usage optimized for monocot expression using methods known to those skilled in the art. Enhancers, promoters, leaders, introns, CTPs, and 3'UTRs were tested in various combinations with the engineered proteins in these DNA constructs. The DNA constructs were used to transform immature maize (LH244) embryos with these vectors using *Agrobacterium tumifaciens* and standard methods known in the art. Regenerated R0 transgenic plantlets were grown in the green house.

[0096] Transgenic R0 maize plants were screened by the application of quizalofop-P (2×) plus 2,4-D (2×) at 7 to 10 days following transplant into plugs (generally corresponding to V3-V4 growth stage). All constructs tested produced plants containing unique events that passed the R0 screen. The R0 plants were selfed to generate R1 homozygous seed and the R0 was also used as the male to cross with inbred plants containing maize event MON89034 to generate segregating F1 hybrid seed for efficacy field trials.

[0097] An efficacy field trial was conducted with segregating F1 hybrid plants, with 50% hemizygous and 50% null for the transgene. Tolerance to quizalofop-P (2×) plus 2,4-D (2×) was assessed using two herbicide application regimens: (1) quizalofop-P (Assure II) at 0.16 lb ai/acre (2×) plus 0.25% v/v non-ionic surfactant (NIS) applied at VE-V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage and (2) 2,4-D amine at 2 lb ae/acre (2×) plus quizalofop-P at 0.04 lb ai/acre (0.5×) plus 0.25% v/v NIS applied at VE-V2 growth stage followed by 2,4-D amine at 2 lb ae/acre (2×) plus 0.25% v/v NIS applied at V4 growth stage followed by the same at V8 growth stage. The 50% of the plants that were null for transgene were removed by the first quizalofop-P application at VE-V2 growth stage. Plots were visually rated 10-14 days after application for crop injury on a scale of 0 to 100 with "0" being none and "100" being complete crop destruction. Table 13 shows the average injury rating at V4 and V8 growth stages for both spray regimens. Injury ratings of <10% were considered very good tolerance and injury ratings of <20% were considered good to fair tolerance. The percentage injury rating with 2× application of 2,4-D at V8 growth stage ranged from a high of 40% to

a low of 0. Similarly, the percentage injury rating with 2× application of quizalofop-P at V8 growth stage ranged from a high of 90% to a low of 0. Variation in injury rating between plants expressing the same protein is likely due to variations in construct design or transgene insertion location. This data confirmed that plants expressing the engineered proteins exhibited tolerance to 2,4-D and quizalofop herbicide application at the 2× rate.

TABLE 13

Protein	CTP	% Injury from V4 Quizalofop	% Injury from V8 Quizalofop	% Injury from V4 2,4-D	% Injury from V8 2,4-D
MON-HT1	A	8.33	8.33	7.5	5.83
MON-HT1	None	7.14	7.86	12.86	10.71
MON-HT1	B	7.5	8.75	10	10
MON-HT1	C	0.45	0.91	14.09	11.36
MON-HT2	A	0	0	20	34.17
MON-HT2	None	0	0	20	33.75
MON-HT2	B	11.67	11.67	10.56	13.33
MON-HT2	C	14.33	14.33	12	12
MON-HT3	A	1.6	0.5	21.7	9.2
MON-HT4	A	6.1	0	22.3	12.3
MON-HT5	A	6.8	1.7	27.2	17.4
MON-HT6	A	8	1.7	27	12.7
MON-HT7	A	31.2	5	21.6	16.6
MON-HT8	A	1.25	1.25	10	10
MON-HT8	None	85	90	0	0
MON-HT8	B	9.17	8.33	12.5	11.67
MON-HT8	C	30	32.5	20	21.25
MON-HT9	A	5	5	20	40
MON-HT10	A	0	0	28	38.33
MON-HT3 + MON-HT1	A + none	6.67	5.56	20.56	18.89
MON-HT4 + MON-HT8	A + none	11.25	11.88	9.38	4.38

[0098] A plant-based enzyme activity assay for heat sensitivity was used to determine the effect of elevated growth temperatures on the herbicide tolerance of transgenic plants containing the optimized engineered proteins. To test for quizalofop-P tolerance at elevated growth temperatures, F1 hybrid (produced by crossing an R1 homozygous plant expressing one of the MON-HT proteins with inbred maize event MON89034) maize seed was grown in a growth chamber for 10 days at a day temperature of 28° C. and a night temperature of 20° C. with 50% humidity. After 10 days, the plants were moved to acclimate for 3 days at one of three different day and night temperature regimens: (1) both day and night temperatures set at 20° C.; (2) day temperature at 28° C. and night temperature at 20° C.; or (3) day temperature at 38° C. and night temperature at 30° C. At the end of the acclimation period, the plants were generally at V4 growth stage and were sprayed with 2× quizalofop-P. Ten days post treatment the plants were scored for injury on a rating scale of 1 to 5 where '0' is no visible injury observed, '1' is chlorotic speckling, '2' is chlorotic streaking, '3' there are leaf gaps or tears, '4' are plants with stunted growth and/or twisted leaves, and '5' are dead plants or no growth observed. Results are presented in FIG. **4**. F1 hybrid maize plants expressing MON-HT55 showed good tolerance (injury ratings of around 2) to the spray treatments when day/night temperatures were 20° C./20° C. (FIG. **4**A) or 28° C./20° C. (FIG. **4**B) relative to the F1 hybrid control plants (maize events NK603×MON89034) (injury rating of 5). When the day/night temperatures were 38° C./30° C. F1 hybrid control plants had an injury rating of 5, F1 hybrid plants expressing MON-HT55 had an average injury rating of 3, and F1 hybrid plants expressing MON-HT1, MON-HT2, MON-HT3, MON-HT4, or MON-HT7 had injury ratings of ≤1 (FIG. **4**C). The engineered proteins optimized for activity in higher temperatures provided AOPP herbicide tolerance when plants expressing these engineered proteins were exposed to high temperatures.

[0099] To test for 2,4-D tolerance at elevated growth temperatures, F1 hybrid plants were produced by crossing an R1 plant containing MON-HT1, MON-HT2, MON-HT3, MON-HT4, MON-HT7, or MON-HT55 with an inbred plant containing maize event MON89034. The F1 hybrid plants were grown in a greenhouse for one week at a minimum temperature of 20° C. and a maximum temperature of 28° C. with 50 to 80% humidity. After 1 week, the plants were moved to acclimate for three days at one of two different day and night temperature regimens: (1) both day and night temperatures set at 20° C. or (2) day temperature at 38° C. and night temperature at 30° C. At the end of the acclimation period, the plants were generally at V4 growth stage and were sprayed with 4× 2,4-D amine Ten days after treatment the plants were scored for injury using an injury scale of 0 to 100 with "0" being no injury and "100" being a dead plant. When plants were acclimated at day/night temperatures of 20° C./20° C. prior to application of 4× 2,4-D amine, F1 plants containing MON-HT1, MON-HT2, MON-HT3, MON-HT4, MON-HT7, or MON-HT55 had injury rating averages of <10%, and the control plants had injury rating averages of <20% (FIG. **4**D). When plants were acclimated at day/night temperatures of 38° C./30° C. prior to application of 4× 2,4-D amine, F1 plants containing MON-HT4 or MON-HT7 had injury rating averages of <20%, F1 plants containing MON-HT1, MON-HT2 or MON-HT3 had injury rating averages of <10% (FIG. **4**E), and the control plants and plants containing MON-HT55 F1 plants had injury rating averages of 50% (FIG. **4**E). These results demonstrated that the engineered proteins optimized for activity in higher temperatures provided 2,4-D herbicide tolerance when plants expressing these engineered proteins were exposed to high temperatures.

[0100] Separate trait efficacy field trials for quizalofop-P and 2,4-D were conducted at two locations each with F1 hybrid transgenic plants produced by crossing an inbred plant containing maize event MON89034 with an R1 plant containing MON-HT55 (with a CTP), MON-HT1 (with or without a CTP), MON-HT2 (with or without a CTP), MON-HT3 (with a CTP), MON-HT4 (with a CTP), MON-HT5 (with a CTP), MON-HT6 (with a CTP), or MON-HT7 (with a CTP). Transgenic F1 hybrid plants containing maize events NK603× MON89034 were used for comparison as a control.

[0101] In the efficacy field trial for quizalofop-P tolerance and clethodim sensitivity, one of four herbicide treatments was used: (1) quizalofop-P (Assure II) at 0.32 lb ai/acre (4×) plus 0.25% v/v non-ionic surfactant (NIS) applied at VE-V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage; (2) quizalofop-P at 0.64 lb ai/acre (8×) plus 0.25% v/v NIS applied at VE to V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage; (3) quizalofop-P at 1.28 lb ai/acre (16×) plus 0.25% v/v NIS applied at VE to V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage; or (4) clethodim at 0.25 lb ai/acre (1×) plus 0.25% v/v NIS applied at V8 growth stage. Plots were visually rated 10-14 days after application for crop injury on a scale of 0 to 100 with "0" being none and "100"

being complete crop destruction. Tables 14 and 15 show the average injury ratings after herbicide application at V4 or V8 growth stage, respectively.

[0102] Plants containing MON-HT1, MON-HT2, MON-HT3, MON-HT4, MON-HT5, MON-HT6, or MON-HT7 (all operably linked to a CTP) showed very good tolerance to quizalofop-P with injury ratings of less than 15% across all application rates and at both V4 and V8 growth stages. Plants containing MON-HT55 operably linked to a CTP showed moderate to poor tolerance with injury ratings from 0.8% to 78.8%. The injury ratings for the control plants after quizalofop-P application were 99.5%. These results indicate that plants containing MON-HT1, MON-HT2, MON-HT3, MON-HT4, MON-HT5, MON-HT6, or MON-HT7 operably linked to a CTP had very good tolerance to sequential applications of quizalofop-P.

[0103] Plants containing MON-HT1 or MON-HT2 with an operably linked CTP had better tolerance to quizalofop-P than plants containing MON-HT1 or MON-HT2 without an operably linked CTP. Plants containing MON-HT1 with an operably linked CTP had 0 to 5.5% injury rating across all quizalofop-P applications compared to the 3.3% to 18.8% injury ratings of plants containing MON-HT1 without an operably linked CTP. Plants containing MON-HT2 with an operably linked CTP had 1.5% to 10% injury rating across all quizalofop-P applications compared to the 16.3% to 82.5% injury ratings of plants containing MON-HT2 without an operably linked CTP. FIG. **5** shows plants containing MON-HT2 operably linked to a CTP (FIG. **5**A) and plants containing MON-HT2 without a CTP (FIG. **5**B) 10 to 14 days after quizalofop-P application (treatment 3) at 1.28 lb ai/acre (16×) plus 0.25% v/v NIS applied at VE to V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage. The control plants did not survive, the plants containing MON-HT2 without a CTP had moderate to poor tolerance, and the plants containing the MON-HT2 operably linked to a CTP had robust tolerance to the quizalofop-P application. These results confirmed that the use of an operably linked CTP greatly improves quizalofop-P tolerance.

[0104] All transgenic plants had injury ratings above 90% to an application of 1× clethodim (0.25 lb ai/acre) applied at the V8 growth stage, demonstrating the use of this herbicide for volunteer control in transgenic plants containing the engineered proteins.

[0105] In the efficacy field trial for 2,4-D tolerance, one of four herbicide treatments was used: (1) 2,4-D amine at 2 lb ae/acre (2×) plus 0.25% v/v non-ionic surfactant (NIS) applied to VE to V2 followed by V4 followed by V8; (2) 2,4-D amine at 4 lb ae/acre (4×) plus 0.25% v/v NIS applied to VE to V2 followed by V4 followed by V8; (3) 2,4-D amine at 8 lb ae/acre (8×) plus 0.25% v/v NIS applied to VE to V2 followed by V4 followed by V8 maize; or (4) 2,4-D amine at 16 lb ae/acre (16×) plus 0.25% v/v NIS applied to VE to V2 followed by V4 followed by V8. Plots were visually rated as above. Tables 14 and 15 show the average injury ratings after herbicide application at V4 or V8 growth stage, respectively.

[0106] Plants containing MON-HT1, MON-HT2, or MON-HT6 (all operably linked to a CTP) showed very good tolerance to 2,4-D with injury ratings of less than 10% and less than 17% across all application rates at V4 and V8 growth stages, respectively. Plants containing MON-HT3, MON-HT4, MON-HT5, or MON-HT7 (all operably linked to a CTP) showed good tolerance to 2,4-D with injury ratings of less than 20% and less than 22% across all application rates at V4 and V8 growth stages, respectively. Plants containing MON-HT55 operably linked to a CTP showed moderate to poor tolerance with injury ratings from 7.5% to 66%. The injury ratings for the control plants after 2,4-D application ranged from 40% to 82.2%. These results indicate that plants containing MON-HT1, MON-HT2, MON-HT3, MON-HT4, MON-HT5, MON-HT6, or MON-HT7 had good tolerance to sequential applications of 2,4-D.

[0107] Plants containing MON-HT1 or MON-HT2 with an operably linked CTP in general did not show the marked differences in tolerance to 2,4-D compared to plants containing MON-HT1 or MON-HT2 without an operably linked CTP as was seen with the quizalofop-P application. Plants containing MON-HT1 with an operably linked CTP had 0 to 16.3% injury rating across all 2,4-D applications compared to the 0 to 13.8% injury ratings of plants containing MON-HT1 without an operably linked CTP. Plants containing MON-HT2 with an operably linked CTP had 1.3% to 15% injury rating across all 2,4-D applications compared to the 1.3% to 21.3% injury ratings of plants containing MON-HT2 without an operably linked CTP. However, the difference was notable after 2,4-D application at V4 growth stage for plants containing MON-HT2 with an operably linked CTP (4.5% injury rating at 8× and 7.5% at 16×) compared to plants without the CTP (13.75% injury rating at 8× and 21.25% at 16×).

TABLE 14

V4 Growth Stage	2x 2,4-D	4x 2,4-D	8x 2,4-D	16x 2,4-D	4x Quizalofop	8x Quizalofop	16x Quizalofop
Control	52.5	64.4	73.8	74.7	99.5	99.5	99.5
MON-HT55	30.0	41.3	43.8	66.3	37.5	67.5	77.5
MON-HT55	20.0	23.1	50.0	55.0	12.5	43.8	62.5
MON-HT55	15.0	30.0	58.8	53.8	2.0	7.5	20.0
MON-HT55	12.5	30.0	48.8	52.5	8.0	18.8	48.8
MON-HT55	13.8	31.3	45.0	61.3	6.0	10.0	21.3
MON-HT55	n/a	n/a	n/a	n/a	0.8	13.8	16.3
MON-HT55	7.5	22.5	53.8	63.8	5.5	12.5	13.8
MON-HT1 (no CTP)	0.0	0.0	5.8	12.5	5.8	11.3	18.8
MON-HT1	0.0	0.0	5.0	6.3	0.0	0.8	4.5
MON-HT2 (no CTP)	1.3	2.5	13.8	21.3	42.5	76.3	82.5
MON-HT2	1.3	0.0	4.5	7.5	1.5	0.8	10.0
MON-HT3	3.8	6.3	8.8	11.3	0.8	0.0	4.3
MON-HT4	0.0	1.3	8.8	18.8	2.8	3.3	13.8
MON-HT5	2.5	2.5	7.5	17.5	1.3	4.5	4.5

[redacted]

TABLE 14-continued

V4 Growth Stage	2x 2,4-D	4x 2,4-D	8x 2,4-D	16x 2,4-D	4x Quizalofop	8x Quizalofop	16x Quizalofop
MON-HT6	0.0	2.0	5.8	8.8	0.0	1.3	2.8
MON-HT7	1.3	1.3	5.8	12.5	0.8	1.3	3.3

TABLE 15

V8 Growth Stage	1x Clethodim	2x 2,4-D	4x 2,4-D	8x 2,4-D	16x 2,4-D	4x Quizalofop	8x Quizalofop	16x Quizalofop
Control	93.6	40.0	52.8	64.1	82.2	99.5	99.5	99.5
MON-HT55	93.3	16.3	28.8	36.3	55.0	25.0	45.0	78.8
MON-HT55	93.3	14.4	20.0	39.4	56.9	10.0	31.3	41.3
MON-HT55	93.3	11.3	16.3	30.0	47.5	3.8	3.8	16.3
MON-HT55	93.3	11.3	20.0	35.0	47.5	3.8	20.0	38.8
MON-HT55	94.5	15.0	18.8	33.8	57.5	4.3	5.0	18.8
MON-HT55	93.3	n/a	n/a	n/a	n/a	2.5	6.3	18.8
MON-HT55	94.5	12.5	13.8	36.3	52.5	2.0	5.5	11.8
MON-HT1 (no CTP)	94.5	2.5	4.0	8.8	13.8	3.3	7.5	16.3
MON-HT1	93.3	1.3	1.5	8.3	16.3	2.0	2.0	5.5
MON-HT2 (no CTP)	92.0	5.0	8.8	13.8	25.0	16.3	52.5	77.5
MON-HT2	93.3	2.5	3.3	10.0	15.0	2.5	3.8	4.8
MON-HT3	94.5	5.5	5.5	11.3	13.8	2.5	2.0	6.0
MON-HT4	94.5	2.0	8.8	12.5	21.3	3.3	3.3	8.0
MON-HT5	92.0	3.3	4.0	10.0	21.3	2.5	0.0	8.0
MON-HT6	93.3	6.3	4.3	11.3	12.5	1.3	2.0	5.0
MON-HT7	93.3	2.0	2.8	9.3	20.0	1.5	0.8	5.5

Example 5

Evaluation of Chloroplast Targeting Peptides on Expression of Optimized Engineered Proteins in Maize

[0108] To evaluate different chloroplast targeting peptides (CTP), plant transformation vectors were constructed, each comprising a recombinant DNA molecule optimized for monocot expression and encoding MON-HT1 (SEQ ID NO:16), MON-HT2 (SEQ ID NO:20), and MON-HT8 (SEQ ID NO:39), MON-HT9 (SEQ ID NO:42), or MON-HT10 (SEQ ID NO:45). The vectors were created using the same combination of promoter, leader, intron, and 3'-UTR, but with one of three separate CTPs (A, B, or C) or without a CTP operably linked to the protein-coding sequence. See Table 16. The DNA constructs were used to transform immature maize (LH244) embryos using *Agrobacterium tumifaciens* and standard methods known in the art. Regenerated R0 transgenic plantlets were grown in the green house. The R0 plants were selfed to generate R1 homozygous seed. The R0 plants were also used as the male to cross with inbred plants containing maize event MON89034 to generate segregating F1 hybrid seed for trait efficacy field trials.

[0109] Separate trait efficacy field trials for quizalofop-P and 2,4-D were conducted at two locations each with the homozygous inbred transgenic plants (R2 or R4 generation). In these field trials, one of two herbicide treatments was used: (1) quizalofop-P (Assure II) at 0.16 lb ai/acre (2×) plus 0.25% v/v non-ionic surfactant (NIS) applied at V4 growth stage followed by the same at V8 growth stage; or (2) 2,4-D amine at 2 lb ae/acre (2×) plus 0.25% v/v non-ionic surfactant (NIS) applied to V4 followed by V8. Injury ratings (crop injury percentage at V4 (CIPV4) or V8 (CIPV8)) were taken 10 to 14 days after the V4 and V8 applications. Error was calculated using LSD (0.05). The results demonstrated that these plants had tolerance to 2× sequential applications of either quizalofop-P or 2,4-D with injury ratings below 10% following V4 and V8 applications. See Table 16.

TABLE 16

		2X Quizalofop-P		2X 2,4-D	
Protein	CTP	CIPV4 LSD (0.05) = 1.45	CIPV8 LSD (0.05) = 2.2	CIPV4 LSD (0.05) = 0.95	CIPV8 LSD (0.05) = 1.6
MON-HT1	A	0	2.125	0	2.5
MON-HT1	B	0	1.75	0	1.25
MON-HT1	C	0	1.75	1.25	1.25
MON-HT1	no CTP	0	1.25	0	1.25
MON-HT2	A	0	1.875	0.875	1.25
MON-HT2	B	0	0	0	2.5
MON-HT2	C	0	2	0	1.25
MON-HT2	no CTP	1.25	3.5	0	2.5
MON-HT8	A	0	1.25	1.75	1.25
MON-HT8	B	0.75	2.5	0	1.25
MON-HT8	C	0	1.25	1.25	0
MON-HT9	A	0	0	3.75	1.25
MON-HT10	A	0	0	0	1.25

[0110] Leaf samples were collected from the plants containing transgene cassettes encoding MON-HT1, MON-HT2, and MON-HT8 with and without CTP sequences to determine expression of the mRNA transcribed from the transgene cassette encoding the engineered proteins. Quantigene® analysis was done on the leaf sample extracts to determine mRNA expression of the transgene cassette. For these assays, the probe was to the common 3'-UTR sequence present in each expression cassette used to generate the transgenic plants. Relative expression was calculated by normal-

izing to maize housekeeping genes. A leaf sample was collected from each of eight plants for each construct configuration used to make the transgenic plants, and the reported relative mRNA expression data is an average of the eight samples with standard error.

[0111] Plants containing the transgene construct encoding either MON-HT1 (SEQ ID NO:14) or MON-HT2 (SEQ ID NO:18) had higher relative transgene mRNA expression for constructs containing either the 'A' or 'B' CTP than for constructs without a CTP or with the 'C' CTP. Plants containing the transgene construct encoding MON-HT8 (SEQ ID NO:37) had similar high relative transgene mRNA expression for constructs containing any of the three CTPs (A, B, or C). See Table17.

TABLE 17

MON-HT variant	CTP	Relative Expression	Standard Error
MON-HT1	A	7.80	1.20
MON-HT1	B	5.95	1.63
MON-HT1	C	3.82	0.44
MON-HT1	no CTP	4.31	0.68
MON-HT2	A	6.96	0.66
MON-HT2	B	6.16	0.65
MON-HT2	C	4.11	0.29
MON-HT2	no CTP	4.66	0.45
MON-HT8	A	8.06	0.48
MON-HT8	B	7.62	0.46
MON-HT8	C	4.87	0.47
LH244 Control	none	0.00	0.00

[0112] Separate trait efficacy field trials for quizalofop-P and 2,4-D pressure screening were conducted at one location each with F1 hybrid transgenic plants produced by crossing an inbred plant containing maize event MON89034 with an R1 plant containing MON-HT1, MON-HT2, MON-HT8, MON-HT9, and MON-HT10 with and without operably linked CTP sequences. Transgenic F1 hybrid plants containing maize events NK603×MON89034 were used for comparison as a control.

[0113] In the trait efficacy field trial for quizalofop-P tolerance, one of three herbicide treatments was used: (1) quizalofop-P (Assure II) at 0.32 lb ai/acre (4×) plus 0.25% v/v non-ionic surfactant (NIS) applied at VE-V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage; (2) quizalofop-P at 0.64 lb ai/acre (8×) plus 0.25% v/v NIS applied at VE-V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage; or (3) quizalofop-P at 1.28 lb ai/acre (16×) plus 0.25% v/v NIS applied at VE-V2 growth stage followed by the same at V4 growth stage followed by the same at V8 growth stage. Plots were visually rated as above. Table 18 shows the average injury ratings after herbicide application at V4 (CIPV4) or V8 (CIPV8) growth stage, respectively. The injury rating for the control plants after all quizalofop-P application was 100%. Error was calculated using LSD (0.05).

TABLE 18

		4X Qizalofop-P		8X Qizalofop-P		16X Qizalofop-P	
MON-HT variant	CTP	CIPV4 LSD (0.05) = 1.92	CIPV8 LSD (0.05) = 2.3	CIPV4 LSD (0.05) = 4.7	CIPV8 LSD (0.05) = 5.3	CIPV4 LSD (0.05) = 5.0	CIPV8 LSD (0.05) = 5.4
none	none	100	100	100	100	100	100
MON-HT1	A	0	7.5	2.5	10	5	15
MON-HT1	B	2.5	5	2.5	7.5	2.5	20
MON-HT1	C	0	5	2.5	10	5	20
MON-HT1	no CTP	2.5	10	10	22.5	22.5	37.5
MON-HT2	A	0	5	2.5	7.5	5	15
MON-HT2	B	0	5	0	10	0	20
MON-HT2	C	0	5	5	12.5	12.5	20
MON-HT2	no CTP	35	45	50	37.5	70	55
MON-HT8	A	15	15	37.5	35	60	50
MON-HT8	B	5	7.5	32.5	35	27.5	30
MON-HT8	C	55	45	75	55	85	72.5
MON-HT9	A	0	5	2.5	15	7.5	20
MON-HT10	A	0	5	2.5	10	5	21

[0114] Plants containing MON-HT1 with any of the three operably linked CTPs (A, B, or C) had better tolerance to quizalofop-P than plants containing MON-HT1 without an operably linked CTP. Plants containing MON-HT1 with the operably linked 'A' CTP had 0 to 15% injury rating across all quizalofop-P applications. Plants containing MON-HT1 with the operably linked 'B' CTP had 2.5% to 20% injury rating across all quizalofop-P applications. Plants containing MON-HT1 with the operably linked 'C' CTP had 0 to 20% injury rating across all quizalofop-P applications compared to plants containing MON-HT1 without an operably linked CTP that had 2.5% to 37.5% injury ratings across all quizalofop-P applications.

[0115] Plants containing MON-HT2 with any of the three operably linked CTPs (A, B, or C) had better tolerance to quizalofop-P than plants containing MON-HT2 without an operably linked CTP. Plants containing MON-HT2 with the operably linked 'A' CTP had 0 to 15% injury rating across all quizalofop-P applications. Plants containing MON-HT2 with the operably linked 'B' CTP had 0 to 20% injury rating across all quizalofop-P applications. Plants containing MON-HT2 with the operably linked 'C' CTP had 0 to 20% injury rating across all quizalofop-P applications compared to plants containing MON-HT2 without an operably linked CTP that had 35% to 70% injury ratings across all quizalofop-P applications.

[0116] Plants containing MON-HT8 with either the operably linked 'A' or 'B' CTP had better tolerance to quizalofop-P than plants containing MON-HT8 with the operably linked 'C' CTP. Plants containing MON-HT8 with the oper-

ably linked 'A' CTP had 15% to 60% injury rating across all quizalofop-P applications. Plants containing MON-HT8 with the operably linked 'B' CTP had 5% to 35% injury rating across all quizalofop-P applications, compared to plants containing MON-HT8 with the operably linked 'C' CTP that had 45% to 85% injury rating across all quizalofop-P applications.

[0117] Plants containing MON-HT1 or MON-HT2 with any of the three operably linked CTPs and plants containing MON-HT9 or MONOHT10 with the operably linked 'A' CTP had better tolerance to quizalofop-P than plants containing compared to plants containing either MON-HT9 or MON-HT10 with an operably linked 'A' CTP. Over the range of 2,4-D applications the relative ranking of tolerance was: plants containing MON-HT1 had better tolerance than plants containing MON-HT2 which were in turn better than plants containing MON-HT8. Consistent with the data from the quizalofop-P pressure testing trial, plants containing MON-HT1, MON-HT2, or MON-HT8 operably linked to the 'A' CTP showed a slight mathematical, but not statistically significant, advantage over the 'B' and 'C' transit peptide. Error was calculated using LSD (0.05).

TABLE 19

		4X 2,4-D		8X 2,4-D		16X 2,4-D	
MON-HT variant	CTP	CIPV4 LSD (0.05) = 3.4	CIPV8 LSD (0.05) = 3.33	CIPV4 LSD (0.05) = 2.1	CIPV8 LSD (0.05) = 3.5	CIPV4 LSD (0.05) = 3.4	CIPV8 LSD (0.05) = 3.5
None - Control	none	80	88.75	82.5	95	87.5	96.25
MON-HT1	A	5	5	5	10	7.5	15
MON-HT1	B	5	5	5	10	10	15
MON-HT1	C	5	5	5	10	10	17.5
MON-HT1	no CTP	5	5	7.5	10	10	22.5
MON-HT2	A	5	5	10	7.5	10	20
MON-HT2	B	5	5	5	10	10	25
MON-HT2	C	10	15	10	10	15	20
MON-HT2	no CTP	7.5	7.5	17.5	42.5	25	45
MON-HT8	A	7.5	5	10	12.5	15	30
MON-HT8	B	5	7.5	10	27.5	10	20
MON-HT8	C	10	7.5	10	17.5	17.5	25
MON-HT9	A	17.5	55	25	75	45	90
MON-T10	A	32.5	70	55	87.5	45	90

MON-HT8 with any of the three operably linked CTPs. Plants containing MON-HT9 or MON-HT10 with the operably linked 'A' CTP had tolerance to quizalofop-P across all applications that was comparable to plants containing MON-HT1 or MON-HT2 with any of the three operably linked CTPs. At the highest rate (16×) of quizalofop application, plants containing MON-HT1 or MON-HT2 with an operably linked 'A' CTP had a slightly higher tolerance than plants containing MON-HT1 or MON-HT2 with an operably linked 'B' or 'C' CTP.

[0118] Three herbicide treatments were used in the trait efficacy field trial for 2,4-D tolerance: (1) 2,4-D amine at 4 lb ae/acre (4×) plus 0.25% v/v NIS applied to VE-V2 followed by V4 followed by V8; (2) 2,4-D amine at 8 lb ae/acre (8×) plus 0.25% v/v NIS applied to VE-V2 followed by V4 followed by V8 maize; or (3) 2,4-D amine at 16 lb ae/acre (16×) plus 0.25% v/v NIS applied to VE-V2 followed by V4 followed by V8. Plots were visually rated as above.

[0119] Table 19 shows the average injury ratings in maize after 2,4-D herbicide application at V4 (CIPV4) or V8 (CIPV8) growth stage, respectively. The injury rating for the control plants after all 2,4-D applications ranged from 80% to 96.25%. At the highest rate of 2,4-D (16—) applied through V8, the plants containing MON-HT1 or MON-HT2 operably linked to any of the three CTPs (A, B, or C) had better tolerance than plants containing MON-HT1 or MON-HT2 not operably linked to a CTP. Plants containing MON-HT1, MON-HT2, or MON-HT8 with or without an operably linked CTP had better tolerance to 2,4-D at all applications tested

Example 6

Expression of Optimized Engineered Proteins in Soy

[0120] Two engineered proteins were selected for analysis in transgenic soybean. DNA constructs were produced for expressing MON-HT1 (SEQ ID NO:14) and MON-HT2 (SEQ ID NO:18) with codon usage optimized for dicot expression using methods known to those skilled in the art. Enhancers, promoters, leaders, introns, CTPs, and 3'UTRs in various combinations were operably linked to the engineered proteins in these DNA constructs. The DNA constructs were used to transform soybean using *Agrobacterium tumifaciens* and standard methods known in the art. Regenerated R0 transgenic plantlets were grown in the green house. Approximately 9 weeks after transformation at the 1-2 trifoliate leaf stage, single copy R0 events were identified and sprayed with 2,4-D herbicide at a rate of 0.5× (0.375 lb ae/acre), 2× (1.5 lb ae/acre), or 4× (3.0 lb ae/acre). Approximately 2 weeks after herbicide application, the plants were rated for herbicide injury on a scale of 1 to 3, where 1=little to no injury (<20%), 2=moderate injury (20-50%) and 3=severe injury (>50%).

[0121] R0 soy plants containing each of the constructs showed tolerance to 2,4-D with little to no injury (<20% injury) or moderate injury (20-50%). Data are provided in Table 20. This indicated that the engineered proteins MON-HT1 and MON-HT2 can confer tolerance to 2,4-D in soybean plants.

TABLE 20

Protein	CTP	2,4-D rate	Single copy events	Events with <20% injury	Events with 20-50% injury	Events with injury >50%
MON-HT1	No	0.5X	130	120	10	0
MON-HT1	No	2X	36	6	30	0
MON-HT1	Yes	2X	136	101	35	0
MON-HT2	No	0.5X	22	3	19	0
MON-HT1	Yes	4X	11	5	6	0

[0122] An additional five engineered proteins optimized for activity for 2,4-D were then selected for analysis in transgenic soybean. DNA constructs were produced for expressing MON-HT13 (SEQ ID NO:47), MON-HT14 (SEQ ID NO:48), MON-HT15 (SEQ ID NO:49), MON-HT17 (SEQ ID NO:51), and MON-HT18 (SEQ ID NO:52) with codon usage optimized for dicot expression. The operably linked expression elements (promoter, leader, intron, CTP, and 3'UTR) were identical in all of the constructs. Leaf samples were taken from R0 plantlets and single copy plants were identified using a PCR-based assay. When the single-copy R0 plants had approximately two to three trifoliate leaves, they were treated with either 1.5 lb ae/acre (2×) or 3.0 lb ae/acre (4×) of 2,4-D. Seven days after herbicide application, the plants were scored for herbicide injury based on the percent area of the plant showing injury, as indicated above.

[0123] At the 2×application rate, soy plants containing any of six MON-HT variants (MON-HT1, MON-HT13, MON-HT14, MON-HT15, MON-HT17, and MON-HT18) showed excellent tolerance to 2,4-D treatment, as evidenced by all but two of the single copy plants having injury rating of <20%; these two events (one for MON-HT13 and one for MON-HT18) had an injury rating of 20-30%. At the 4×application rate, of the eleven single-copy plants containing MON-HT1, five plants had an injury score of <20%, and six plants had an injury score of 20-50%. Of the eleven single-copy plants containing MON-HT13, ten plants had an injury score of <20% and one plant had an injury score of 20-50%. Of the eight single-copy plants containing MON-HT14, six plants had an injury score of <20%, one plant had an injury score of 20-50%, and one plant had an injury score of >50%. Of the seven single-copy plants containing MON-HT15, five plants had an injury score of <20%, one plant had an injury score of 20-50%, and one plant had an injury score of >50%. Of the eleven single-copy plants containing MON-HT17, all eleven plants had an injury score of <20%. Of the twelve single-copy plants containing MON-HT18, nine plants had an injury score of <20% and three plants had an injury score of 20-50%. These results indicate that soy plants containing MON-HT1 (SEQ ID NO:14), MON-HT13 (SEQ ID NO:47), MON-HT14 (SEQ ID NO:48), MON-HT15 (SEQ ID NO:49), MON-HT17 (SEQ ID NO:51), or MON-HT18 (SEQ ID NO:52) had tolerance to 2,4-D at the 4× application rate. Furthermore, this demonstrated that soy plants containing MON-HT13 (SEQ ID NO:47), MON-HT14 (SEQ ID NO:48), MON-HT15 (SEQ ID NO:49), MON-HT17 (SEQ ID NO:51), or MON-HT18 (SEQ ID NO:52) had improved 2,4-D tolerance at the 4× application rate compared to MON-HT1 (SEQ ID NO:14). Based on the percentage of single-copy plants with an injury score of <20%, soy plants containing either MON-HT13 or MON-HT17 had better tolerance to 2,4-D applied at the 4× rate compared to soy plants containing MON-HT1, MON-HT14, MON-HT15, or MON-HT18. See Table 21.

TABLE 21

MON-HT	SEQ ID NO	2,4-D rate	Single copy events	Events with <20% injury	Events with 20-50% injury	Events with injury >50%
MON-HT1	14	2X	12	12	—	—
MON-HT13	47	2X	13	12	1	—
MON-HT14	48	2X	9	9	—	—
MON-HT15	49	2X	5	5	—	—
MON-HT17	51	2X	12	12	—	—
MON-HT18	52	2X	12	11	1	—
MON-HT1	14	4X	11	5	6	—
MON-HT13	47	4X	11	10	1	—
MON-HT14	48	4X	8	6	1	1
MON-HT15	49	4X	7	5	1	1
MON-HT17	51	4X	11	11	—	—
MON-HT18	52	4X	12	9	3	—

Example 7

Tolerance to Synthetic Auxins Fluroxypyr, Triclopyr, and MCPA

[0124] Tolerance of maize and soy plants containing MON-HT1 to applications of 2,4-D, fluroxypyr, triclopyr, and MCPA was determined F1 hybrid maize seed for three unique events containing MON-HT1 with the 'A' CTP and R2 soy seed were planted in pots. Hybrid maize seed containing NK603×MON89034 and the same soybean germplasm used for plant transformation were used as controls. Plants were grown in a green house and four plants were used for each treatment. The plants were sprayed with herbicide in a growth chamber when the plants were between 6-8 inches (soy) and 10-12 inches (corn) tall, then transferred to a greenhouse programmed to maintain optimum growth conditions.

[0125] For soy, a 2× herbicide application rate of each of the following was used: (1) 2,4-D Amine 4 (1680 g ae/ha) (2) triclopyr (840 g ae/ha, GARLON®); (3) fluroxypyr (840 g ae/ha, Starane®); or (4) MCPA (g ae/ha 1680). Following application of triclopyr, fluroxypyr, or MCPA the primary symptomology on soy was severe necrosis and epinasty. Visual plant injury ratings were made for all treatments on a rating scale from 0% to 100%, where 0% represented plants equivalent to untreated controls and 100% represented plants that were completely dead. All ratings were taken at seven days after treatment. Plants for all three soy MON-HT1 events showed good tolerance to 2,4-D Amine (2,4-D) averaging less than 7% crop injury compared to controls at 90-97% crop injury. No soybean events showed tolerance to triclopyr or fluroxypyr, with injury ratings across all three events averaged 81-97% crop injury compared to controls at 91% crop injury. One of the three soy events showed a low level of tolerance to MCPA with an average injury rating of 72% while the other two events had 90% crop injury compared to the controls at 90% injury. See Table 22.

TABLE 22

			Average soy % crop injury for 4 reps			
TRT #	Herbicide	2X Rate (g ae/ha)	MON-HT1 Event 1	MON-HT1 Event 2	MON-HT1 Event 3	Control
1	2,4-D Amine	1680	4.5	6.5	5.3	90.0
2	triclopyr	840	81.3	90.0	90.0	91.3
3	fluroxypyr	840	96.3	93.8	97.5	91.3
4	MCPA	1680	90.0	90.0	72.5	90.0
5	Controls	0	0.0	0.0	0.0	0.0

[0126] For maize, a 4× herbicide application rate of each of the following was used: (1) 2,4-D Amine 4 (3360 g ae/ha); (2) triclopyr (1680 g ae/ha, GARLON®); (3) fluroxypyr (1680 g ae/ha, Starane®); or (4) MCPA (g ae/ha 3360). Following application of triclopyr, fluroxypyr, or MCPA to maize, the primary symptomology was lodging. Plants for all three maize MON-HT1 events were tolerant of 2,4-D averaging less than 15% injury compared to controls at 43% crop injury. The three MON-HT1 maize events appeared to show some low level tolerance to triclopyr with crop injury averaging 26%-37% compared to controls with 47% crop injury. The three MON-HT1 maize events appeared to show some low level tolerance to fluroxypyr with crop injury averaging 20%-21% compared to controls with 55% crop injury. Two of the MON-HT1 maize events showed good tolerance to MCPA with an average crop injury of less than 6% compared to controls at 31% crop injury. The third maize MON-HT1 event had an average injury rating of 20%. See Table 23. These results of low tolerance to triclopyr and fluroxypyr and good tolerance to MCPA were consistent with the in vitro enzymatic data with purified MON-HT1 enzyme.

TABLE 23

			Average maize % crop injury for 4 reps			
TRT #	Herbicide	4X Rate (g ae/ha)	MON-HT1 Event 1	MON-HT1 Event 2	MON-HT1 Event 3	LH244
1	2,4-D Amine	3360	5.8	15.0	5.3	43.8
2	triclopyr	1680	26.3	37.5	35.0	47.5
3	fluroxypyr	1680	20.0	20.0	21.3	55.0
4	MCPA	3360	3.8	5.0	20.0	31.3
5	Controls	0	0.0	0.0	0.0	0.0

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<400> SEQUENCE: 7

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Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
```

-continued

```
1               5                   10                  15

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

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

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

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

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

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

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

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

Thr Ala Tyr Asp Ala Leu Ser Asp Gly Leu Lys Lys Leu Ile Ser Gly
145                 150                 155                 160

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

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

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

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

Ile Glu Gly Met Ser Glu Lys Glu Ser Glu Pro Leu Leu Ser Phe Leu
225                 230                 235                 240

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

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

Ile Asn Asp Tyr His Gly Gln Thr Arg Ile Leu His Arg Thr Thr Val
        275                 280                 285

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

<210> SEQ ID NO 8
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 8

```
atgcatgctg cactgtcccc cctctcccag cgctttgagc gcatcgcggt ccagccgctg     60

accggcgtcc tgggcgccga gatcaccggc gtcgacctgc gcgagccgct cagcgacagc    120

acctggaacg aaatcctcga cgcgttccac acttaccagg tcatctattt tcccggccag    180

gcgatcacca acgaacagca catcgccttc agccggcgct tcggccccgt cgatcccgtg    240

cccctgctca agagcatcga agggtatcca gaggtgcaga tgatccgccg cgaagccaac    300
```

-continued

gaaagcgggc gtgtgatcgg tgatgactgg cacagcgaca gcaccttcct ggacgcaccg 360

ccggccgccg tggtgatgcg cgcgatcgac gtgcccgagc atggcggcga caccggtttt 420

ctgagcatgt acaccgcgta tgatgcgctg tcggatggcc tgaagaaact gatcagcggg 480

ttgaacgtag tgcacagcgc cacgcgtgtg ttcggctcgc tctaccaggc ccagaaccgg 540

cgcttcagca acaccagcgt caaggtgatg gacgtcgacg cgggcgaccg tgaaaccgtg 600

cacccctgg tggtgaccca tccgggcagc ggccgcaagg gcctgtacgt gaaccaggtc 660

tattgccagc gcatcgaggg catgagcgaa aaagaaagcg aaccgctgct gagcttcctg 720

tttgcgcatg cgacaaaacc ggaattcacc tgccgcgtgc gctggaagaa ggaccaggtc 780

ctggtctggg acaacctgtg cacgatgcac tatgccatta acgactacca tggccagacc 840

cgcattctgc atcgcaccac ggtcggtggc gtgcgcccgg cgcgccatca tcaccatcat 900

cactag 906

<210> SEQ ID NO 9
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 9

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

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

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

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

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

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

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

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

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

Thr Ala Tyr Asp Ala Leu Ser Asp Gly Leu Lys Lys Leu Ile Ser Gly
145 150 155 160

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

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

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

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

Ile Glu Gly Met Ser Glu Lys Glu Ser Glu Pro Leu Leu Ser Phe Leu
225 230 235 240

-continued

```
Phe Ala His Ala Thr Lys Pro Glu Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

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

Ile Asn Asp Tyr His Gly Gln Thr Arg Ile Leu His Arg Thr Thr Val
        275                 280                 285

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

<210> SEQ ID NO 10
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 10

```
atgcatgctg cactgtcccc cctctcccag cgctttgagc gcatcgcggt ccagccgctg     60

accggcgtcc tgggcgccga gatcaccggc gtcgacctgc gcgagccgct cgacgacagc    120

acctggaacg aaatcctcga cgcgttccac acttaccagg tcatctattt tcccggccag    180

gcgatcacca acgaacagca catcgccttc agccggcgct tcggccccgt cgatcccgtg    240

cccctgctca agagcatcga agggtatcca gaggtgcaga tgatccgccg cgaagccaac    300

gaaagcgggc gtgtgctggg tgatgactgg cacaccgaca gcaccttcct ggacgcaccg    360

ccggccgccg tggtgatgcg cgcgatcgac gtgcccgagc atggcggcga caccggtttt    420

ctgagcatgt acaccgcgta tgatgcgctg tcggatggcc tgaagaaact gatcagcggg    480

ttgaacgtag tgcacagcgc cacgcgtgtg ttcggctcgc tctaccaggc ccagaaccgg    540

cgcttcagca acaccagcgt caaggtgatg gacgtcgcag atggcgaccg tgaaaccgtg    600

cacccccctgg tggtgaccca tccgggcagc ggccgcaagg gcctgtacgt gaaccaggtc    660

tattgccagc gcatcgaggg catgagcgaa aaagaaagcg aaccgctgct gagcttcctg    720

tttgcgcatg cgacaaaacc ggaattcacc tgccgcgtgc gctggaagaa ggaccaggtc    780

gtggtctggg acaacctgtg cacgatgcac tatgccatta acgactacca tggccagacc    840

cgcattctgc atcgcaccac ggtcggtggc gtgcgcccgg cgcgccatca tcaccatcat    900

cactag                                                               906
```

<210> SEQ ID NO 11
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 11

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

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

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

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

-continued

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

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

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

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

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

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

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

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

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

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

Ile Glu Gly Met Ser Glu Lys Glu Ser Glu Pro Leu Leu Ser Phe Leu
225                 230                 235                 240

Phe Ala His Ala Thr Lys Pro Glu Phe Thr Cys Arg Val Arg Trp Gln
                245                 250                 255

Glu Gly Asp Val Leu Val Trp Asp Asn Leu Cys Thr Gln His Tyr Ala
            260                 265                 270

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

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

<210> SEQ ID NO 12
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 12

```
atgcatgctg cactgtcccc cctctcccag cgctttgagc gcatcgcggt ccagccgctg     60

accggcgtcc tgggcgccga gatcaccggc gtcgacctgc gcgagccgct cgacgacagc    120

acctggaacg aaatcctcga cgcgttccac acttaccagg tcatctattt tcccggccag    180

gcgatcacca acgaacagca catcgccttc agccggcgct tcggccccgt cgatcccgtg    240

cccctgctca agagcatcga agggtatcca gaggtgcaga tgatccgccg cgaagccaac    300

gaaagcgggc gtgtgatcgg tgaaaactgg cacaccgaca gcaccttcct ggacgcaccg    360

ccggccgccg tggtgatgta tgcgaaagaa attccccccgt atggcggcga caccctgttt    420

accagcatgt acaccgcgtg ggagacgctg tcgcccacca tgcaggccac catcgaaggg    480

ttgaacgtag tgcacagcgc cacgcgtgtg ttcggctcgc tctaccaggc ccagaaccgg    540

cgcttcagca acaccagcgt caaggtgatg gacgtcgacg cgggcgaccg tgaaaccgtg    600
```

-continued

```
caccccctgg tggtgaccca tccggaaacc ggccgcaagg gcctgtacgt gaaccaggtc    660

tattgccagc gcatcgaggg catgagcgaa aaagaaagcg aaccgctgct gagcttcctg    720

tttgcgcatg cgacaaaacc ggaattcacc tgccgcgtgc gctggcagga aggcgatgtc    780

ctggtctggg acaacctgtg cacgcagcac tatgccgtac ccgactacgc gggcaagttc    840

cgctacctga cgcgcaccac ggtcggtggc gtgcgcccgg cgcgccatca tcaccatcat    900

cactag                                                               906


<210> SEQ ID NO 13
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 13

atgcacgccg ctctgagccc gcttagccag cgcttcgagc gcatcgccgt gcagccgctg     60

accggcgtgc taggcgctga gatcaccggc gttgacctga gggagccgct tgacgactcc    120

acctggaacg agatcctcga cgccttccac acttaccaag ttatctactt cccaggacag    180

gctatcacga acgaacagca catcgccttc tcgcggaggt tcgggccagt ggacccagtc    240

ccgctgctta agtctatcga aggctaccct gaggtgcaaa tgatccgccg cgaggcgaac    300

gaatccggga gggttattgg cgagaactgg cacactgact ccaccttcct cgatgctcct    360

ccagcagccg tcgtgatgta cgccaaggag atcccgccct acggcggcga tacgctcttc    420

acctccatgt acactgcttg ggagaccctt tctccgacca tgcaagccac catcgaggga    480

ctcaatgtgg tccactctgc gacccgtgtc tttggctcgc tctatcaggc gcagaatagg    540

cgcttcagca acacctccgt gaaggtcatg gacgtggacg ccggagacag ggagactgtc    600

cacccgctcg tcgttactca ccctgagacc ggccgtaagg gtctgtacgt gaaccaggtg    660

tactgtcagc gaattgaggg tatgagtgag aaggagtccg agccgctgct cagtttcctc    720

ttcgcgcacg ccaccaagcc cgagttcacc tgccgcgtcc gctggcaaga gggcgacgtc    780

ctggtgtggg acaacctctg cacccagcac tacgccgtgc cggactatgc cgggaagttc    840

cgctacctta cccgcacaac ggttggcggc gtgagacctg cccggtga                 888


<210> SEQ ID NO 14
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 14

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                  10                  15

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

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

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

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

-continued

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 15
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 15

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atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgattctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctagcc gttttattgg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aaccaaagtg ttcggcagcc tgtaccaggc aaccaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtaactca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccaga aaattcaggg catgactgat gcagagtcaa aatctctgct ccaatttctg    720
```

-continued

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta 780

ctggtatggg ataatctgtg tacgatgcac cgcgccgtac ctgattatgc cggcaaattt 840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat 900

cattag 906

<210> SEQ ID NO 16
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 16

atgcacgcgg cgctgactcc tctcaccaac aagtatcgct ttatcgacgt gcagccgctg 60

acaggcgtcc tcggtgcaga gattacaggc gtggatctgc gggagcctct cgatgacagc 120

acttggaatg agatcctgga cgcctttcac acctaccaag tgatctactt tccgggtcaa 180

gctatcacta acgagcagca catcgcgttc tcccgccggt tcggccctgt ggacccggtg 240

ccgatcttaa agagtatcga gggctatcca gaggtgcaga tgatacggcg cgaggcgaac 300

gagagcagcc ggttcatcgg agatgactgg cacaccgatt ccaccttcct ggacgctccg 360

cctgccgccg tggtgatgag agctatcgaa gtgccggagt atggaggtga cacaggcttc 420

ctctccatgt acagtgcctg ggagacactc tcgcctacga tgcaagctac catcgaaggc 480

ttaaacgtgg tccactcggc gacgaaggtc ttcgggtcat tgtaccaggc gactaattgg 540

cgcttctcga acaccagcgt gaaagtgatg gacgtggacg ccggagatag agagactgtg 600

cacccactcg tcgtgacgca tcctgttacg ggaaggcgcg cactctactg caaccaggtg 660

tactgccaga agatccaggg aatgacggac gcggagtcga agtccctgtt gcaattcctt 720

tacgagcacg ccaccaagtt cgacttcacc tgccgggtcc ggtggaagaa ggaccaagtc 780

ctggtgtggg acaacctgtg taccatgcac cgcgccgtcc cggactacgc tgggaaattc 840

agatacctga cccgcaccac cgtggcggga gacaagccgt cgcgttga 888

<210> SEQ ID NO 17
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 17

atgcacgcgg ctttgacacc tttgaccaac aagtatcggt tcatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggttcatagg cgacgattgg cacacagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

-continued

aggttcagta acaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccaggtt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

tacgaacacg ccaccaaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 18
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 18

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

-continued

Ala Gly Asp Lys Pro Ser Arg
290 295

<210> SEQ ID NO 19
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 19

atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg 60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct 120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag 180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt 240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac 300

gaatctagcc gtgttattgg tgacgattgg cacagcgact ccaccttcct ggacgcgccg 360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc 420

ctgtccatgt actctgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt 480

ctgaacgttg tgcactccgc aaccaaagtg ttcggcagcc tgtaccaggc aaccaactgg 540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg 600

cacccgctgg tggtaactca cccggttacc ggccgtcgtg cgctgtactg caaccaggta 660

tattgccaga aaattcaggg catgactgat gcagagtcaa aatctctgct ccaatttctg 720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta 780

ctggtatggg ataatctgtg tacgatgcac cgcgccgcgc ctgattatgc cggcaaattt 840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat 900

cattag 906

<210> SEQ ID NO 20
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 20

atgcacgcgg cgctgactcc tctcaccaac aagtatcgct ttatcgacgt gcagccgctg 60

acaggcgtcc tcggtgcaga gattacaggc gtggatctgc gggagcctct cgatgacagc 120

acttggaatg agatcctgga cgcctttcac acctaccaag tgatctactt tccgggtcaa 180

gctatcacta acgagcagca catcgcgttc tcccgccggt tcggccctgt ggacccggtg 240

ccgctgttaa agagtatcga gggctatcca gaggtgcaga tgatacggcg cgaggcgaac 300

gagagcagcc gggtgatcgg agatgactgg cactccgatt ccaccttcct ggacgctccg 360

cctgccgccg tggtgatgag agctatcgaa gtgccggagt atggaggtga cacaggcttc 420

ctctccatgt acagtgcctg ggagacactc tcgcctacga tgcaagctac catcgaaggc 480

ttaaacgtgg tccactcggc gacgaaggtc ttcgggtcat tgtaccaggc gactaattgg 540

cgcttctcga acaccagcgt gaaagtgatg gacgtggacg ccggagatag agagactgtg 600

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cacccactcg tcgtgacgca tcctgttacg ggaaggcgcg cactctactg caaccaggtg    660

tactgccaga agatccaggg aatgacggac gcggagtcga agtccctgtt gcaattcctt    720

tacgagcacg ccaccaagtt cgacttcacc tgccgggtcc ggtggaagaa ggaccaagtc    780

ctggtgtggg acaacctgtg taccatgcac cgcgccgccc cggactacgc tgggaaattc    840

agatacctga cccgcaccac cgtggcggga gacaagccgt cgcgttga                 888


<210> SEQ ID NO 21
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 21

atgcacgcag cccttactcc actgacgaac aagtatcgct tcatcgacgt gcagccactc     60

acgggtgtac tcggagccga gatcacggga gtggatctgc gcgagccgct cgatgactct    120

acatggaacg agatcctaga cgctttccac acttatcaag ttatctactt tccaggacaa    180

gccatcacta acgagcaaca catcgcgttc tcacgtcggt tcgggcctgt tgatcctgtg    240

ccgctcctca agtcaatcga gggttatcca gaagttcaga tgatccggcg cgaggctaat    300

gagtcatccc gtgttatcgg tgatgactgg cactcggaca gtacattcct cgacgcacca    360

ccggccgcag ttgtgatgag ggctatcgaa gtgccggaat acggtggtga cactgggttc    420

ctgtcaatgt actctgcatg ggagaccctt agtcccacta tgcaagcaac catcgaaggg    480

ctcaacgttg tgcattcagc tactaaagta ttcggttccc tttatcaggc gacaaactgg    540

cggttcagca ataccagtgt taaagttatg gatgtggatg ctggagacag ggaaacggtc    600

caccctcttg tcgtcacgca cccagttaca gggcgtcgag cgctttactg caatcaggtg    660

tactgtcaga agattcaagg aatgaccgat gcggagtcca aatcactgtt acaattcttg    720

tacgagcacg ccactaagtt cgatttcacg tgccgagtgc gctggaagaa ggatcaagtg    780

ctcgtttggg acaacttgtg caccatgcac cgggcagctc ccgactacgc gggcaagttc    840

cgttatctca ctcgcacaac tgtcgctgga gacaagccct cccgatga                 888


<210> SEQ ID NO 22
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 22

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

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

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

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

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

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

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

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

Lys Glu Val Pro Pro Tyr Gly Gly Asp Thr Leu Phe Ala Ser Met Tyr
    130                 135                 140

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

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

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

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

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

Ile Glu Gly Met Ser Glu Lys Glu Ser Glu Pro Leu Leu Ser Phe Leu
225                 230                 235                 240

Phe Ala His Ala Thr Lys Pro Glu Phe Thr Cys Arg Val Arg Trp Gln
                245                 250                 255

Glu Gly Gln Val Leu Val Trp Asp Asn Leu Cys Thr Gln His Phe Ala
            260                 265                 270

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

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 23
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 23

atgcacgcgg ctctgagccc gcttagccag cgtttcgaac gtatcgcggt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgactgg cacaccgact ctaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgta cgctaaagaa gtgccgccat acggtggcga caccctgttc    420

gcgtccatgt acaccgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aactcgtgtt ttcggctccc tgtaccaggc acagaaccgt    540

cgcttcagca acactagcgt taaagtgatg gatgtggatg caggcgatcg tgaaactgtg    600

cacccgctgg tggtaactca cccggaaact ggccgtaaag gcctgtacgt gaaccaggta    660

tattgccagc gtattgaagg catgagtgag aaagagtcgg agccgctgct ctcatttctg    720

tttgcacacg ccactaaacc agagtttact tgccgtgtcc gttggcaaga gggccaggta    780
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ctggtatggg ataatctgtg tacgcaacac ttcgccgtac ctgattatgc cggcaagttt 840

cgctatttga cgcgcacgac agtcggcggg gtccgccctg cccgccatca ccatcaccat 900

cattag 906

<210> SEQ ID NO 24
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 24

atgcacgcgg ccctgtctcc tctgtcccag cggttcgagc gcatcgcggt ccagccgcta 60

acgggtgtcc tgggcgcgga aatcaccgga gttgatctga gagagccttt agacgacagc 120

acctggaacg agatcctcga tgcctttcac acataccaag ttatctactt tcccggccaa 180

gccatcacga acgagcagca catcgcgttt agccggaggt ttggcccggt tgatccggtt 240

cctctgctta agtcaattga gggttaccca gaggtgcaga tgatccgccg cgaggccaac 300

gaatctgggc gtgtcatagg cgacgattgg catacggaca gcacctttct cgacgctcct 360

ccggccgcag tcgtgatgta cgcgaaggag gtgccgcctt acggcggcga taccctgttc 420

gcgtcgatgt acacggcctg ggagacgctc agcccgacca tgcaagccac aatagagggt 480

ctaaatgtgg tccactccgc gacgcgggtg ttcgggagcc tctaccaggc gcagaacaga 540

cggttctcga acacatcagt caaggtgatg gacgtggatg ccggagatcg tgaaactgtt 600

cacccacttg tggtcaccca tccagagacg ggaaggaaag gactttacgt gaaccaggtg 660

tactgccagc ggatcgaggg catgtccgag aaggagagtg agccattgct gagctttcta 720

ttcgcgcacg caactaagcc cgagttcacg tgccgcgtcc gatggcaaga gggccaagtt 780

ctcgtctggg ataacttgtg cacacagcac ttcgcggttc ccgattacgc cggaaagttc 840

cgctatctca cacgcaccac tgtgggaggc gttcgtcccg cgcggtga 888

<210> SEQ ID NO 25
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 25

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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            100                 105                 110

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

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

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

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

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

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

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 26
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 26

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atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgta cgctcgtgaa gttccgccgt acggtggcga caccggtttc    420

ctgtccatgt acaccgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aacccgtgtg ttcggcagcc tgtaccaggc acagaaccgt    540

cgctacagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtatctca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccagc gtattgaggg catgactgat gcagagtcaa aatctctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcgccgtac ctgattatgc cggcaaattt    840
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cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat 900

cattag 906

<210> SEQ ID NO 27
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 27

atgcacgctg ctctgactcc actcacaaac aagtaccggt tcatcgacgt gcaaccgctg 60

accggcgtct tgggtgcgga aatcaccggc gtggacttgc gggagccgct ggatgacagc 120

acatggaacg agatcctcga tgcgttccac acctaccaag tgatctattt cccaggccag 180

gccattacga acgagcagca catcgcgttc agtcgaaggt tcgggcctgt ggacccggtt 240

ccgctgctta agagtatcga gggctacccg gaagtacaga tgattcgccg cgaagcgaat 300

gagtccgggc gagtgatcgg cgatgactgg cacaccgaca gcacgttcct cgacgcgccg 360

cctgccgctg tcgtgatgta cgcacgggag gtgccaccct acggcggaga tacgggattc 420

ctttcaatgt acacggcatg ggagacactc tctccgacca tgcaagcaac gatagagggc 480

ttgaacgtgg tgcactccgc cacgagggtc ttcggcagcc tctaccaagc ccagaaccgc 540

cggtactcca acactagcgt gaaagtgatg gacgtggatg cgggcgaccg ggagaccgtg 600

catcctctag ttgtgagcca cccggtgact ggccgacggg cgctgtactg caaccaggtc 660

tattgccagc gcatcgaggg catgaccgac gcggagtcga aatctctgct ccaattcctg 720

tacgagcacg ccacgaagtt cgacttcacc tgccgggttc gctggaagaa ggatcaagtg 780

ctagtgtggg acaacctctg cactatgcac agggctgtgc cggactatgc tggcaaattc 840

cgttaccta cccggaccac tgtggcgggc gacaagccaa gcagatga 888

<210> SEQ ID NO 28
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 28

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala

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        115                 120                 125

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 29
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 29

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atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aaccaaagtg ttcggcagcc tgtaccaggc aaccaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtaactca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccaga aaattcaggg catgactgat gcagagtcaa aatctctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcgccgtac ctgattatgc cggcaaattt    840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat    900

cattag                                                               906
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<210> SEQ ID NO 30
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 30

atgcacgcgg cgctgactcc tctcaccaac aagtatcgct ttatcgacgt gcagccgctg 60

acaggcgtcc tcggtgcaga gattacaggc gtggatctgc gggagcctct cgatgacagc 120

acttggaatg agatcctgga cgcctttcac acctaccaag tgatctactt tccgggtcaa 180

gctatcacta acgagcagca catcgcgttc tcccgccggt tcggccctgt ggacccggtg 240

ccgctgttaa agagtatcga gggctatcca gaggtgcaga tgatacggcg cgaggcgaac 300

gagagcggcc gggtgatcgg agatgactgg cacaccgatt ccaccttcct ggacgctccg 360

cctgccgccg tggtgatgag agctatcgaa gtgccggagt atggaggtga cacaggcttc 420

ctctccatgt acagtgcctg ggagacactc tcgcctacga tgcaagctac catcgaaggc 480

ttaaacgtgg tccactcggc gacgaaggtc ttcgggtcat tgtaccaggc gactaattgg 540

cgcttctcga acaccagcgt gaaagtgatg gacgtggacg ccggagatag agagactgtg 600

cacccactcg tcgtgacgca tcctgttacg ggaaggcgcg cactctactg caaccaggtg 660

tactgccaga agatccaggg aatgacggac gcggagtcga agtccctgtt gcaattcctt 720

tacgagcacg ccaccaagtt cgacttcacc tgccgggtcc ggtggaagaa ggaccaagtc 780

ctggtgtggg acaacctgtg taccatgcac cgcgccgtcc cggactacgc tgggaaattc 840

agatacctga cccgcaccac cgtggcggga gacaagccgt cgcgttga 888

<210> SEQ ID NO 31
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 31

Met His Ala Ala Leu Ser Pro Leu Ser Gln Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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

Arg Glu Val Pro Pro Tyr Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr

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    130                 135                 140

Ser Ala Trp Asp Thr Leu Ser Asp Thr Met Lys Ala Thr Ile Glu Gly
145                 150                 155                 160

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

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

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

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295


<210> SEQ ID NO 32
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 32

atgcacgcgg ctctgagccc gcttagccag aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttaccctgc gtgaaccgct ggacgacaac    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgta cgctcgtgaa gttccgccgt acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggacaccctg tccgacacca tgaaagctac cattgaaggt    480

ctgaacgttg tgcactccgc aacccgtgtg ttcggcagcc tgtaccaggc acagaaccgt    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtatctca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccagc gtattgaggg catgactgat gcagagtcaa aaccactgct ccaatttctg    720

tatgagcacg ccactcgttt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcgccgtac ctgattatgc cggcaaattt    840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat    900

cattag                                                               906


<210> SEQ ID NO 33
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<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 33

atgcacgccg ccctctctcc tcttagccag aagtaccgat tcatcgacgt ccagccgctc 60

actggcgtac tgggcgctga gataacgggt gtgaccctga gggagccgct ggacgacaac 120

acttggaacg agatccttga cgccttccat acttatcaag ttatctactt tcctggacaa 180

gcgattacga atgagcagca catcgcgttc tcccggaggt tcgggccagt cgatccggtg 240

ccgctactca agtccatcga aggataccca gaagtccaga tgatccgtcg tgaggcaaac 300

gagtccggcc gggtcatcgg cgacgattgg cacaccgact ctaccttcct tgacgcgcct 360

ccggccgcag tggtcatgta cgcccgcgag gtgcctccct acggcggcga tacgggcttc 420

ctcagtatgt actctgcatg ggacacccta agcgacacca tgaaggccac catcgaaggt 480

ctgaacgtcg tgcactcggc aacacgagtc ttcggatcac tctaccaagc acagaatcgc 540

cgcttctcga acacctcggt taaggtgatg gacgtggatg cgggtgacag ggaaaccgtt 600

catcctctgg tggtctcgca tccggttacg ggacgccgcg ctctctactg caaccaggtg 660

tactgtcaaa ggatcgaagg aatgacagat gctgagagca agccgttgct ccaattcctc 720

tatgaacacg caacaaggtt cgacttcacc tgccgggttc gatggaagaa ggatcaagtg 780

ctagtctggg acaacctctg caccatgcac cgggccgtgc cggactatgc tgggaaattc 840

cgttacctga cccgcacgac cgtcgcggga gacaagccgt cgagatga 888

<210> SEQ ID NO 34
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 34

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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

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

Ser Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly

-continued

```
145                 150                 155                 160

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 35
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 35

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atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgattgg cacgcggact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aaccaaagtg ttcggcagcc tgtaccaggc aaccaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtaactca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccaga aaattcaggg catgactgat gcagagtcaa aatctctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcagcgtac ctgattatgc cggcaaattt    840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat    900

cattag                                                              906
```

<210> SEQ ID NO 36
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 36

atgcacgcgg cgctgactcc tctcaccaac aagtatcgct ttatcgacgt gcagccgctg 60

acaggcgtcc tcggtgcaga gattacaggc gtggatctgc gggagcctct cgatgacagc 120

acttggaatg agatcctgga cgcctttcac acctaccaag tgatctactt tccgggtcaa 180

gctatcacta acgagcagca catcgcgttc tcccgccggt tcggccctgt ggacccggtg 240

ccgctgttaa agagtatcga gggctatcca gaggtgcaga tgatacggcg cgaggcgaac 300

gagagcggcc gggtgatcgg agatgactgg cacgccgatt ccaccttcct ggacgctccg 360

cctgccgccg tggtgatgag agctatcgaa gtgccggagt atggaggtga cacaggcttc 420

ctctccatgt acagtgcctg ggagacactc tcgcctacga tgcaagctac catcgaaggc 480

ttaaacgtgg tccactcggc gacgaaggtc ttcgggtcat tgtaccaggc gactaattgg 540

cgcttctcga acaccagcgt gaaagtgatg gacgtggacg ccggagatag agagactgtg 600

cacccactcg tcgtgacgca tcctgttacg ggaaggcgcg cactctactg caaccaggtg 660

tactgccaga agatccaggg aatgacggac gcggagtcga agtccctgtt gcaattcctt 720

tacgagcacg ccaccaagtt cgacttcacc tgccgggtcc ggtggaagaa ggaccaagtc 780

ctggtgtggg acaacctgtg taccatgcac cgctccgtcc cggactacgc tgggaaattc 840

agatacctga cccgcaccac cgtggcggga gacaagccgt cgcgttga 888

<210> SEQ ID NO 37
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 37

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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

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

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

Leu Asn Val Val His Ser Ala Thr Lys Val Phe Gly Ser Leu Tyr Gln

-continued

```
               165                 170                 175

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 38
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 38

```
atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgattctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtgttattgg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggaaaccctg tccccgacca tgcaggctac cattgaaggt    480

ctgaacgttg tgcactccgc aaccaaagtg ttcggcagcc tgtaccaggc aaccaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtaactca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccaga aaattcaggg catgactgat gcagagtcaa aatctctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcagcgtac ctgattatgg caatgcgttt    840

cgctatttga cgcgcacgac agtcgcgggg gacaaaacctt ctcgccatca ccatcatcat    900

cattag                                                               906
```

<210> SEQ ID NO 39
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

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<400> SEQUENCE: 39

atgcacgcgg cgctgactcc tctcaccaac aagtatcgct ttatcgacgt gcagccgctg 60

acaggcgtcc tcggtgcaga gattacaggc gtggatctgc gggagcctct cgatgacagc 120

acttggaatg agatcctgga cgcctttcac acctaccaag tgatctactt tccgggtcaa 180

gctatcacta acgagcagca catcgcgttc tcccgccggt tcggccctgt ggacccggtg 240

ccgatcttaa agagtatcga gggctatcca gaggtgcaga tgatacggcg cgaggcgaac 300

gagagcggcc gggtgatcgg agatgactgg cacaccgatt ccaccttcct ggacgctccg 360

cctgccgccg tggtgatgag agctatcgaa gtgccggagt atggaggtga cacaggcttc 420

ctctccatgt acagtgcctg ggagacactc tcgcctacga tgcaagctac catcgaaggc 480

ttaaacgtgg tccactcggc gacgaaggtc ttcgggtcat tgtaccaggc gactaattgg 540

cgcttctcga acaccagcgt gaaagtgatg gacgtggacg ccggagatag agagactgtg 600

cacccactcg tcgtgacgca tcctgttacg ggaaggcgcg cactctactg caaccaggtg 660

tactgccaga agatccaggg aatgacggac gcggagtcga agtccctgtt gcaattcctt 720

tacgagcacg ccaccaagtt cgacttcacc tgccgggtcc ggtggaagaa ggaccaagtc 780

ctggtgtggg acaacctgtg taccatgcac cgctccgtcc cggactacgg caacgccttc 840

agatacctga cccgcaccac cgtggcggga gacaagccgt cgcgttga 888

<210> SEQ ID NO 40
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 40

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1 5 10 15

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

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

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

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

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

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

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

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

Ser Ala Trp Asp Thr Leu Ser Asp Thr Met Lys Ala Thr Ile Glu Gly
145 150 155 160

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

Ala Gln Asn Trp Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val

-continued

```
            180                 185                 190

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

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

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

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

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

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

Ala Gly Asp Arg Pro Ala Arg
    290                 295
```

<210> SEQ ID NO 41
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 41

```
atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

accggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccggcgcag    180

cagatcacca acgaacagca catctctttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtatcctggg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggacaccctg tccgacacca tgaaagctac cattgaaggt    480

ctgaacgttg tgcactccgc aacccgtgtg ttcggcagcc tgtaccaggc acagaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtatctca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccagc gtattgaggg catgactgat gcagagtcaa aatgcctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac cgcgccgtac ctgattatgc cggcaaattt    840

cgctatttga cgcgcacgac agtcgcgggg gaccgccctg cccgccatca ccatcatcat    900

cattag                                                               906
```

<210> SEQ ID NO 42
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 42

```
atgcacgccg ccttaacgcc actcacgaac aagtatcgct tcatcgacgt ccagccgctc     60
```

-continued

```
actggcgtgc taggtgctga gatcaccggc gttgatctcc gcgagcctct tgacgactcg    120

acctggaacg agatcctgga tgccttccac acttaccaag tgatctactt cccggcccaa    180

cagatcacaa acgagcagca catctccttt agtaggcgat tcggtccagt cgatccggtg    240

ccgctcctca agtcgattga gggctacccg gaggtccaga tgattcgtag ggaagccaac    300

gagtcaggcc gtattctggg cgacgactgg catacggact ccactttcct agacgcacct    360

ccggctgccg tcgtcatgag ggctattgaa gtgccggagt acggcggcga taccggattc    420

ctctctatgt actccgcctg ggacacgctc tcagacacca tgaaggccac gatagagggc    480

ctgaacgttg tgcactccgc aacgagagta ttcggatctc tgtaccaggc acagaactgg    540

cggttcagta acacgagcgt gaaagtcatg gacgtggacg ctggcgacag ggaaactgtt    600

cacccgttgg tcgtttccca ccctgtcact ggtcgccggg cactttactg caaccaggtg    660

tactgtcagc gcattgaggg catgacggat gctgagtcga agtgccttct ccaattcttg    720

tacgagcacg cgacaaagtt cgatttcacg tgccgggtcc gatggaagaa ggaccaagtg    780

ctagtgtggg acaacctttg cacgatgcac cgcgctgtcc cggattacgc cggaaagttc    840

cgttacctca ccagaacaac agttgccggt gacagacccg cgagatga                888
```

<210> SEQ ID NO 43
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 43

```
Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                  10                  15

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

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

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

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

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

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

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

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

Ser Ala Trp Asp Thr Leu Ser Asp Thr Met Lys Ala Thr Ile Glu Gly
145                 150                 155                 160

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

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

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Ser His Pro
```

[redacted]

-continued

```
        195                 200                 205

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 44
<211> LENGTH: 906
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 44

```
atgcacgcgg ctctgacccc gcttaccaac aaataccgtt tcatcgacgt tcagccgctg     60

tgcggtgttt taggtgctga aatcaccggt gttgacctgc gtgaaccgct ggacgactct    120

acctggaacg aaatcctgga cgcgttccac acctaccagg ttatctactt cccgggtcag    180

gcgatcacca acgaacagca catcgcgttc tctcgtcgct tcggtccggt tgacccggtt    240

ccgctgctca aatctatcga aggttacccg gaagttcaga tgatccgtcg cgaagcgaac    300

gaatctggtc gtatcctggg tgacgattgg cacaccgact ccaccttcct ggacgcgccg    360

ccagctgcag ttgtgatgcg tgctattgaa gttccggaat acggtggcga caccggtttc    420

ctgtccatgt actctgcttg ggacaccctg tccgacacca tgaaagctac cattgaaggt    480

ctgaacgttg tgcactccgc aacccgtgtg ttcggcagcc tgtaccaggc acagaactgg    540

cgcttcagca acactagtgt gaaagtgatg gatgtggatg caggcgatcg tgagactgtg    600

cacccgctgg tggtatctca cccggttacc ggccgtcgtg cgctgtactg caaccaggta    660

tattgccagc gtattgaggg catgactgat gcagagtcaa aatgcctgct ccaatttctg    720

tatgagcacg ccactaaatt tgattttact tgccgtgtcc gttggaaaaa ggatcaagta    780

ctggtatggg ataatctgtg tacgatgcac ctggccgtac ctgattatga cggcaaattt    840

cgctatttga cgcgcacgac agtcgcgggg gacaaacctt ctcgccatca ccatcatcat    900

cattag                                                               906
```

<210> SEQ ID NO 45
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 45

```
atgcacgcgg ctctcacacc gctcacaaac aagtaccgct tcattgacgt gcagccgctg     60

tgtggcgtcc tgggcgcaga aatcacgggc gtggatctcc gcgagcctct ggacgacagc    120
```

-continued

```
acctggaacg aaatcctgga tgctttccac acttaccaag tgatctactt tcccggacaa    180

gccatcacta acgagcagca catcgctttc tcacggcgct tcggcccggt agatccggtg    240

ccgctactca agtcaattga aggctatccg gaggtgcaaa tgattcgccg cgaagctaac    300

gagagcgggc gcatactggg cgacgactgg catactgact ccaccttcct cgatgctcca    360

ccagccgcag tggtgatgcg tgccatcgaa gttcccgagt atggtggcga cacgggtttc    420

ctgtctatgt actccgcttg ggatacactg tctgacacga tgaaggctac catcgagggc    480

ctgaatgtcg tccacagtgc tacgcgcgtg ttcggctcac tgtaccaggc gcagaactgg    540

aggttcagca acaccagtgt caaggtgatg gacgttgatg ctggagacag ggaaactgtg    600

catcctcttg tggtctccca tccagttacc gggaggagag cactctactg caaccaggtt    660

tactgccagc gcatcgaggg catgaccgat gcggagtcga agtgtctgtt acagttcttg    720

tacgagcacg cgacgaagtt cgacttcacg tgccgggtgc ggtggaagaa ggaccaagtt    780

ctcgtctggg acaatctctg cacgatgcac ctcgccgttc ccgactacga cggcaaattc    840

agatacttga cccgaacaac agtagcggga gataagcctt cccgttga                888
```

<210> SEQ ID NO 46
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 46

```
Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                  10                  15

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295


<210> SEQ ID NO 47
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 47

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                   10                  15

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295


<210> SEQ ID NO 48
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 48

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg His Ile Asp
1               5                   10                  15

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
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<210> SEQ ID NO 49
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 49

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg His Ile Asp
1 5 10 15

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

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

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

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

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

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

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

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

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

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

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

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

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Ile Tyr Cys Gln Lys
210 215 220

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
290 295

<210> SEQ ID NO 50
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 50

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Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                  10                  15

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

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

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

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

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

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

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

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

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

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

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

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

Val Thr Gly Arg Arg Ala Leu Tyr Cys Asn Gln Ile Tyr Cys Gln Lys
    210                 215                 220

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295


<210> SEQ ID NO 51
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 51

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg Phe Ile Asp
1               5                  10                  15

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

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
35                  40                  45
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Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
50                  55                  60

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
290                 295


<210> SEQ ID NO 52
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 52

Met His Ala Ala Leu Thr Pro Leu Thr Asn Lys Tyr Arg His Ile Asp
1               5                   10                  15

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

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

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

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

Pro Ile Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95
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```
Arg Glu Ala Asn Glu Ser Ser Arg Tyr Ile Gly Asp Asp Trp His Ala
            100                 105                 110

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

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

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

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

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

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

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

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

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

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

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

Ala Gly Asp Lys Pro Ser Arg
    290                 295
```

<210> SEQ ID NO 53
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 53

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atgcacgcgg ctttgacacc tttgaccaac aagtatcggt tcatcgacgt tcaaccactc     60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc    120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag    180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt    240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac    300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct    360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc    420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt    480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg    540

aggttcagta acaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg    600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccaggtt    660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc    720

tacgaacacg ccaccaaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg    780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc    840
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cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 54
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 54

atgcacgcgg ctttgacacc tttgaccaac aagtatcggt tcatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

aggttcagtg gaaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccaggtt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

tacgaacacg ccacccaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 55
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 55

atgcacgcgg ctttgacacc tttgaccaac aagtatcggc atatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

aggttcagtg gaaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccaggtt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

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tacgaacacg ccacccaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 56
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 56

atgcacgcgg ctttgacacc tttgaccaac aagtatcggc atatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccagcta tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

aggttcagta acaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccagatt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

tacgaacacg ccacccaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 57
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 57

atgcacgcgg ctttgacacc tttgaccaac aagtatcggt tcatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

aggttcagta acaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

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catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccagatt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

tacgaacacg ccacccaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 58
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 58

atgcacgcgg ctttgacacc tttgaccaac aagtatcggt tcatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

gcgatcacca acgagcagca cattgccttc tcaaggaggt tcggaccggt agatccagtt 240

ccaattctca aatccattga gggttatccc gaggtgcaga tgattagacg agaagccaac 300

gagtcctcac ggtacatagg cgacgattgg cacgcagaca gcaccttcct tgacgctcct 360

ccggctgccg tggttatgcg cgcaatagag gtgccggagt acggcggcga taccggtttc 420

ctatcaatgt actctgcatg ggagacgctc tcaccaacga tgcaagccac cattgaaggt 480

ctaaacgtgg ttcactcagc tactaaggtc ttcggaagtc tttaccaggc gacgaattgg 540

aggttcagta acaccagtgt gaaggtgatg gatgtggacg ctggagacag ggagacggtg 600

catccactcg tagttacaca ccctgtaact ggacgcagag ccctttactg caaccaggtt 660

tactgccaga agatccaggg aatgactgat gcggagtcta agtccctgct tcaattcctc 720

tacgaacacg ccacccaatt cgacttcact tgtcgtgttc ggtggaagaa ggaccaagtg 780

ctcgtgtggg ataacctttg caccatgcac cgagcagtac cagactacgc cgggaaattc 840

cgctatctca cccgcactac agtggccgga gacaagccta gccgctga 888

<210> SEQ ID NO 59
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant

<400> SEQUENCE: 59

atgcacgcgg ctttgacacc tttgaccaac aagtatcggc atatcgacgt tcaaccactc 60

acaggcgtgc tcggcgcaga gattaccgga gtggacctga gggagccctt agacgactcc 120

acttggaacg agatcctcga cgcctttcac acctaccaag ttatctactt tcctggacag 180

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Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
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Xaa Gly Xaa Xaa Pro Xaa Arg
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What is claimed is:

1. A recombinant DNA molecule comprising a nucleic acid sequence encoding a polypeptide having at least 92% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52.

2. The recombinant DNA molecule of claim **1**, wherein the nucleic acid sequence is selected from the group consisting of SEQ ID NO:2, 3, 5, 6, 8, 10,12, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41, 42, 44, 45 and 53-59.

3. The recombinant DNA molecule of claim **1**, wherein the recombinant DNA molecule is operably linked to a heterologous promoter functional in a plant cell.

4. The recombinant DNA molecule of claim **3**, wherein the recombinant DNA molecule is further operably linked to a DNA molecule encoding a chloroplast transit peptide.

5. A DNA construct comprising a heterologous promoter functional in a plant cell operably linked to the recombinant DNA molecule of claim **1**.

6. The DNA construct of claim **5**, further comprising a DNA molecule encoding a chloroplast transit peptide operably linked to the recombinant DNA molecule.

7. The DNA construct of claim **5**, wherein the nucleic acid sequence encodes a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43 and 46-52.

8. The DNA construct of claim **5**, wherein the DNA construct is present in the genome of a transgenic plant.

9. A plant, seed, plant tissue, plant part, or cell comprising the recombinant DNA molecule of claim **1**.

10. The plant, seed, plant tissue, plant part, or cell of claim **9**, wherein the plant, seed, plant tissue, plant part, or cell comprises tolerance to at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

11. A plant, seed, plant tissue, plant part, or cell comprising the DNA construct of claim **5**.

12. A plant, seed, plant tissue, plant part, or cell comprising the polypeptide encoded by the recombinant DNA molecule of claim **1**.

13. A polypeptide having at least 92% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52.

14. The polypeptide of claim **13**, wherein the polypeptide has oxygenase activity against at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

15. A method for conferring herbicide tolerance to a plant, seed, cell, or plant part comprising expressing in said plant, seed, cell, or plant part the polypeptide of claim **13**.

16. The method of claim **15**, wherein said plant, seed, cell, or plant part comprises a DNA construct comprising a heterologous promoter functional in a plant cell operably linked to a recombinant DNA molecule comprising a nucleic acid sequence encoding a polypeptide having at least 92% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52.

17. The method of claim **15**, wherein the plant, seed, cell, or plant part comprises tolerance to at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

18. A method for producing an herbicide tolerant transgenic plant comprising transforming a plant cell or tissue with a recombinant DNA molecule comprising a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52;

b) a nucleic acid sequence selected from the group consisting of SEQ ID NO:2, 3, 5, 6, 8, 10,12, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41, 42, 44, 45 and 53-59; and

c) a nucleic acid sequence encoding a polypeptide having at least 92% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52, and capable of conferring herbicide tolerance to a transgenic plant;

and regenerating an herbicide tolerant transgenic plant from the transformed plant cell or tissue.

19. The method of claim **18**, wherein said herbicide tolerant transgenic plant comprises tolerance to at least one her-

[redacted] COR-AAD1-00385780

bicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides.

20. A method for controlling weeds in a plant growth area, comprising contacting a plant growth area comprising a plant or seed comprising a recombinant DNA molecule comprising a nucleic acid sequence encoding a protein having at least 92% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:1, 4, 7, 9, 11, 14, 18, 22, 25, 28, 31, 34, 37, 40, 43, and 46-52 with at least one herbicide selected from the group consisting of AOPP herbicides, phenoxy acid herbicides, and pyridinyloxy acid herbicides, wherein the plant or seed is tolerant to the at least one herbicide.

* * * * *

EXHIBIT 12

US007838733B2

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

(10) **Patent No.: US 7,838,733 B2**
(45) **Date of Patent: Nov. 23, 2010**

(54) **HERBICIDE RESISTANCE GENES**

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

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

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 360 days.

(21) Appl. No.: **11/587,893**

(22) PCT Filed: **May 2, 2005**

(86) PCT No.: **PCT/US2005/014737**

§ 371 (c)(1),
(2), (4) Date: **May 22, 2008**

(87) PCT Pub. No.: **WO2005/107437**

PCT Pub. Date: **Nov. 17, 2005**

(65) **Prior Publication Data**

US 2009/0093366 A1 Apr. 9, 2009

Related U.S. Application Data

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

(51) **Int. Cl.**
C12N 15/82 (2006.01)
C12N 15/52 (2006.01)

(52) **U.S. Cl.** **800/300**; 435/419; 536/23.2; 800/288; 800/298

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Kohler, H.P.E. "*Sphingomonas herbicidovorans* MH: A versatile phenoxyalkanoic acid herbicide degrader" *Journal of Industrial Microbiology & Biotechnology*, Oct. 1999, pp. 336-340, vol. 23, No. 4-5.

Database EMBL, Accession No. AF516751, Schleinitz, K.M. et al., "Rhodoferax sp. P230 R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds.", Aug. 6, 2002, pp. 1-2.

Database EMBL, Accession No. AF516752, Schleinitz, K.M. et al., "*Sphingobium herbicidovorans* R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds.", Aug. 6, 2002, pp. 1-2.

Schleinitz, K.M. "Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1" *Applied and Environmental Microbiology*, Sep. 2004, pp. 5357-5365, vol. 70, No. 9.

Supplemental European Search Report dated Mar. 28, 2008.

Genbank Accession No. M16730, Apr. 1993.

Horvath, M. et al., "Isolation and characterization of a 2-(2,4-dichlorophenoxy)propionic acid-degrading . . . ", Appl. Microbiol Biotechnol.,1990, pp. 33;213-216. (Abstract only).

(Continued)

Primary Examiner—David H Kruse
(74) *Attorney, Agent, or Firm*—Kenneth B. Ludwig; Baker & Daniels LLP

(57) **ABSTRACT**

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

62 Claims, 24 Drawing Sheets

OTHER PUBLICATIONS

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Lyon, B.R. et al.,"Cotton plants transformed with a bacterial degradation gene are protected from accidental . . . "; Transgenic Res.; 1993; pp. 2:166-169. (Abstract only).

Smejkal, C.W. et al., "Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria . . . "; Biol. Fertil., 2001; pp. 33:507-513.

Streber, W. et al., "Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D"; Bio/Technology; 1989; pp. 7:811-816. (Abstract only).

Streber, W.R. et al., "Analysis, cloning, and high-level expression of 2,4-dichlorophenixyacetic monooxygenase gene . . . "; J. Bacteriol; 1987; pp. 169:2950-2955.

Westendorf, A. et al., "The two enantiospecific dichlorprop/alpha-ketoglutarate-dioxygenases . . . "; Microbiol.Res.; 2002; pp. 157:317-22. (Abstract only).

Westendorf, A., "Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* . . . "; Acta Biotechnol.; 2003; pp. 23:3-17.

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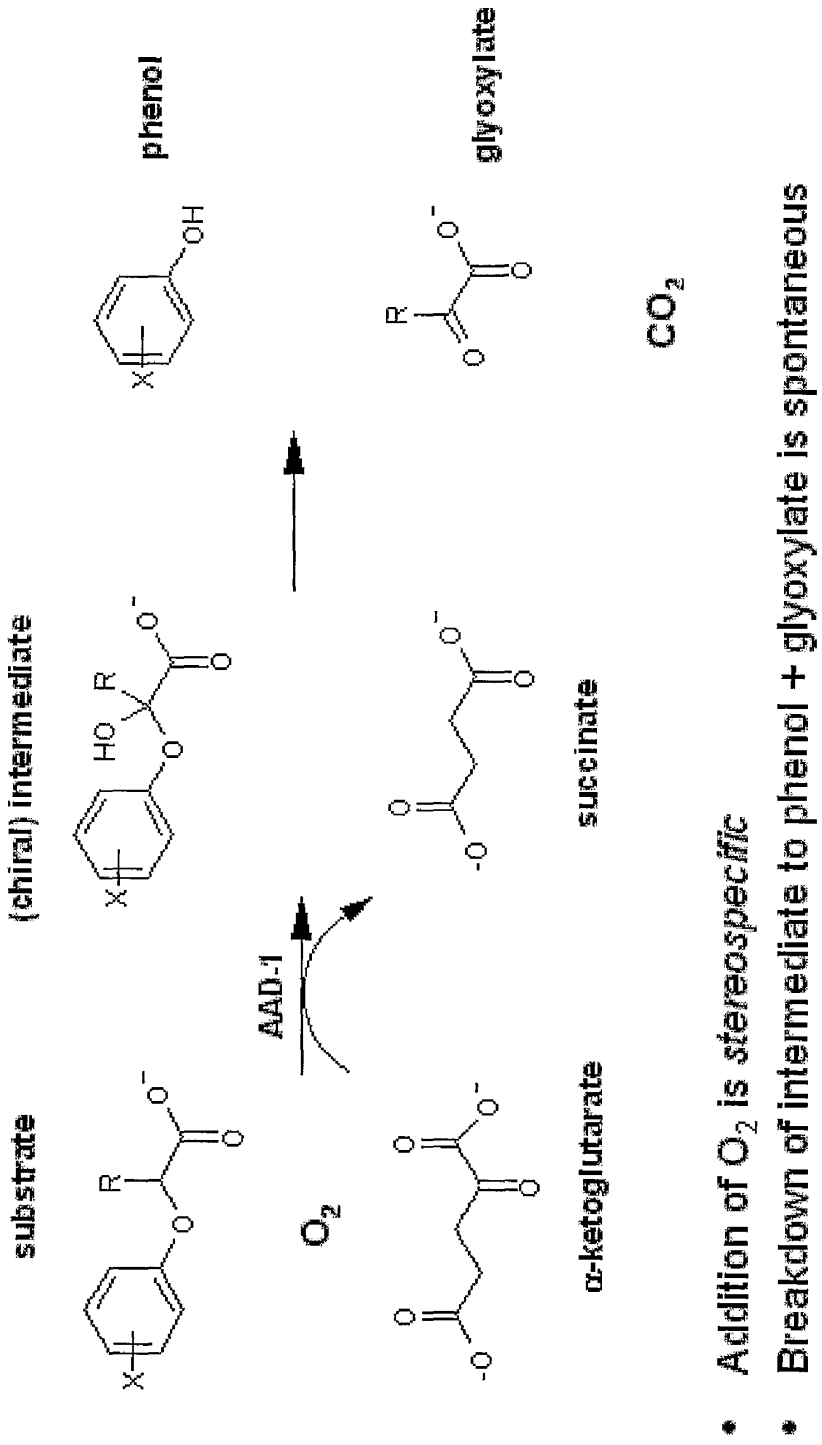

substrate
(chiral) intermediate
phenol
glyoxylate
AAD-1
O_2
α-ketoglutarate
succinate
CO_2
• Addition of O_2 is *stereospecific*
• Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

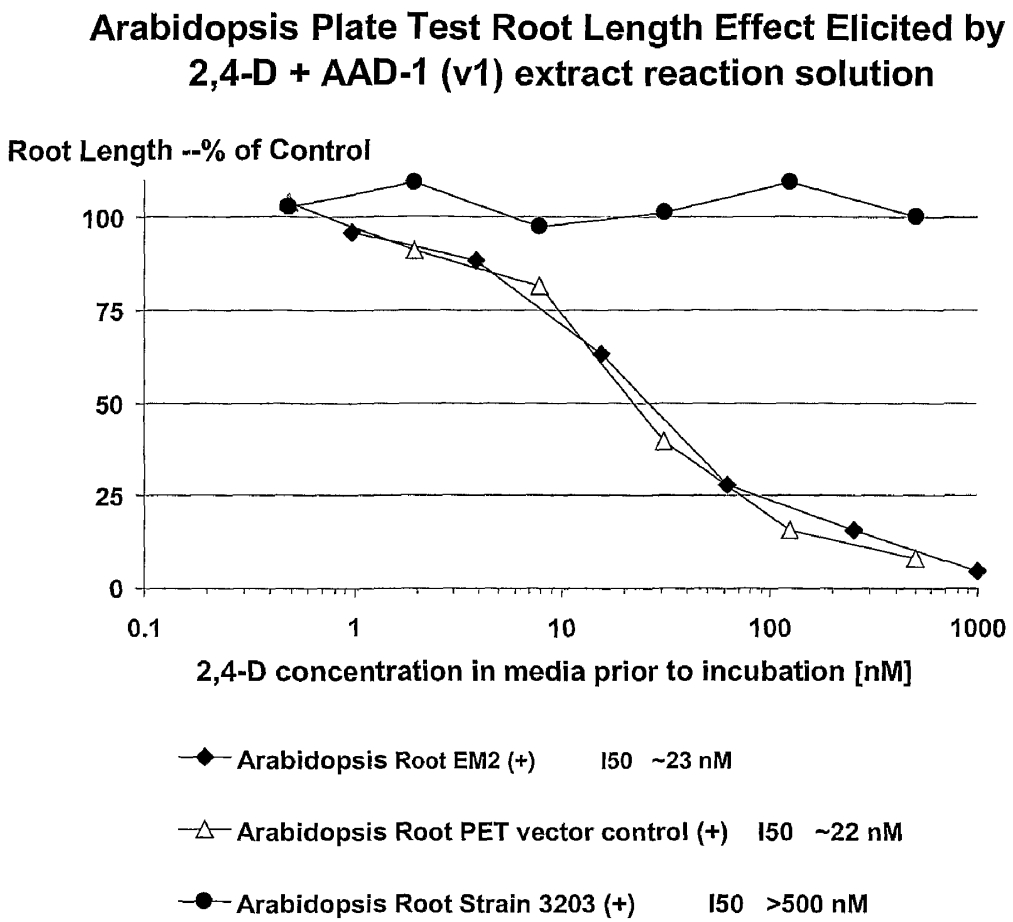

Arabidopsis Plate Test Root Length Effect Elicited by 2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis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

Fig. 2

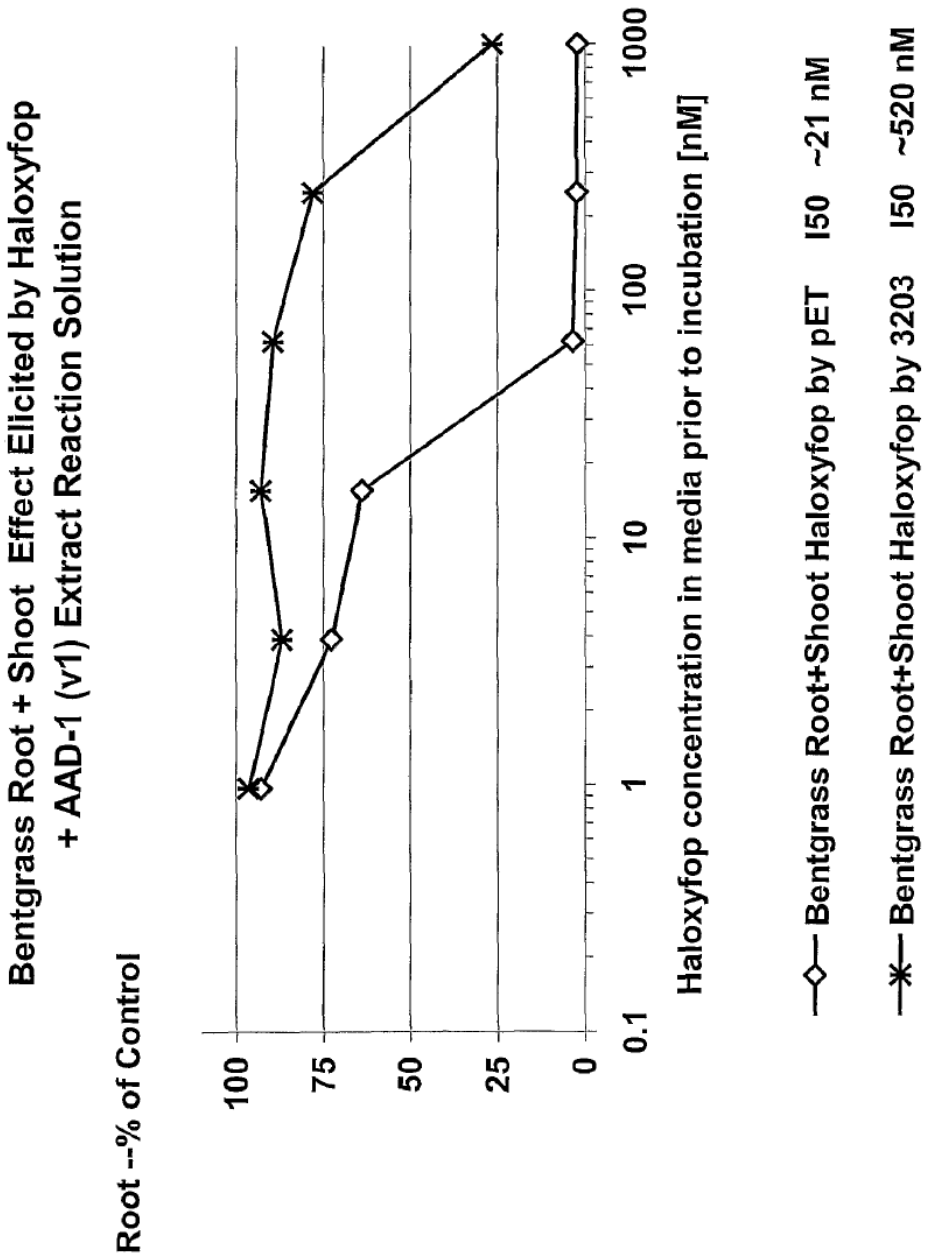

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

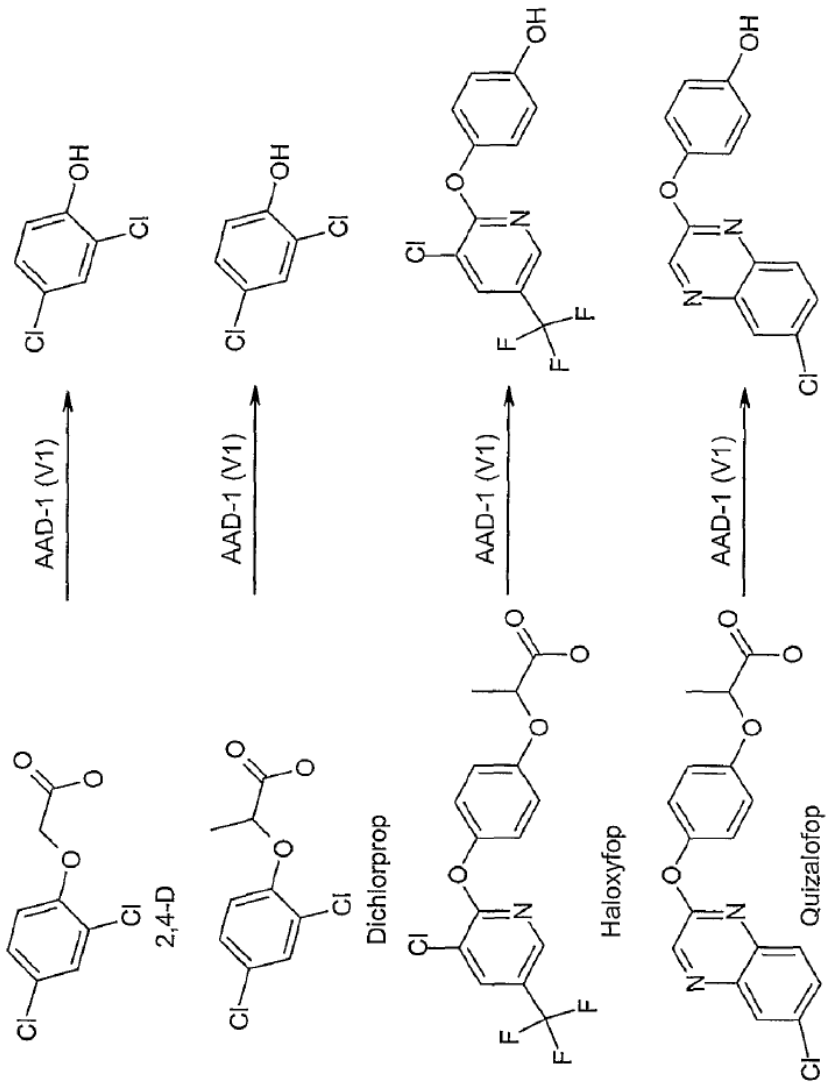

AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
2,4-D
Dichlorprop
Haloxyfop
Quizalofop

Fig. 4

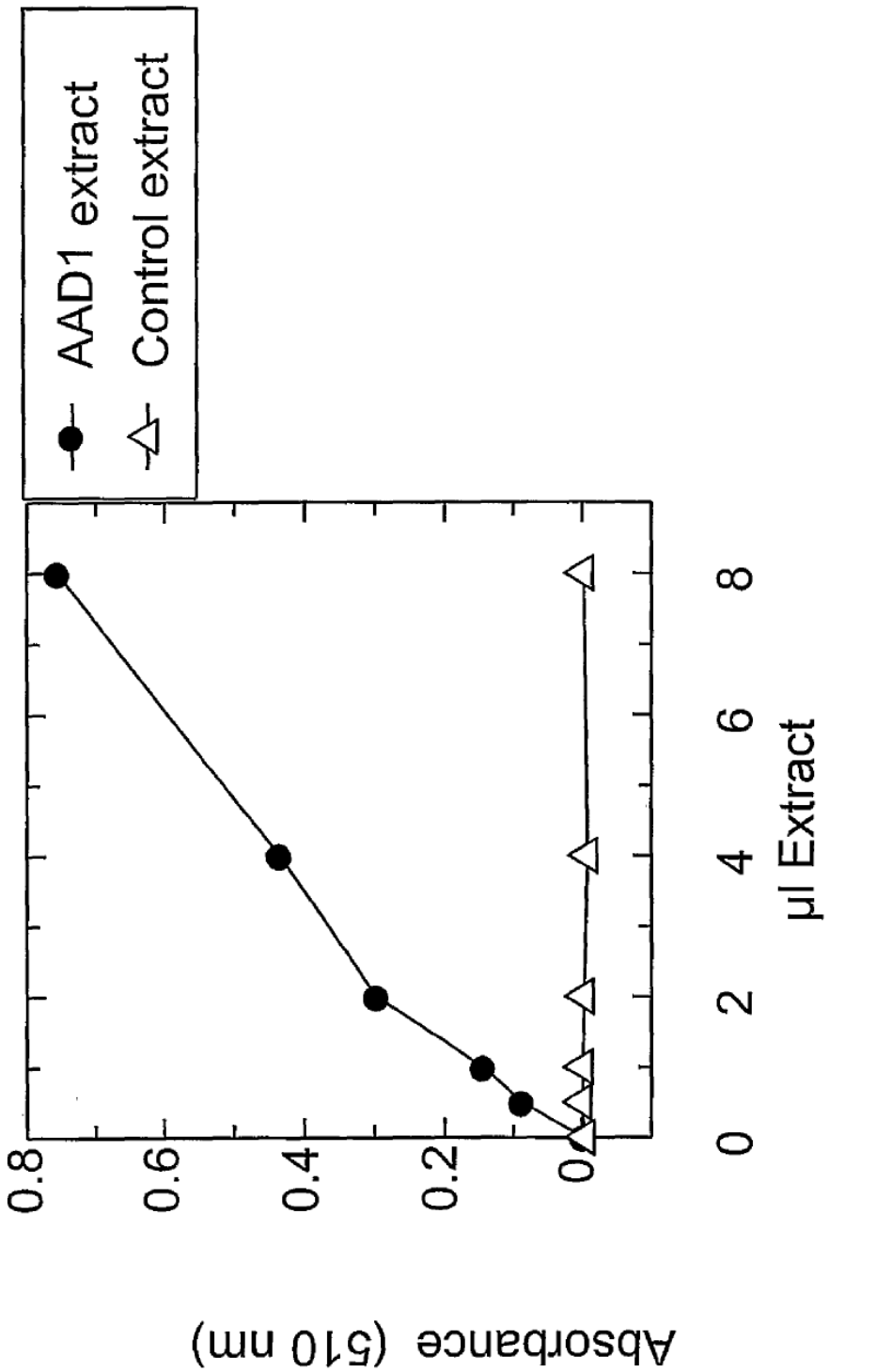

AAD1 extract
Control extract
Absorbance (510 nm)
0.8
0.6
0.4
0.2
0
0
2
4
6
8
µl Extract

Fig. 5

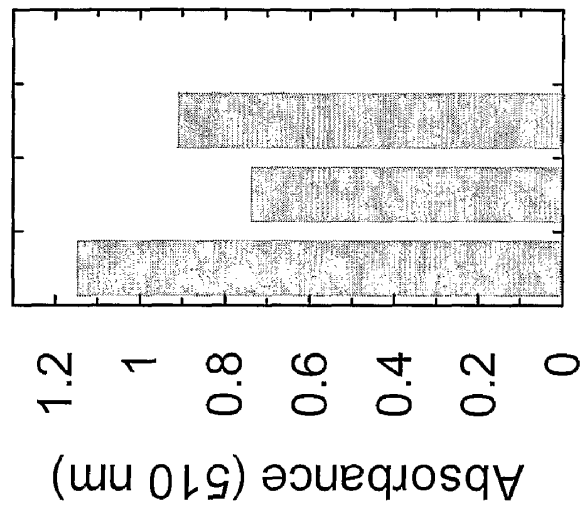

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

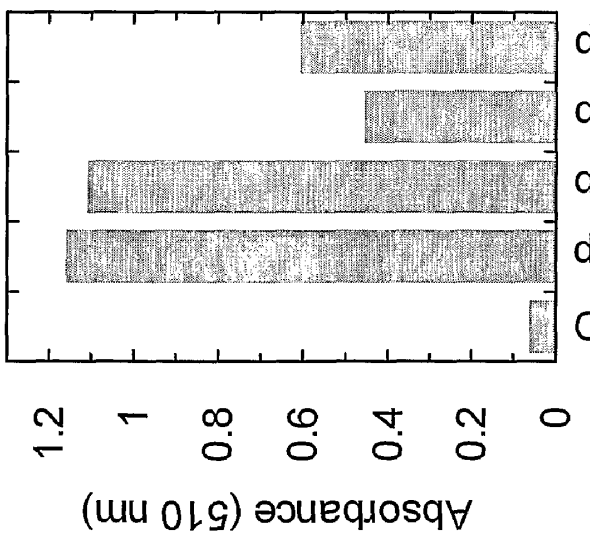

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

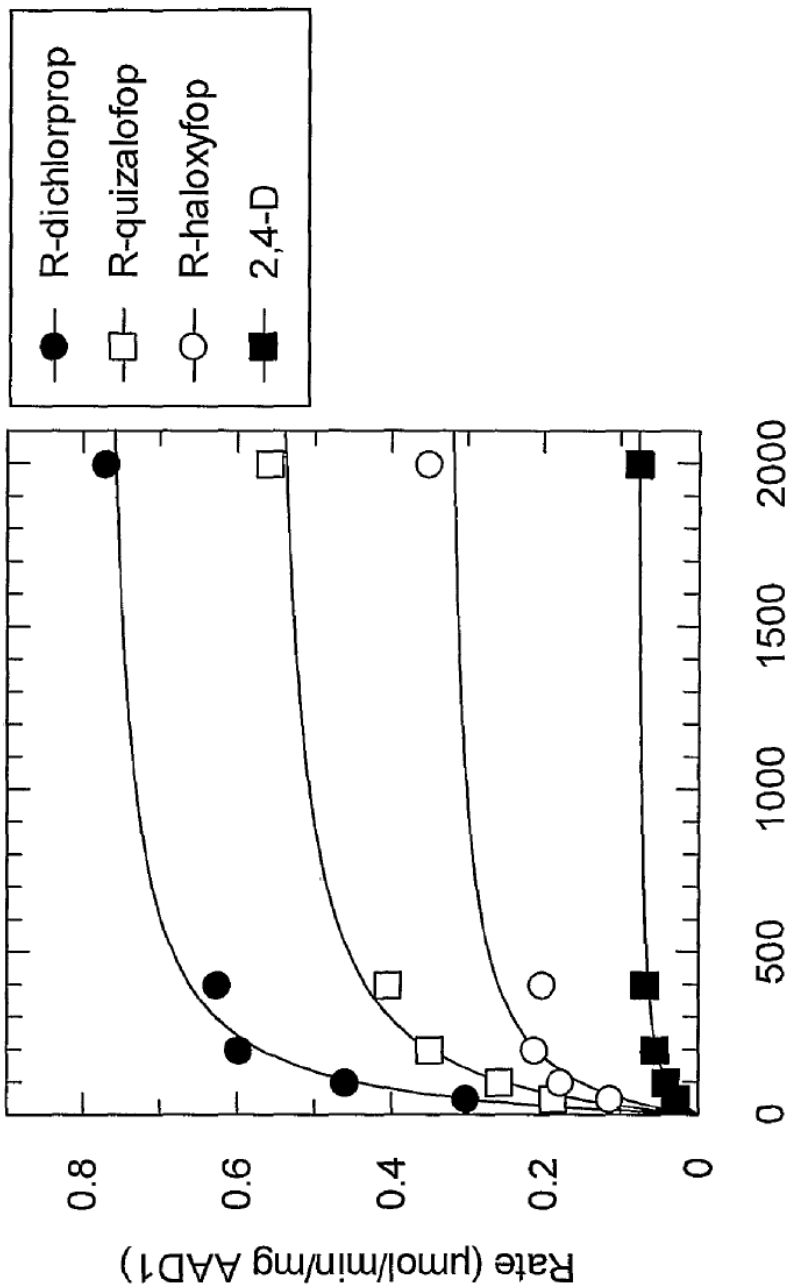

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (μmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (μM)

Fig. 7

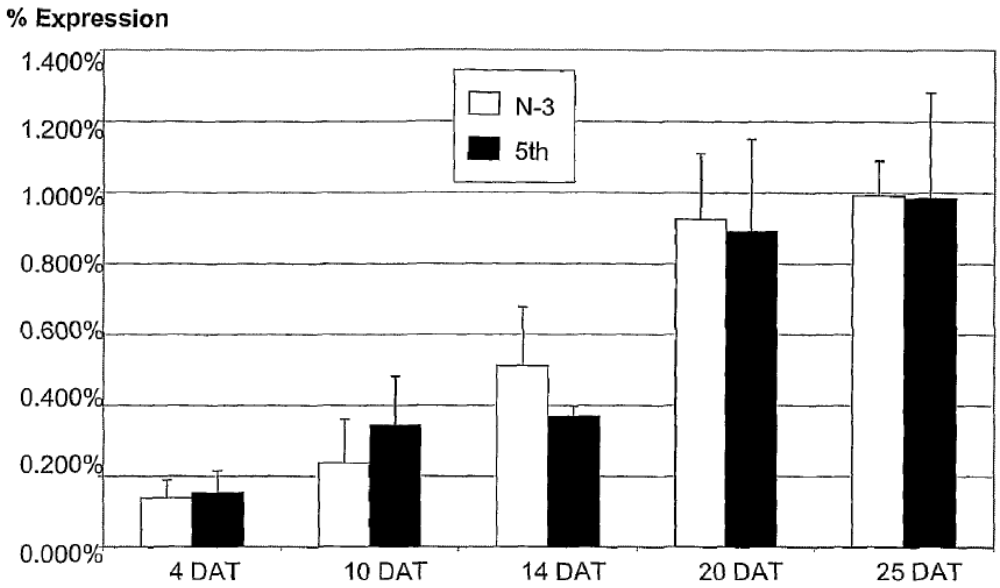

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

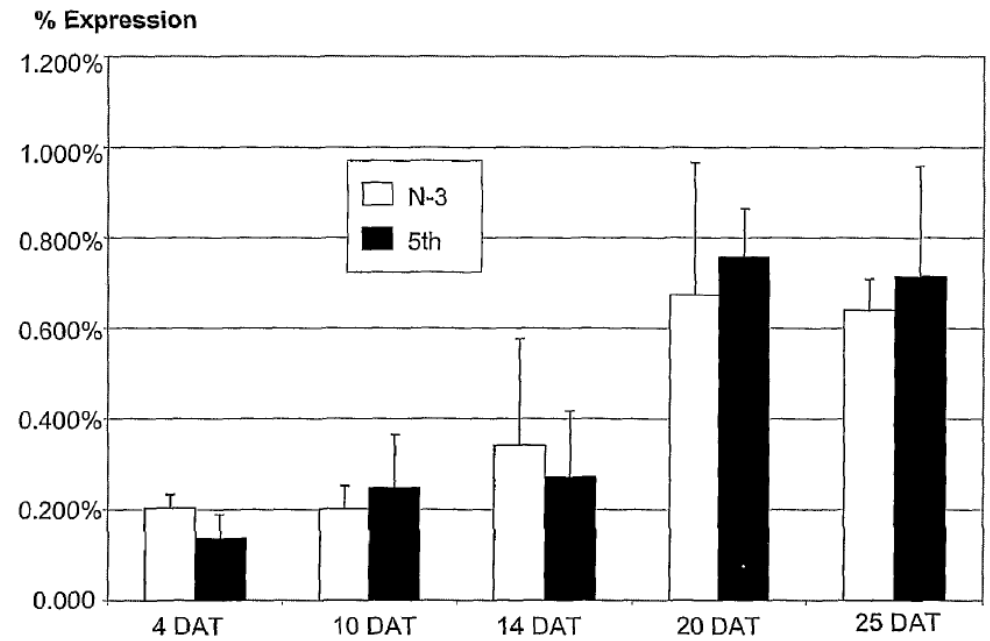

% Expression
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

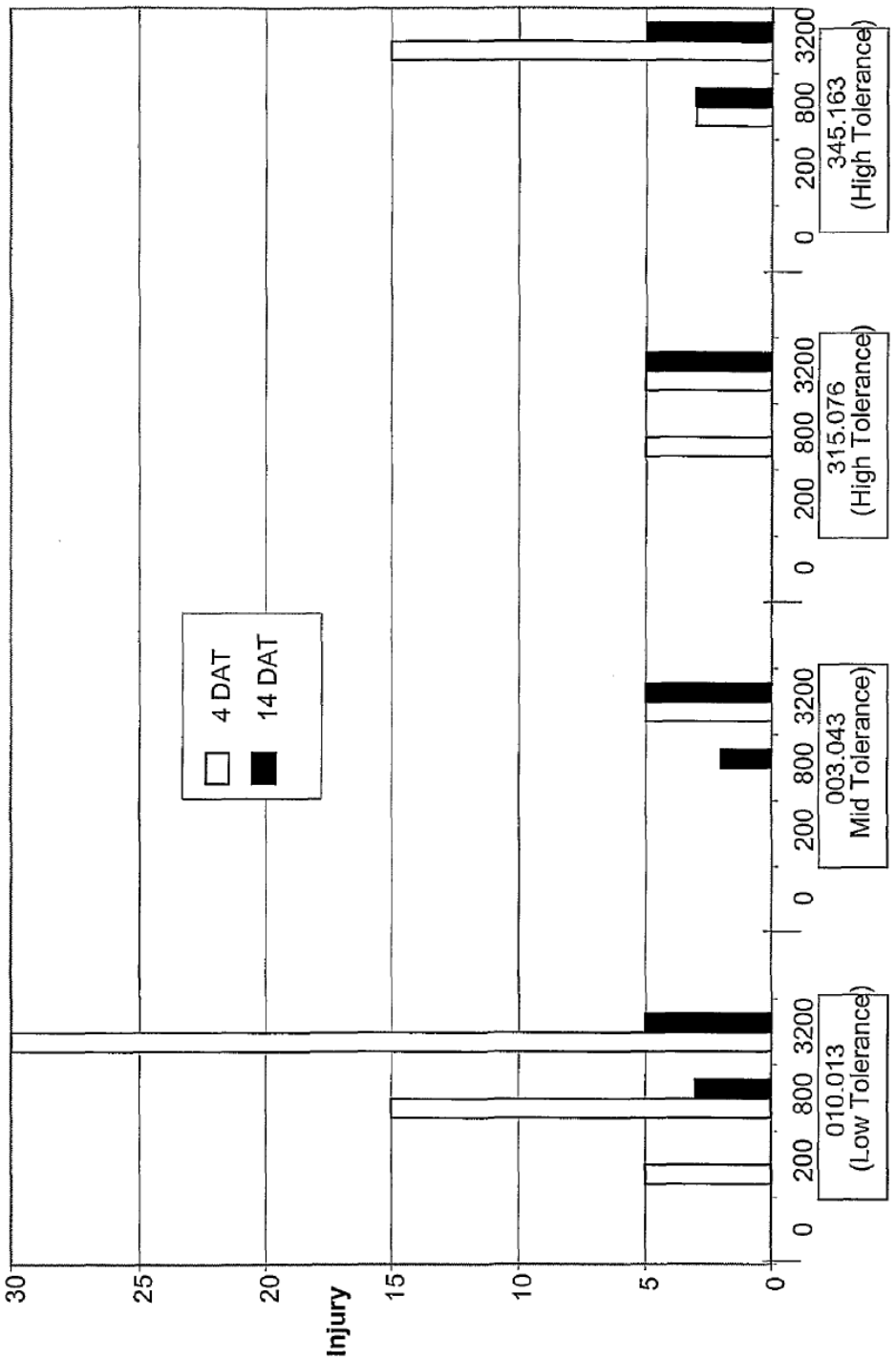

Injury
30
25
20
15
10
5
0
4 DAT
14 DAT
0
200
800
3200
010.013
(Low Tolerance)
003.043
Mid Tolerance)
315.076
(High Tolerance)
345.163
(High Tolerance)

Fig. 9A

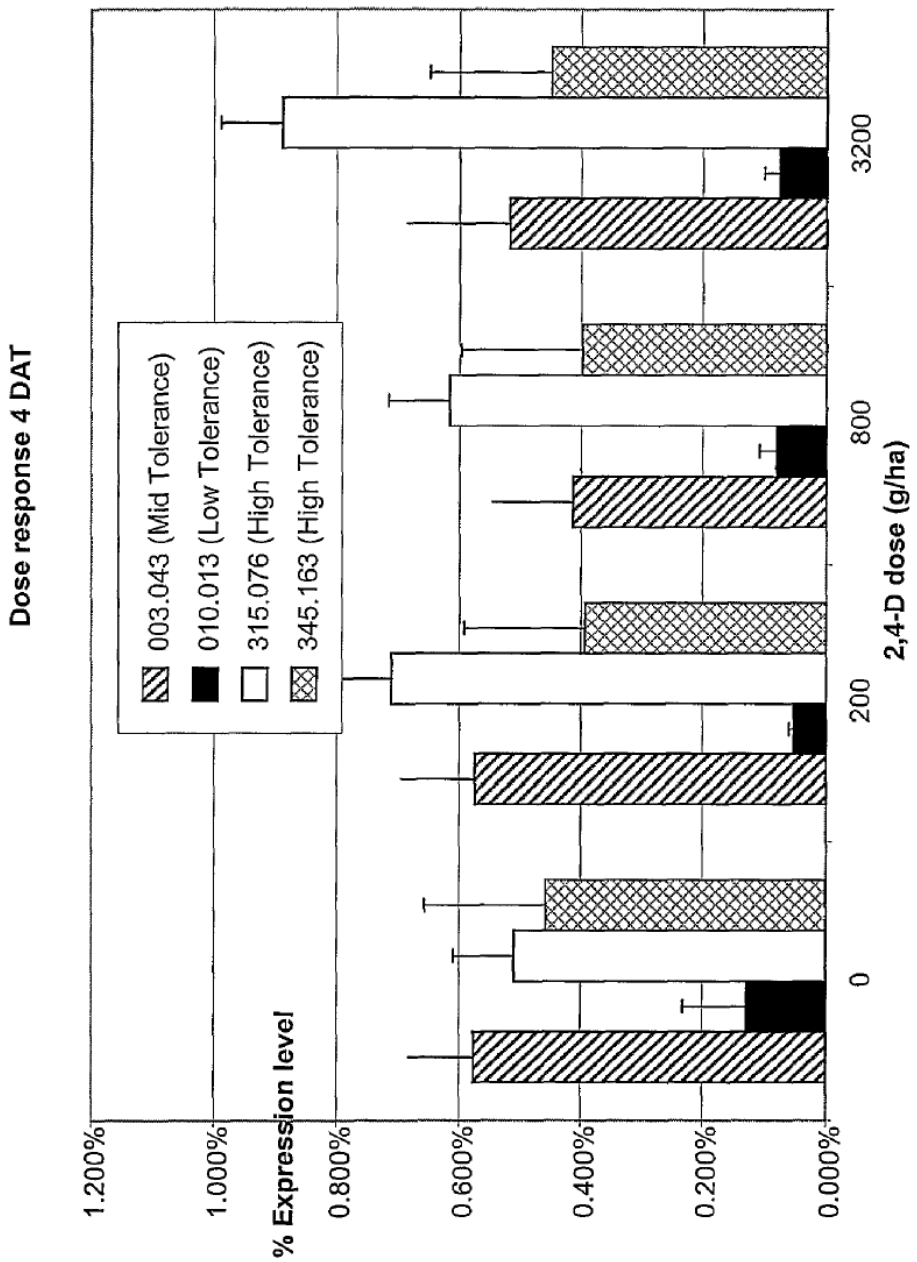

Dose response 4 DAT
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

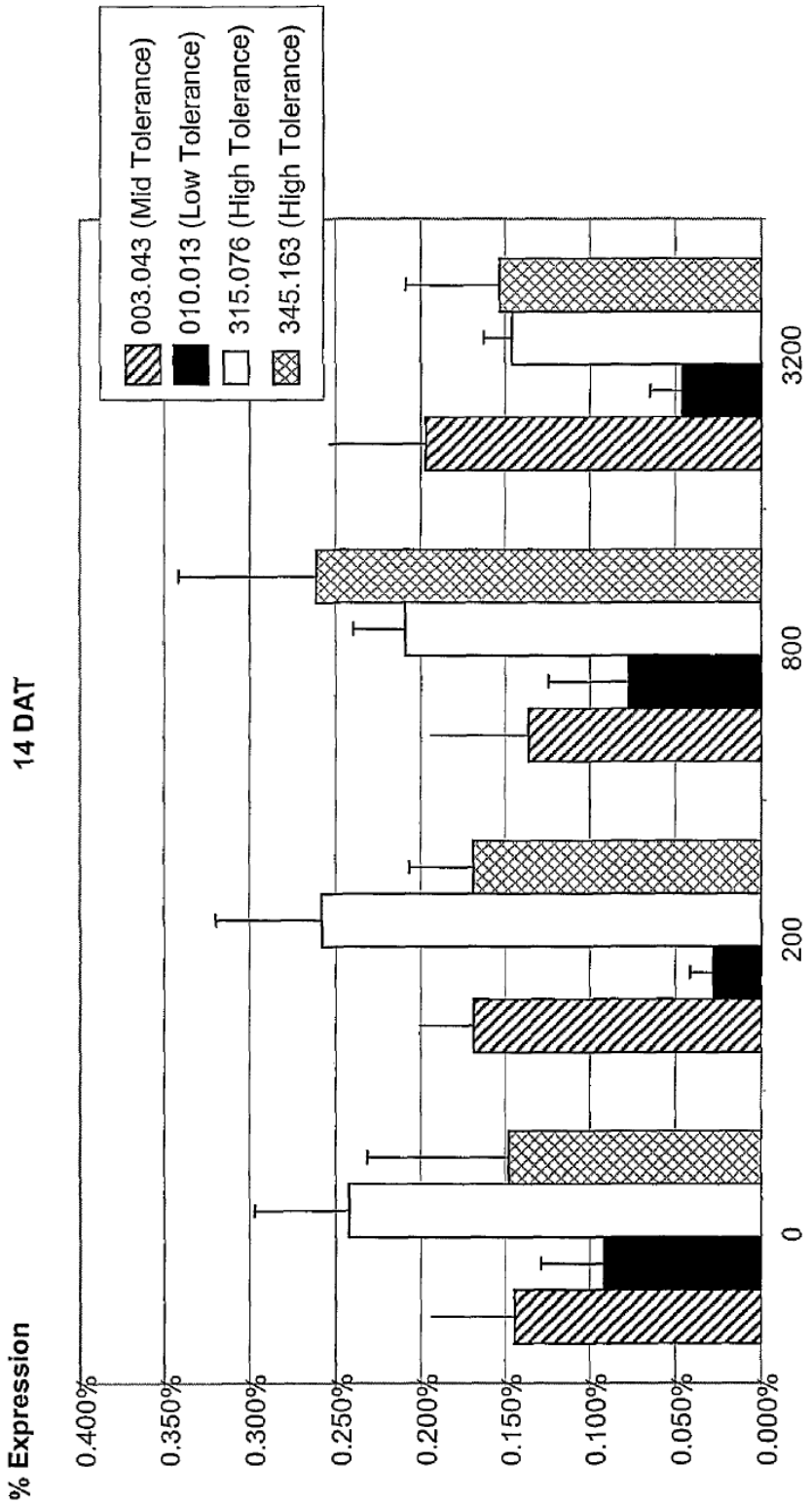

003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
14 DAT
% Expression
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

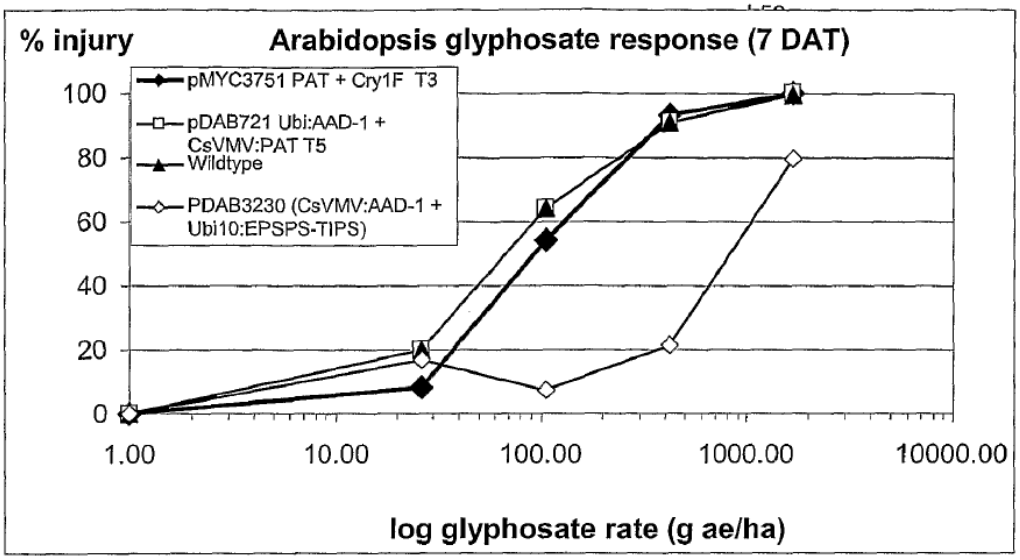

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

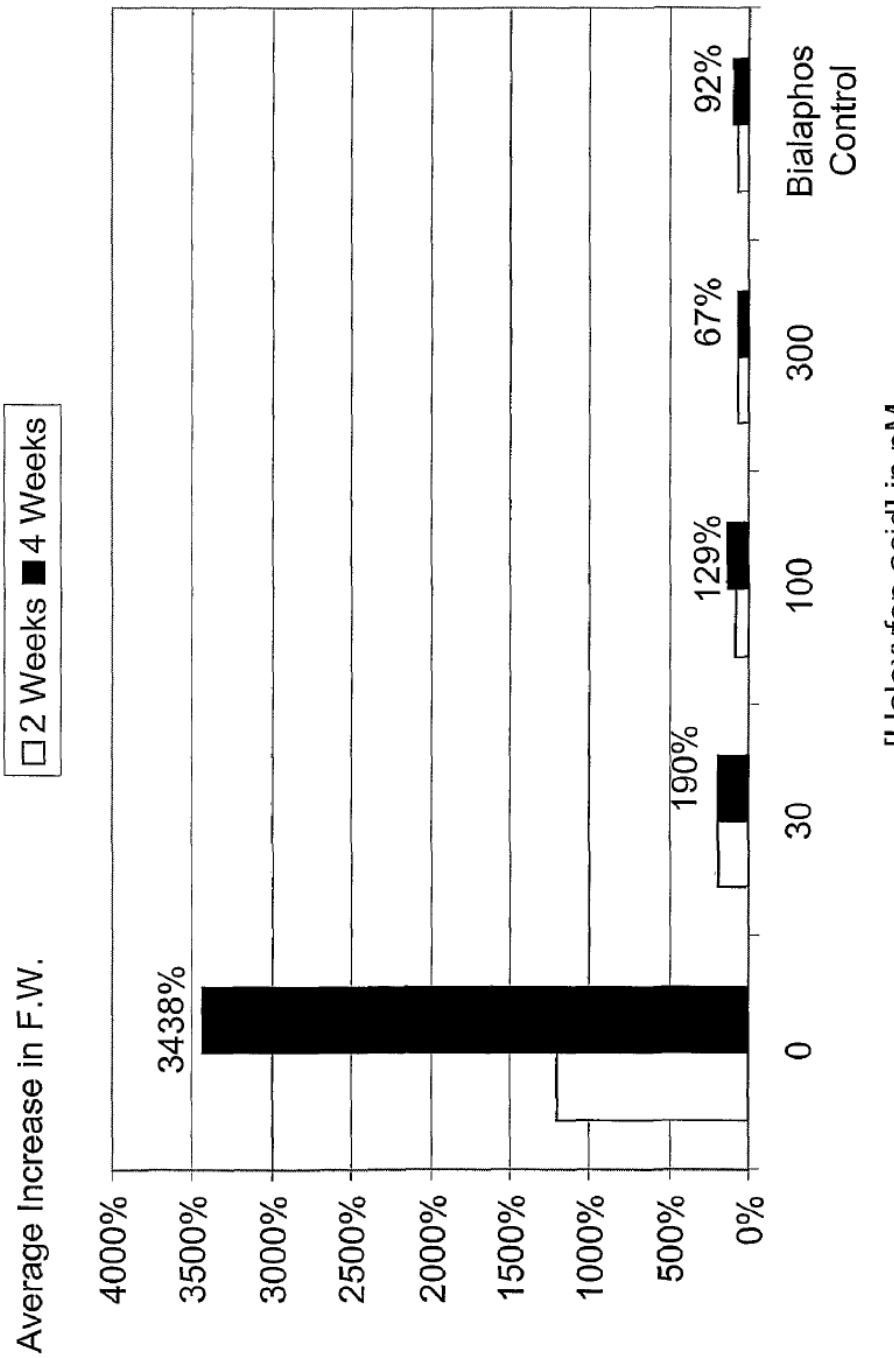

Average Increase in F.W.
□2 Weeks ■4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

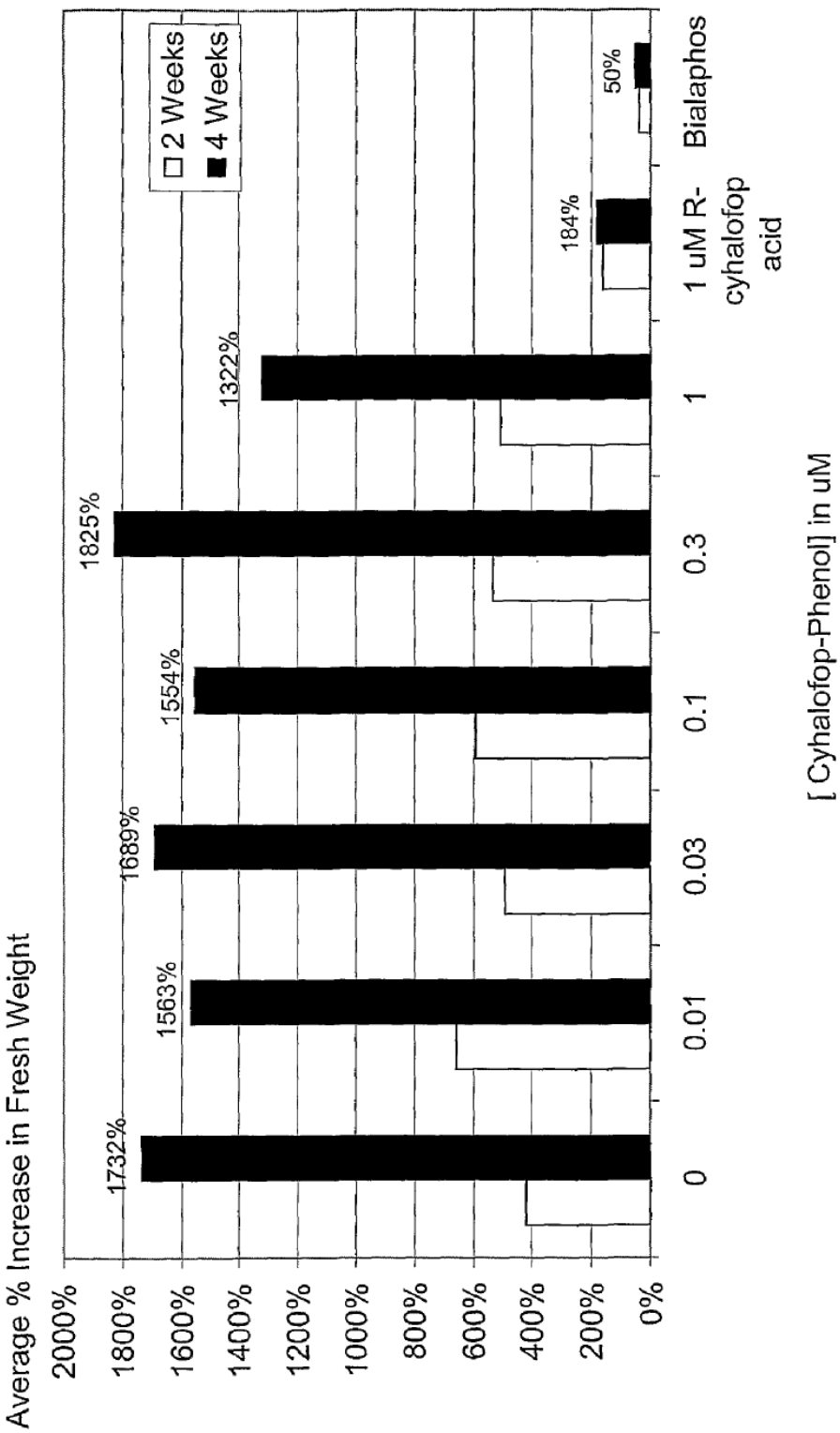

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
2 Weeks
4 Weeks
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

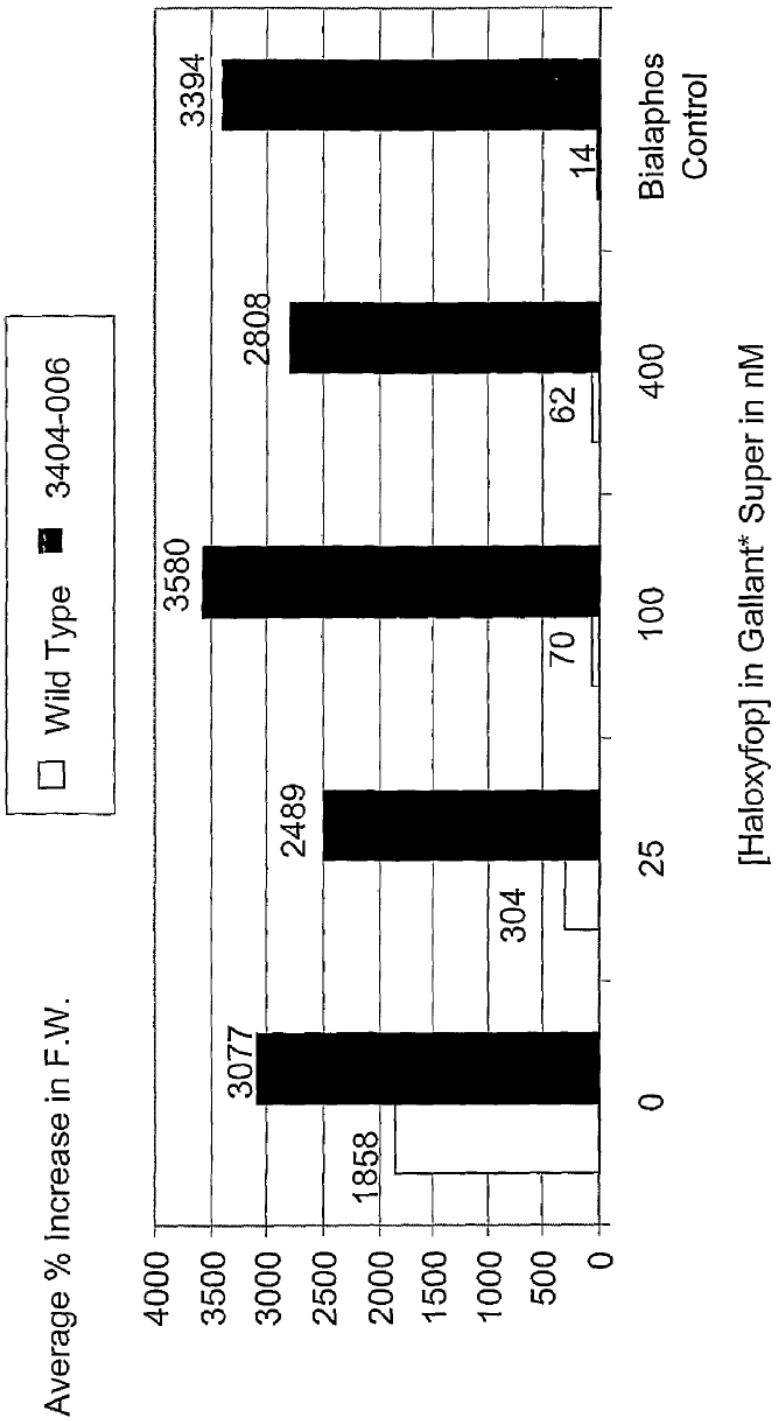

Wild Type
3404-006
Average % Increase in F.W.
4000
3500
3000
2500
2000
1500
1000
500
0
3077
1858
2489
304
3580
70
2808
62
3394
14
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

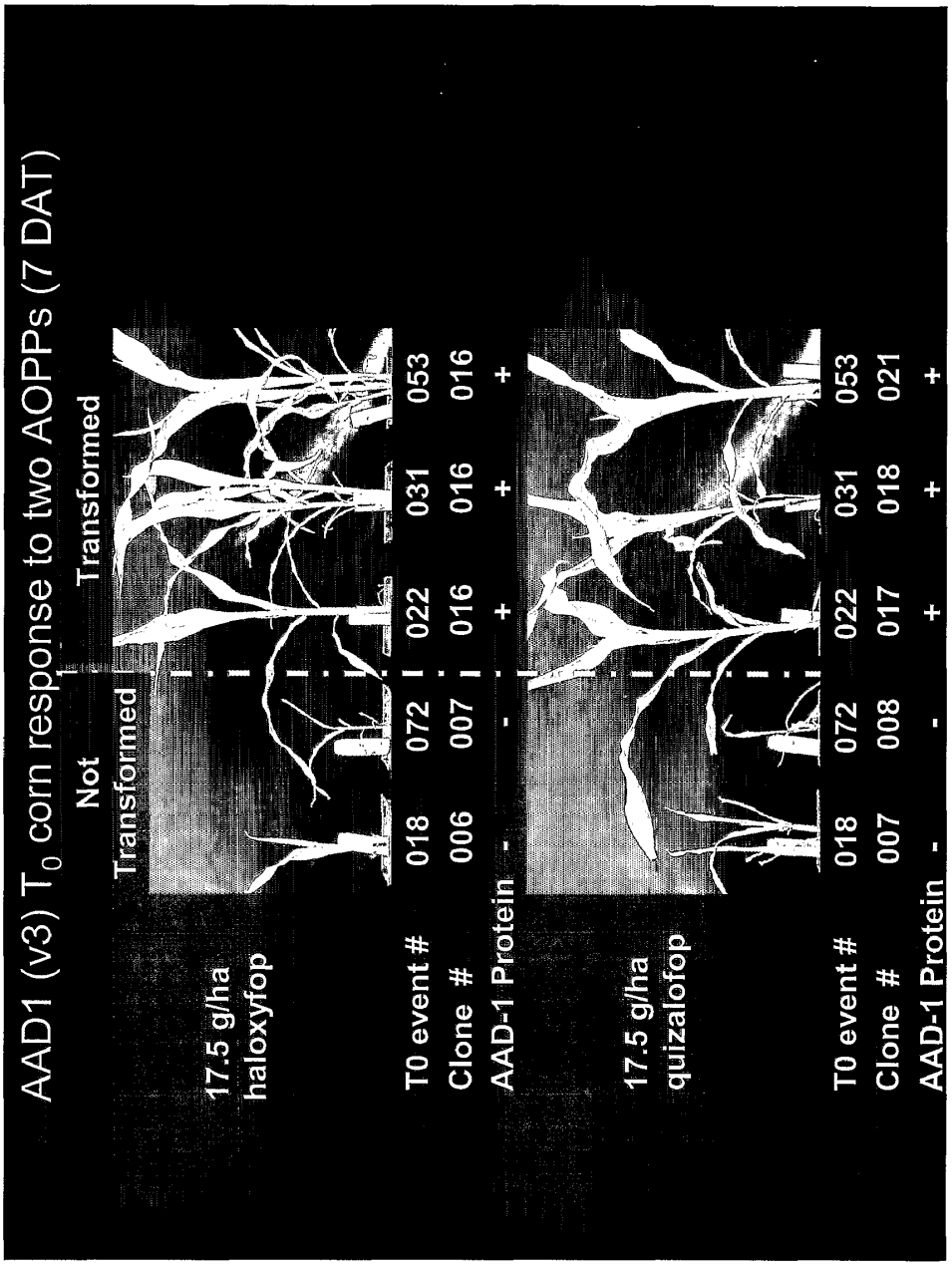

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

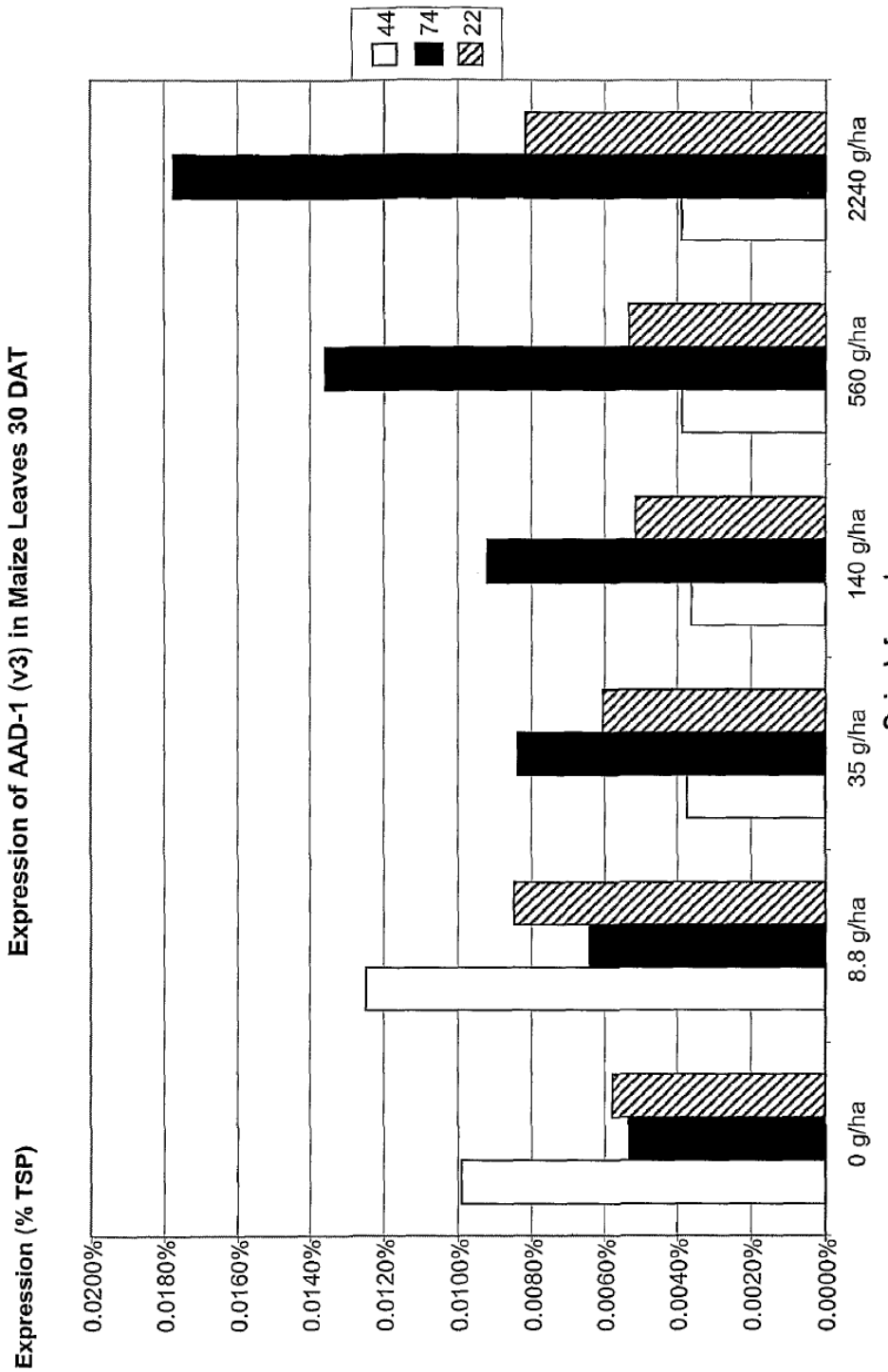

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

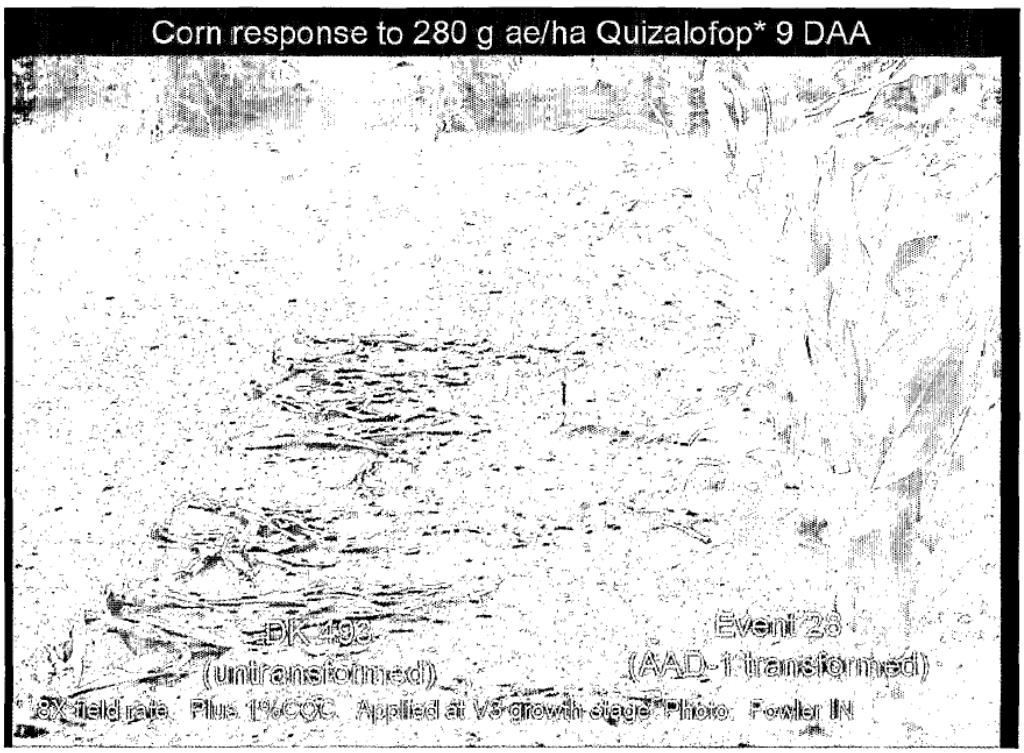

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 2s
(AAD-1 transformed)
2X field rate. Plus 1%COC. Applied at V3 growth stage. Photo: Fowler IN

Fig. 16

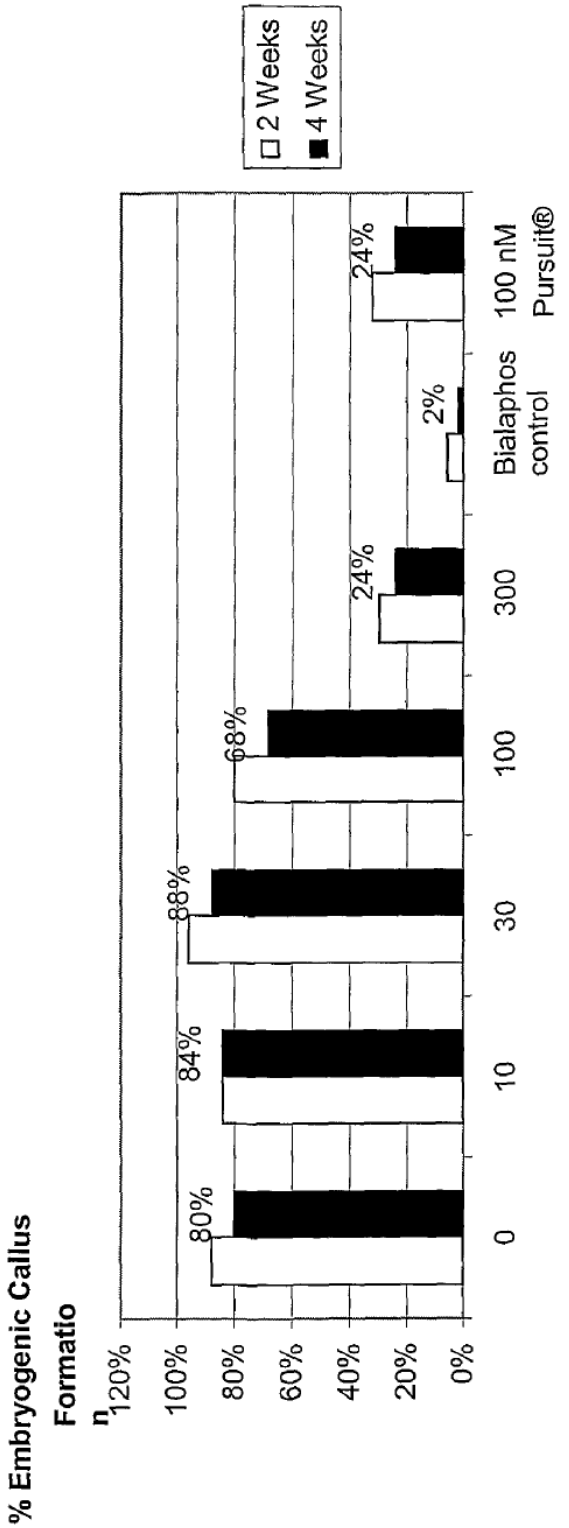
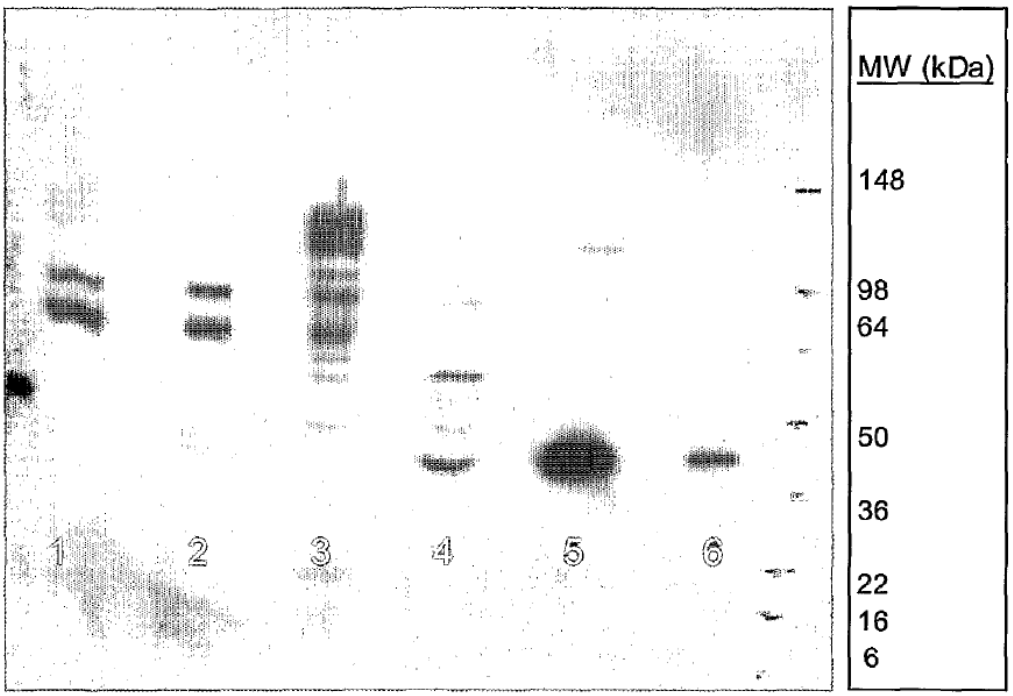

2 Weeks
4 Weeks
% Embryogenic Callus Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
[R-cyhalofop ACID] in nM

Fig. 17



Western

Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

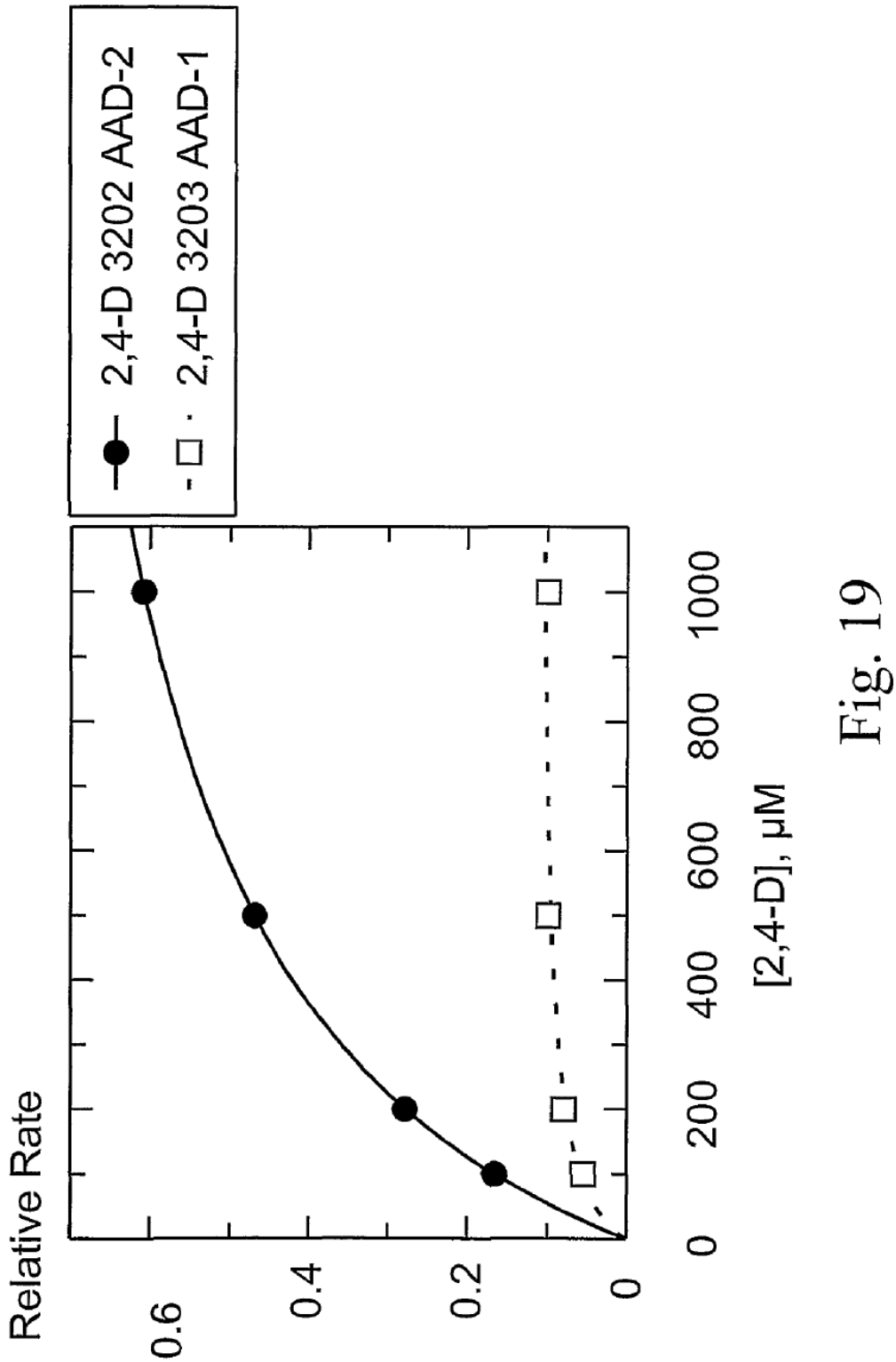

2,4-D 3202 AAD-2
2,4-D 3203 AAD-1
Relative Rate
0
0.2
0.4
0.6
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

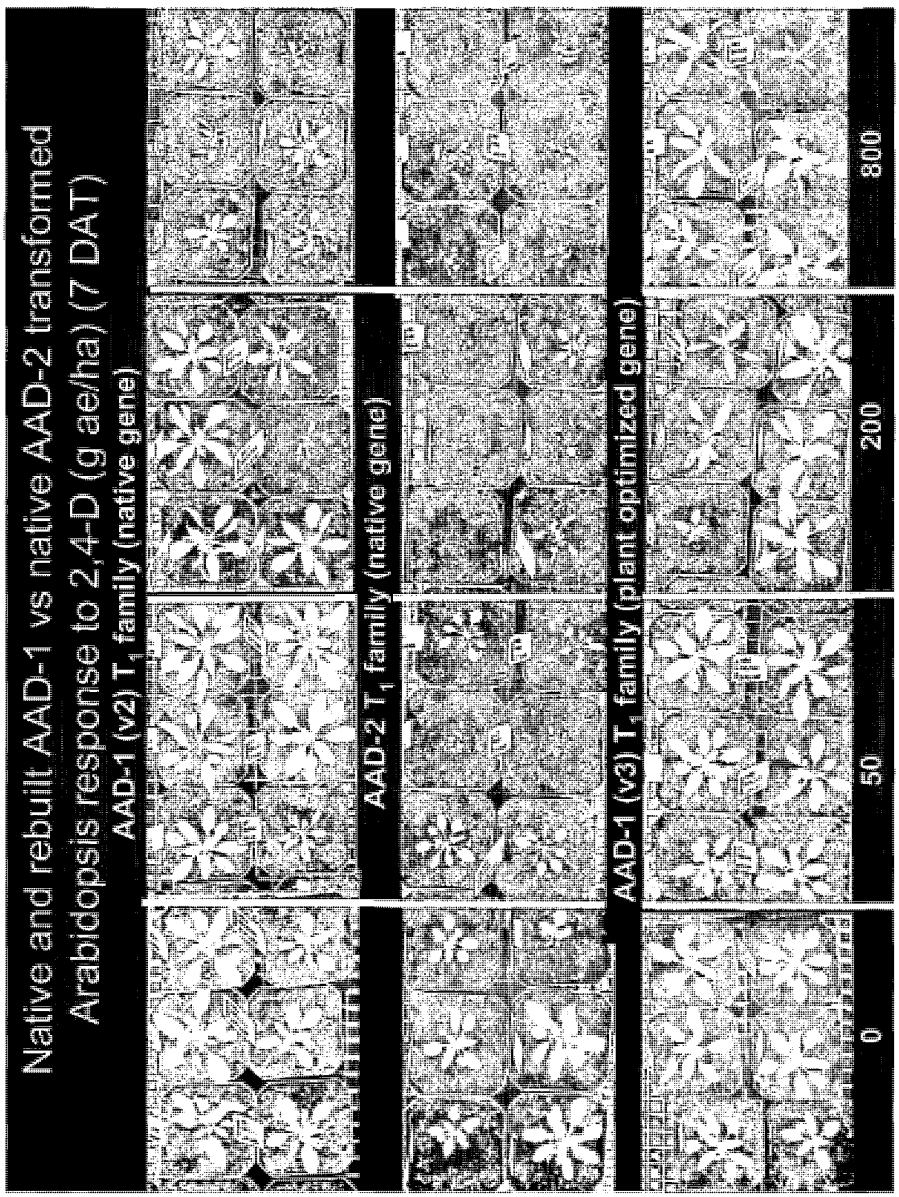

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

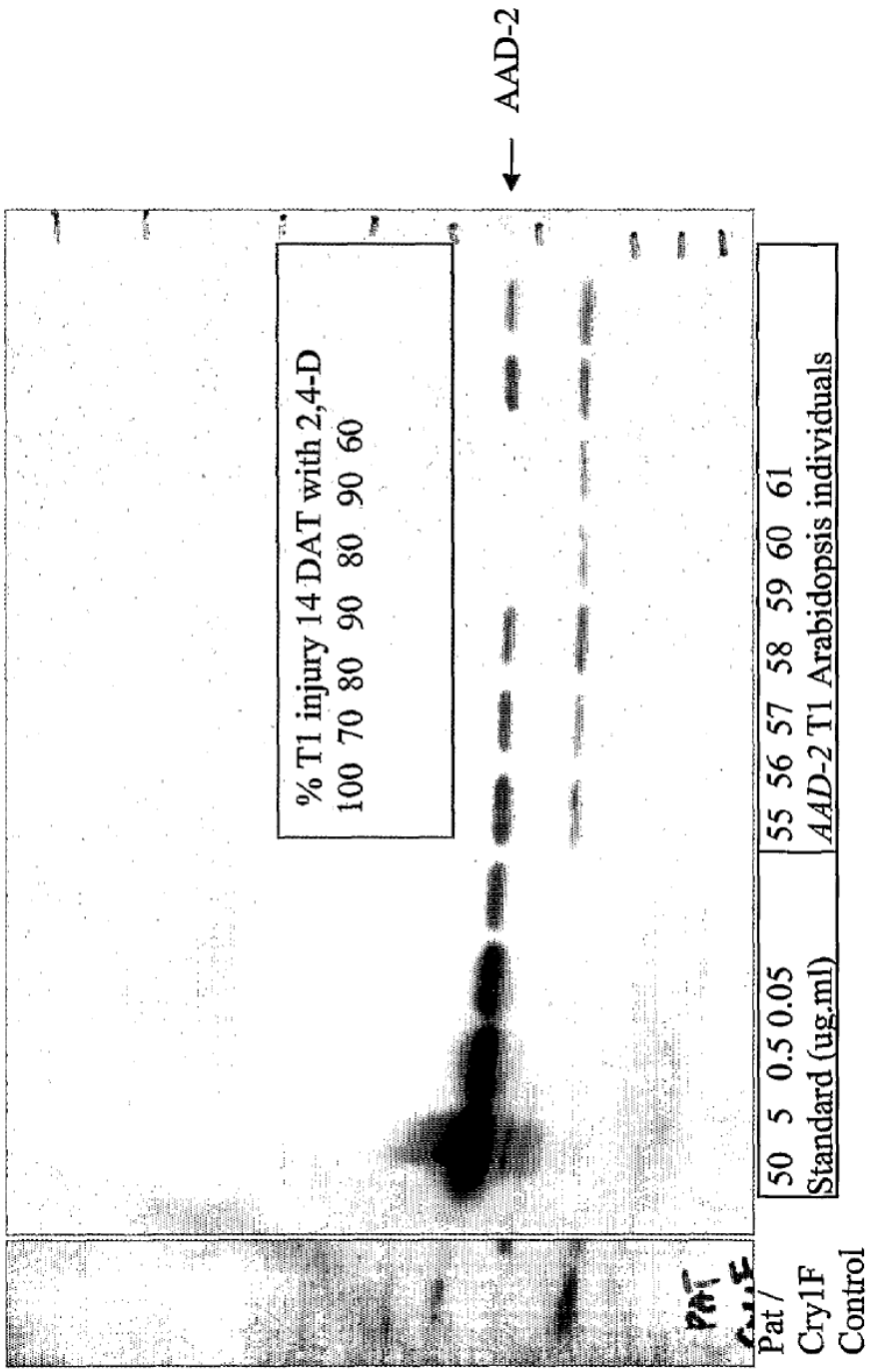

AAD-2
% T1 injury 14 DAT with 2,4-D
100 70 80 90 80 90 60
50 5 0.5 0.05
Standard (ug.ml)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
Pat /
Cry1F
Control

Fig. 21

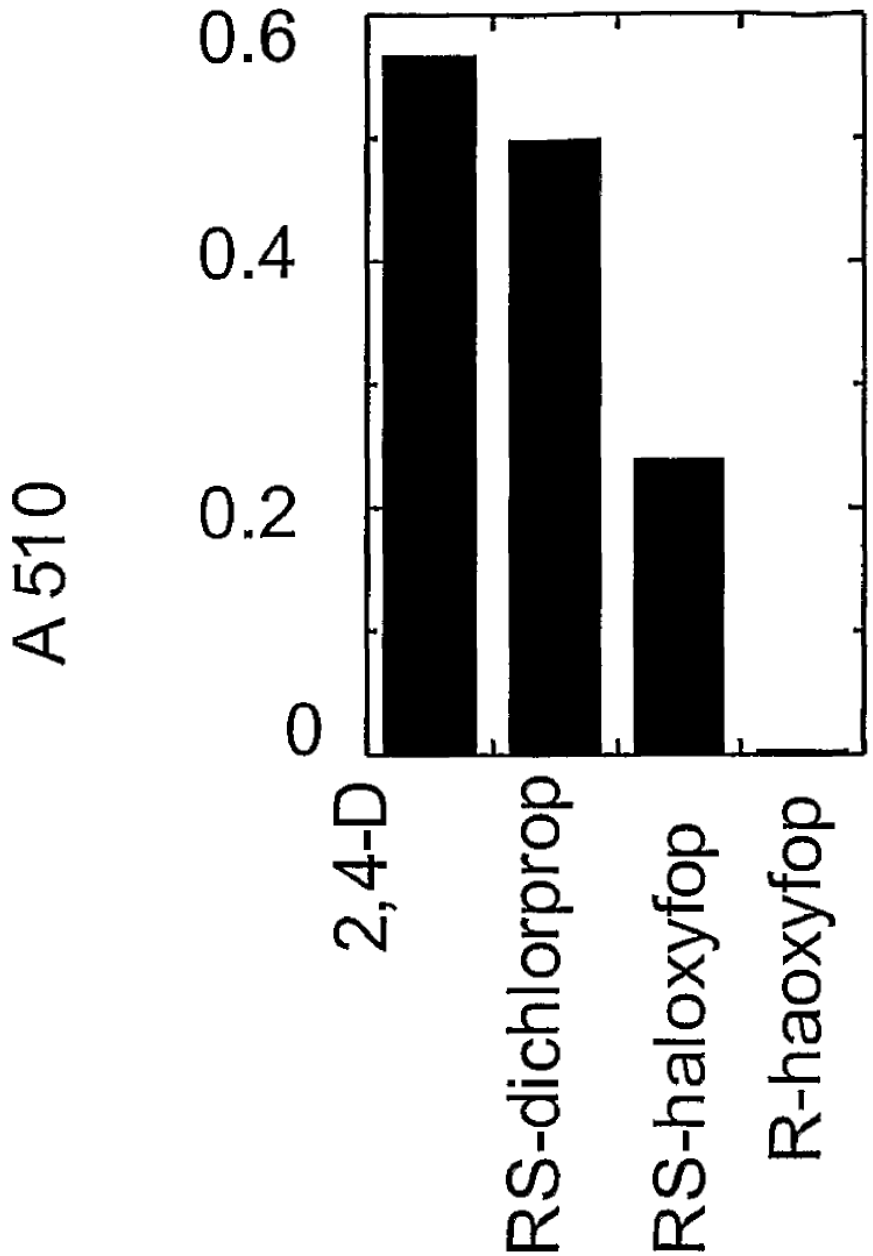


Fig. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCES TO RELATED APPLICATIONS

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

BACKGROUND OF THE INVENTION

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

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

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

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

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

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

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

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

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

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstoizia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the mineralization pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. TfdA catalyzes the con-

version of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, there are a number of homologues that have a significantly lower identity to tfdA (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

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

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

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

BRIEF SUMMARY OF THE INVENTION

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

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

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

There was no prior motivation to produce plants comprising an AAD-1 gene (preferably an AAD-1 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, et al.). Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an AAD-1 gene. The subject invention also provides methods of using an AAD-1 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-1, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of

preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

BRIEF DESCRIPTION OF THE FIGURES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

BRIEF DESCRIPTION OF THE SEQUENCES

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

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

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

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

SEQ ID NO:5 is a "plant-optimized" DNA sequence AAD-1 (v3). This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

SEQ ID NO:6 ("rdpA(ncoI)") and SEQ ID NO:7 ("3'saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

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

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

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

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

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

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

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

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

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

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

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

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

SEQ ID NO:21 is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threo-

nine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

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

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

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

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

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

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

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

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

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-1 gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

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

TABLE 1

Commercially available phenoxy auxins.

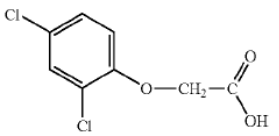
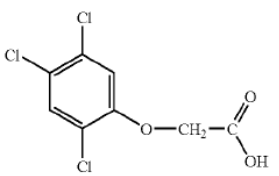
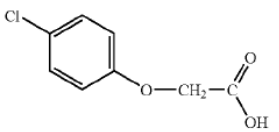
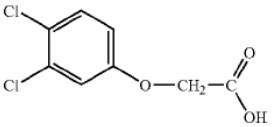
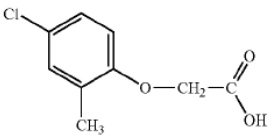
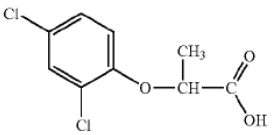
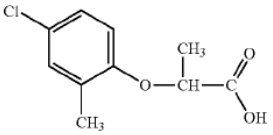
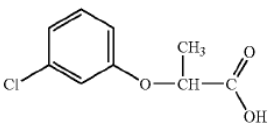
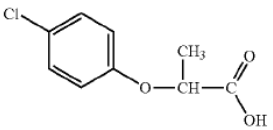
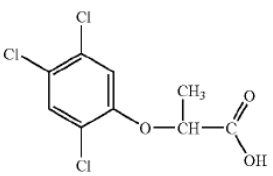
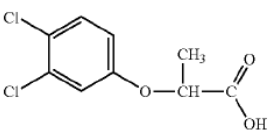
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Dichlorprop	120-36-5	25-12000	100-2240	
Mecoprop	7085-19-0	25-4000	250-3360	
Cloprop	101-10-0	25-4000	25-4000	
4-CPP	3307-39-9	25-4000	25-4000	
Fenoprop	93-72-1	25-4000	25-4000	
3,4-DP	3307-41-3	25-4000	25-4000	

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

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

TABLE 2

AOPP graminicidal compounds listed by accepted common names.

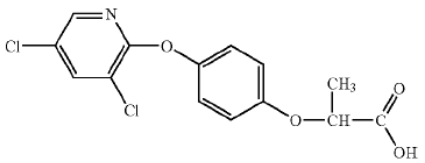
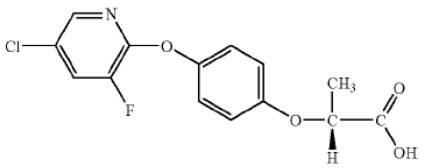
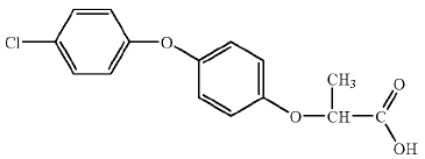
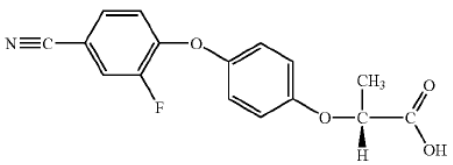
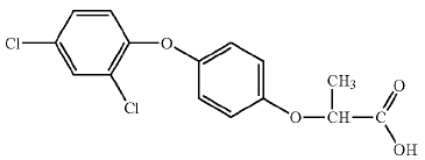
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

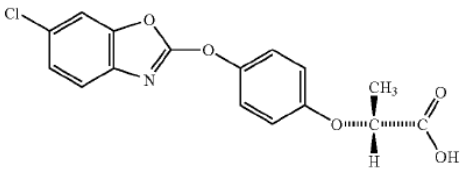
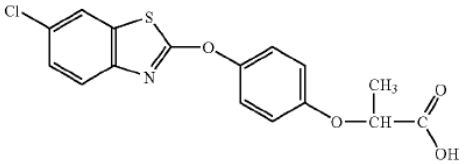
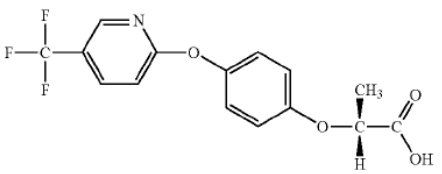
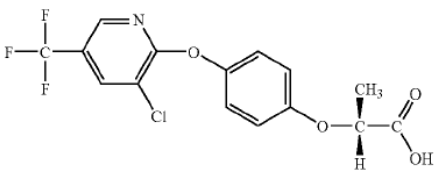
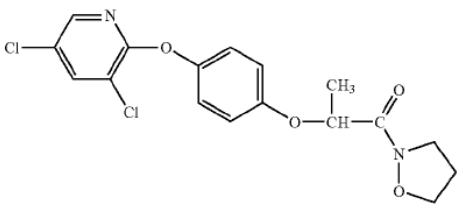
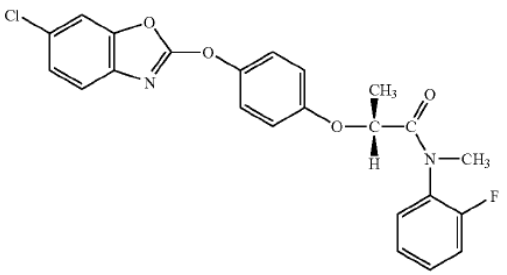
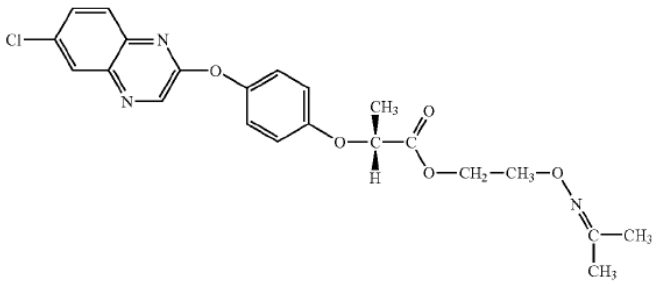
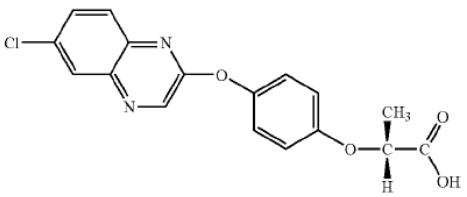
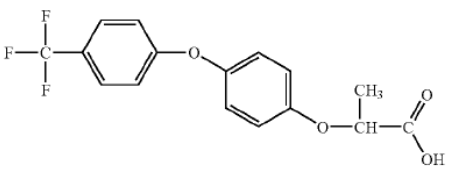
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop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

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

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

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-1 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and AOPP herbicide(s) (e.g., quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications

in a crop with a glyphosate resistance gene/AAD-1 stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

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

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

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

Additionally, AAD-1 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

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

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-1 (AryloxyAlkanoate Dioxygenase) genes and proteins.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100% conversion) in vitro. An additional advantage that AAD-1-transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

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

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

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

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

acid identity) and have similar enzymatic properties to tfdA. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to tfdA.

RdpA, from *Sphingobium herbicidovorans* (Westendorf et al, 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath et al., 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf et al., 2003). Heterologous expression of rdpA in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of tfdA that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that rdpA or AAD-1 genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

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

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

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

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

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

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

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

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

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

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

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

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

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

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

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

TABLE 3

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

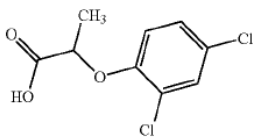
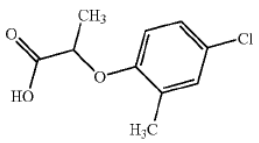
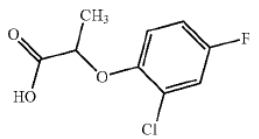
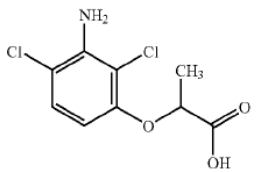
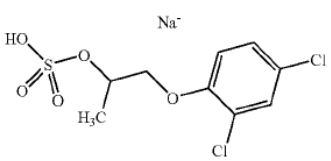
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro,4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687

TABLE 3-continued

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

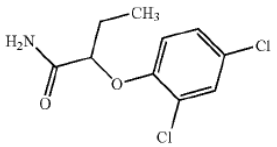
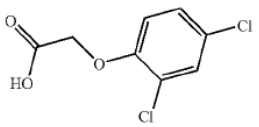
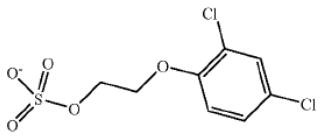
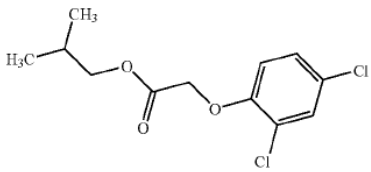
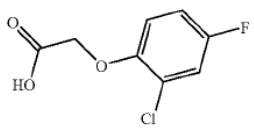
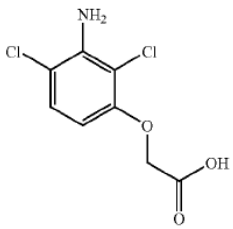
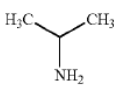
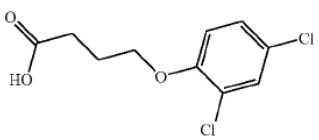
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
				

TABLE 3-continued

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

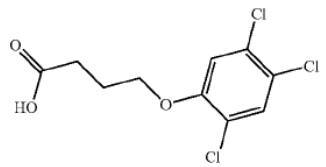
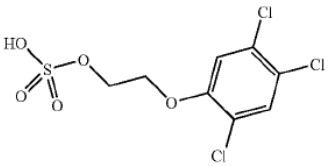
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
Na⁻	178587		0.003	0.02
				
Na⁻	188527		0.003	0.036
				

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

TABLE 4

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

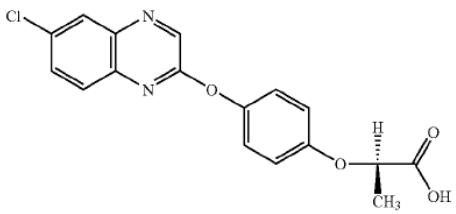
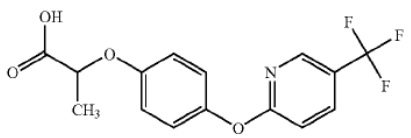
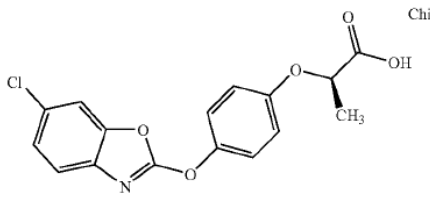
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
	Chiral 11044492	(R)-fenoxaprop	0.408	0.597

TABLE 4-continued

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

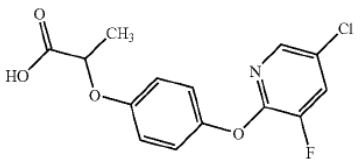
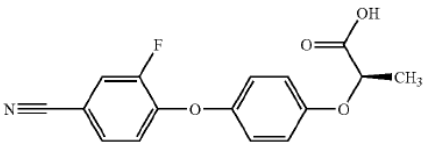
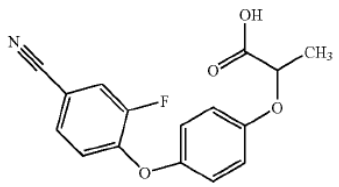
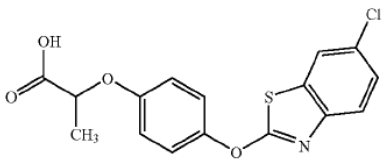
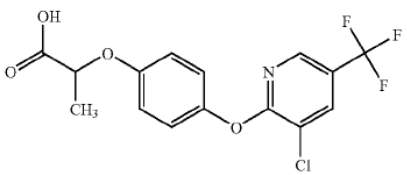
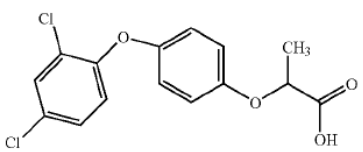
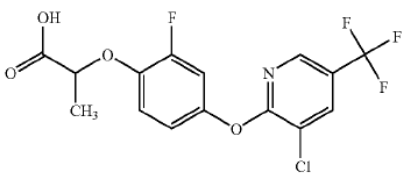
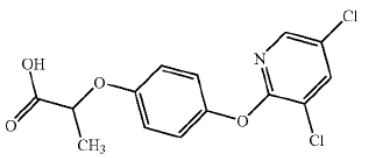
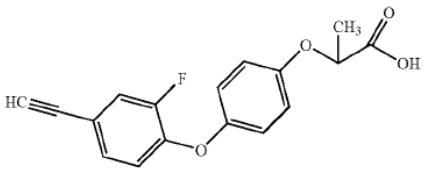
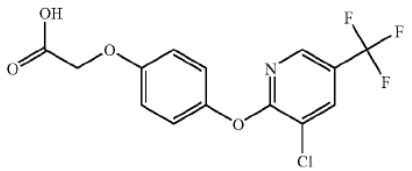
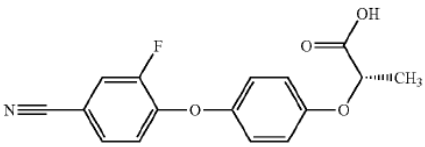
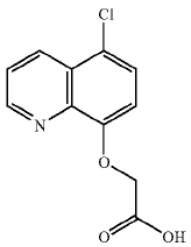
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69

TABLE 4-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

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

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional

activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

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

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

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known rdpA's, such as *Sphingobium, Delftia, Rodoferax*, and *Comamonas* for example, can be used as source isolates.

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

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

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

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

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-1 gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein in vivo, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

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

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

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide

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

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

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

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

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

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

Washes can typically be carried out as follows:

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

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

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

$$Tm(° C.) = 2(\text{number } T/A \text{ base pairs}) + 4(\text{number } G/C \text{ base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

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

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

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

Low: 1 or 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

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

PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683, 195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential

accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

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

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

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

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

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

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

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40,

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

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

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

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 5 provides a listing of examples of amino acids belonging to each class.

TABLE 5

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

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

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

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

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

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

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

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

Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject

invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

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

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

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

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

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

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

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. 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. 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, mono-

cotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

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

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

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

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

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

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

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

such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

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

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

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

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

EXAMPLE 1

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

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with <50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

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

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

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

Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following conditions: 94° C. 45 sec, 52° C. 1.5 minute, 72° C. 1.5 minute, for 30 cycles, followed by a final cycle of 72° C. 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

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

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

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

EXAMPLE 2

Expression and Testing

2.1—HPLC Analysis.

Plasmid pDAB 3203 was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

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

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

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

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

2.2—Plate Test Bioassays for Herbicide Degradation.

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

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

Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×5-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

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

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

TABLE 6

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

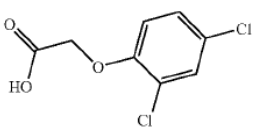
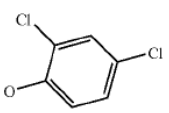
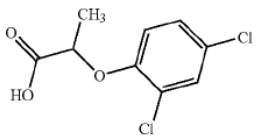
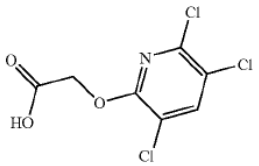
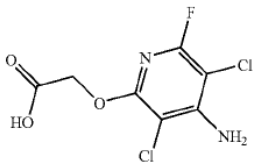
	GR_{50} (nM)				
Chemical tested	Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR_{50} ratio**	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	

TABLE 6-continued

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

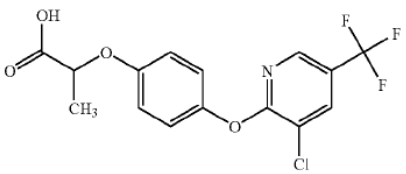
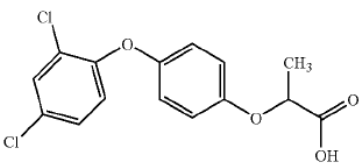
Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR_{50} ratio**	Structure
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

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

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

TABLE 7

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

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

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

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

2.3—HPLC Results.

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

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

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

ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (FIG. **3**) and diclofop. These results were also confirmed by the calorimetric assay, which was also used to test a wider sampling of these compounds.

2.4—Plate Test Bioassays for Herbicide Degradation.

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

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

EXAMPLE 3

In Vitro Assay of AAD-1 (v1) Activity via Colorimetric Phenol Detection

3.1—AAD-1 (v1) Assay.

AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily detected. Pyridinols derived from fluoroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to 500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

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

3.2—Extraction.

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

eluant stored in aliquots at −70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding AAD-1 (v1) was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without AAD-1 (v1) using 1 mM 2,4-D and is shown in FIG. **5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

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

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

EXAMPLE 4

Substrate Specificity of AAD-1 (v1)

4.1—Additional Substrates of AAD-1 (v1).

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

Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (Table 8). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7-fold better than 2,4-D as a substrate (Table 3). The 5-F substitution appears to decrease the effectiveness of X1115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

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

TABLE 8

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

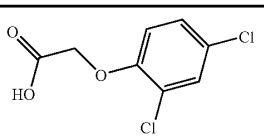
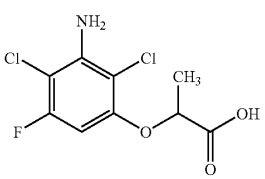
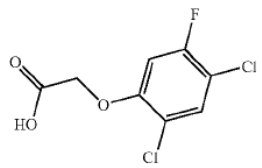
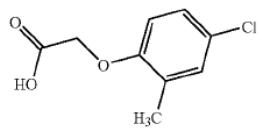
Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125

TABLE 8-continued

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

Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

4.2—Kinetic Characterization.

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

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

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

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

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

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

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

EXAMPLE 5

Optimization of Sequence for Expression in Plants

5.1—Background.

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

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant

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optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

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

TABLE 10

Compilation of G + C contents of protein coding regions of maize genes

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

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

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

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 11

Preferred amino acid codons for proteins expressed in maize

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

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

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

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5)

was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

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

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

5.3—Completion of Binary Vectors.

5.3.1—Rebuilt AAD-1 (v3. The plant optimized gene AAD-1 (v3) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-1 (v3) DNA sequence. The AAD-1 (v3) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB720, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-1 (v3): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: ADD-1 (v3): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

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

The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17)

primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

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

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

A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 AAD-1 (v2) construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

The modified AAD-1 (v2) gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI-NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: AAD-1 (v2): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: 0RF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

5.3.3—Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID NO:21.

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

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

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

TABLE 13

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

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	DNU	9.1
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	DNU*	8.5		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	DNU	9.8
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	DNU	8.2	LYS (K)	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	DNU	6.0	MET (M)	ATG	100.0	100
	CGT	16.9	14.5	PHE (F)	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	PRO (P)	CCA	39.8	36.5

TABLE 13-continued

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

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	DNU	8.3
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	SER (S)	AGC	16.0	15.1
END	TAA	DNU	40.7		AGT	18.2	17.1
	TAG	DNU	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	DNU	6.1
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	THR (T)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	DNU	8.3
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	TRP (W)	TGG	100.0	100
	GGT	30.4	30.4	TYR (Y)	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	VAL (V)	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

*DNU = Do Not Use

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

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

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

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

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species* Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	—	—	—
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—

TABLE 14-continued

Binary constructs used in transformation of various plant species.

3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—
3403		Cn, R	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3404		Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3415	1283	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3602	1421	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	—	—

pDAB #	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721	Erythromycin	—	pat	CsVMV	Agro binary
3230	Spectinomycin	—	AAD1 v3	CsVMV	Agro binary
3289	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291	Spectinomycin	—	HptII	AtUbi3	Agro binary
3295	Spectinomycin	—	pat	AtUbi10	Aerosol beam
3297	Spectinomycin	—	pat	AtUbi10	Agro binary
3403	Ampicillin	—	Same as GOI		Whiskers/ Gun
3404	Ampicillin	—	pat	OsAct1	Whiskers
3415	Ampicillin	—	AHAS v3	OsAct1	Whiskers
3602	Spectinomycin	—	AHAS v3	OsAct1	Agro Superbinary
3757	Spectinomycin	—	pat	AtUbi11	Agro binary
3705	Erythromycin	—	pat	CsVMV	Agro binary

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola
CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region
ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase
(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

EXAMPLE 6

Transformation into *Arabidopsis* and Selection

6.1—*Arabidopsis thaliana* Growth Conditions.

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

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

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

6.2—*Agrobacterium* Transformation.

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

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with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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

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

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

6.3—*Arabidopsis* Transformation.

Arabidopsis was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700×g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 µM benzylamino purine (10 µl/liter of 1 mg/ml stock in DMSO) and 300 µl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

6.4—Selection of Transformed Plants.

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

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

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

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

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young,

tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of 7-8.

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

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

6.5—Results of Selection of Transformed Plants.

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

TABLE 15

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

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

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

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

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

TABLE 16

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

	% Injury			% Injury	Std
Averages	<20%	20-40%	>40%	Ave	Dev
Native AAD-1 (v2) gene					
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2

TABLE 16-continued

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

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

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some AAD1 (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first in planta data that AAD-1 (v3) will provide AOPP resistance. No resistance was observed with fluoroxypyr (a pryridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with in vitro work using heterologously expressed enzyme.

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

TABLE 17

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

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

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

TABLE 18

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

Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev	Range
AAD-1 v3						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20

TABLE 18-continued

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

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

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

The ability to use AAD-1 (v3) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed transformed with PAT and AAD1 (v3) (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50 g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active PAT gene.

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

6.7—Heritability.

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

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

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

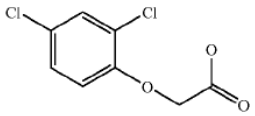
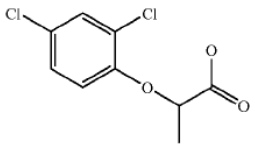
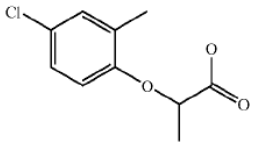
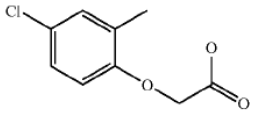
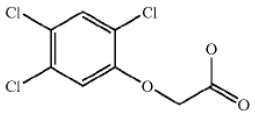
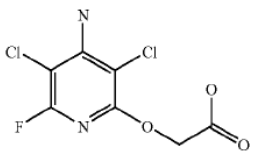
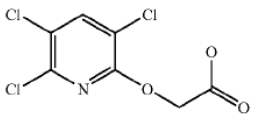
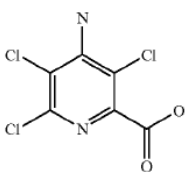
The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified in vitro plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4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

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

Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (150) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorpropand mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluoroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

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

Compound	Structure	wt ARBTH shoot 150 ppm	PAAD1.315.064 T3 ARBTH shoot 150 ppm	wt ARBTH root 150 ppm	PAAD1.315.064 ARBTH root 150 ppm	shoot ratio	shoot ratio
Phenoxy auxins							
2,4-D		0.2	10	<0.01	0.04	50	>4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

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

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

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

TABLE 20

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

Herbicide Treatment	Ave % Injury 14DAT: AAD1.01.315.076.T_4 homozygous AAD1 plants	Ave % Injury 14DAT: PatCry1f - Control
Phenoxypropionic auxins		
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100
Pyridyloxyacetic auxins		
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
Inactive DCP metabolite		
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

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

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

In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (FIG. **9**A), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

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

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-1 (v3) homozygous lines to determine stable integration of the plant transformation unit

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

For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling r×n (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

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

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

T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (AAD-1 (v3)+ EPSPS) coding for a putative glyphosate resistance trait. T_1 transformants were selected using AAD-1 (v3) as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-1 (v3)+PAT T_5 homozygous lines (pDAB721-transformed), and PAT+Cry1F homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. 150 values were calculated and show >14 fold level of tolerance imparted by EPSPS molecularly stacked with AAD-1 (v3) (see FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

EXAMPLE 7

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

7.1—Cloning of AAD-1 (v3).

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

7.2—Callus/Suspension Initiation.

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

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

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

the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+medium. Once the suspensions were fully established, they were cryopreserved for future use.

7.3—Cryopreservation and Thawing of Suspensions.

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

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100×25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

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

Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60×20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6(1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in FIG. **11**.

Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see FIG. **12**) shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

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

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

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

After 1 week, filter papers were transferred to 60×20 mm plates of GN6(1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L bialaphos; 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

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

Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60×20 mm plates. If sustained growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28(1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36(1H), which

was identical to 28(1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150×25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

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

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

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

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

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

TABLE 21

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

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

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

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

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

TABLE 22

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
1283[1]-001	2	–
1283[1]-002	206	+++
1283[1]-003	1	–
1283[1]-004	90	+++
1283[1]-005	1	–
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	–
1283[1]-011	4	–
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	–
1283[1]-015	194	+++
1283[1]-016	4	–
1283[1]-017	196	+++
1283[1]-018	3	–
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++

TABLE 22-continued

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	–
1283[1]-027	1006	++
Neg Control	3	–
Neg Control	3	–
Standard (0.5 μg/mL)		++
Standard (5 μg/mL)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

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

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

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

7.10.4—Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set up to obtain a gravity gel to nylon membrane (Millipore IYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling r×n (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at –80° C.

EXAMPLE 8

In Vivo Tolerance and Field Tolerance Data Generated from PAT-Selected (pDAB3404) AAD-1 (v3) Events

8.1—Tolerance of T_0 Corn Plants to AOPP Herbicides.

If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assured II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1× lethal dose (~⅛× field dose) up to 8× field doses (64× lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each AAD-1 (v3)-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8× field dose (64× lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did

incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 in vitro.

FIG. **14** shows the responses of several AAD-1 (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

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

TABLE 23

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

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

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

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

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

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

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

Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100%

scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

8.2.1—Results.

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

Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

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

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

TABLE 24

	Treatment (rate) =												
	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results			Resistance Segregation Ratios following Liberty Spray		
	Evaluation =											
Event or	% Injury 3 WAA (0-100 scale)		% Leaning 2 DAA (0-100 scale)		Braceroot Deformation 3-4 WAA (0-10 scale)		AAD-1 copy number leaf (Southern	AAD-1 leaf Western	AAD-1 leaf ELISA	T_1 Population Average		T_2 Population Average
Hybrid	IN	HI	IN	HI	IN	HI	analysis)	Blot T_0	T_0	IN	HI	HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/−	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51%	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/−	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493	100	100	20	23	8	9						
HI-II X 5XH571	100	100	13	34	7	9						
CROPLAN 585SR	80	96	11	33	9	9						

*Fits single locus dominant trait segregation as determined by chi square analysis ($P > 0.05$)

2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all AAD-1 (v3) events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all AAD-1 (v3) events except one lineage of 3404.001.

NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, AAD-1 (v3), and glufosinate resistance.

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

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

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

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

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

TABLE 25

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

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

8.3.1—Resistance of AAD-1 (v3) corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.00R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

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

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

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

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

Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see FIG. **15**). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1× field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

EXAMPLE 9

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

9.1—Plant Material.

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

9.2—Tissue Source.

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

9.3—Preparation of a Superbinary Vector.

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

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 ($\sim 1\times10^9$ cfu/ml) and 100 μM acetosyringone added to the solution.

9.5—Infection and Cocultivation.

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

9.6—Dose Response Using Immature Embryos.

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

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

9.8—Regeneration and Seed Production.

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

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

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

TABLE 26

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

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

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

EXAMPLE 10

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

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

EXAMPLE 11

Recombinant AAD1 Purification and Antibody Production

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

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

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

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

Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1% Triton

X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000×g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

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

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

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

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

11.4—Western Blotting Analysis.

Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

EXAMPLE 12

Tobacco Transformation

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

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

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

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

dards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (Table 27).

TABLE 27

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

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

nd = not done

Legend:

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

Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

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

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

T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line IY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5× field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6× field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (Table 27).

Verification of High 2,4-D Tolerance.

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

TABLE 28

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

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

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

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

EXAMPLE 13

Soybean Transformation

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

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

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

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

13.1.1—*Agrobacterium* preparation. The plasmid, pDAB721, contains the AAD-1 (v3) gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the PAT gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in

the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

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

13.1.2—Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C., with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg et al. (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100×25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N)HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee et al., 1988).

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

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

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

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

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

13.2—Transformation Method 2: "No-shake" *Agrobacterium* Mediated Transformation of Non-regenerable Soybean Suspension Cells.

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

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

13.2.1—Selection of soybean suspension cells and isolation of transformed colonies. After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100×15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (Table 29) and Western Blotting (FIG. **18**) analysis on two separate soybean calli transformed with AAD-1 (v3) gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not

in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

TABLE 29

PCR and ELISA data for transgenic soybean events.

Event	AAD-1 Coding region PCR	PAT Coding region PCR	T SP (µg/mL)	AAD-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	−	−	2074.63	−1.22	−0.0001%

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

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

Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

Variations of media referred to in Table 30 were tested, e.g., B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/1 myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/1 myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

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

EXAMPLE 14

AAD-1 (V3) Enablement in Cotton

14.1—Cotton Transformation Protocol.

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

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

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

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (Table 31), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 31) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

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

TABLE 31

Media for Cotton Transformation

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

14.2—Experiment Specifics.

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

14.3—Callus Analysis Results.

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

TABLE 32

Analysis of cotton callus

Line	Copy #	PTU	Western
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**

TABLE 32-continued

Analysis of cotton callus			
Line	Copy #	PTU	Western
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*
standard	AAD1	5 ug/ml	*****
standard	AAD1	0.5 ug/ml	**

14.4—Plant Regeneration.

Two AAD-1 (v3) cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the AAD-1 (v3) gene provides resistance to 2,4-D in cotton, both the AAD-1 (v3) cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the AAD-1 (v3) plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the AAD-1 (v3) plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

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

EXAMPLE 15

Agrobacterium Transformation of Other Crops

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

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

EXAMPLE 16

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

Stacking AAD-1 (v3) with an AHAS herbicide resistance gene is described in Example 7.9.

EXAMPLE 17

Further Evidence of Surprising Results: AAD-1 vs. AAD-2

17.1—AAD-2 (v1) Initial Cloning.

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

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was

isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO: 15 (added a Xho I site)].

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

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

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

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium.*

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

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

TABLE 33

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

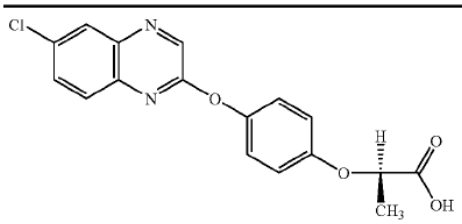
				A510	
STRUCTURE	Reg ID	Compound	Enantiomer	AAD1	AAD2
	18706	quizalofop	R	0.27	0.01

TABLE 33-continued

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

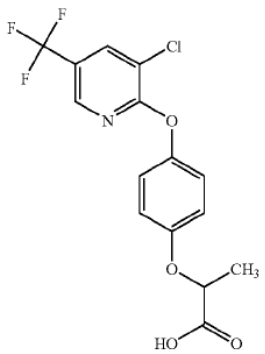
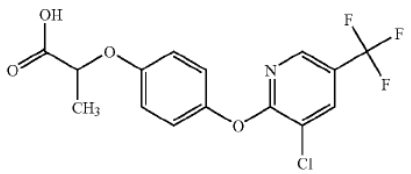
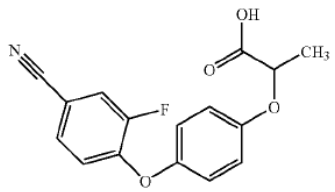
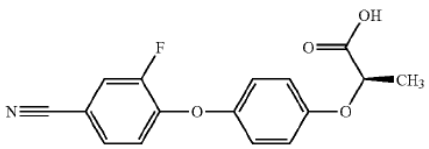
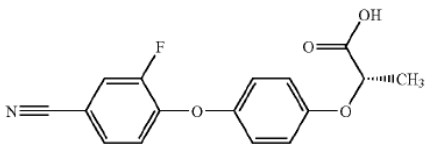
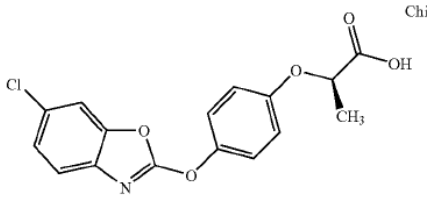
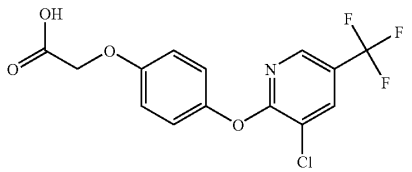
					A510	
STRUCTURE	Reg ID	Compound	Enantiomer		AAD1	AAD2
	8671	haloxyfop	R		0.12	0
	66905	haloxyfop	R, S		0.1	0.3
	14623	cyhalofop	R, S		0.12	0.1
	14603	cyhalofop	R		0.14	0
	7466	cyhalofop	S		0	0.15
	Chiral 11044492	fenoxaprop	R		0.14	0

TABLE 33-continued

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

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	43865	haloxyfop-acetate	—	0	0.22

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **19**. The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (Table 34). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D in vitro than AAD-1 (v1). This is in surprising contrast to the in planta findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

TABLE 34

Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ± SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes:

Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

17.4—Evaluation of Transformed *Arabidopsis*.

Freshly harvested T_1 seed transformed with a native [AAD-1 (v2)], plant optimized [AAD-1 (v3)], or native AAD-2 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

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

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

Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, Del.) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0).

17.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15 in Example 6.5 above). A small lot of native AAD-1 (v2) transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native AAD-2(v1) gene provided a very low transformation frequency when selected for glufosinate tolerance (PAT selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection fre-

quency of 0.018% (see Table 15). Transformation frequency for native AAD-2 (v1) was only 6% of that of native AAD-1 (v2). The native AAD2 (v1) gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into *Arabidopsis* using methods previously described (see Example 5). The plant optimized AAD2 (v2) (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (see Table 15).

T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 16 (in Example 6.5 above) compares the response of AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) and AAD-1 (v2) genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 16 simply to demonstrate the significant difference between the plants transformed with AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) or AAD-2 (v2) versus the wildtype or PAT/Cry1F transformed controls.

Surprisingly, AAD-2 (v1) transformants were far less resistant to 2,4-D than either AAD-1 (v2) or AAD-1 (v3) genes (Table 16) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v4) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) AAD-1 (v2)-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average=34%), and >73% (11 of 15) AAD-1 (v3) T_1 plants were uninjured (population injury average=14%). See FIG. **20**. Tolerance improved slightly for plant-optimized AAD2 (v2) versus the native gene; however, comparison of both AAD-1 and -2 plant optimized genes indicates a significant advantage for AAD-1 (v3) in planta (see Table 16).

These results are unexpected given that in vitro comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. See FIG. **21**. No large difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) in planta for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected.

EXAMPLE 18

Preplant Burndown Applications

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

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

EXAMPLE 19

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

AAD-1 (v3) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D, dichlorprop, MCPA, et al.) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot

crops which are naturally tolerant to these herbicides. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3)-transformed dicot crop (i.e., soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

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

Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of AAD-1 (v3)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 20

In-Crop Use of Phenoxy Auxins and AOPP Herbicides in AAD-1 (v3) Only Transformed Corn, Rice, and Other Monocot Species

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

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the AAD-1 (v3) gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar

compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 21

AAD-1 (v3) Stacked with Glyphosate Tolerance Trait in Any Crop

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

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

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

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

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 22

AAD-1 (v3) Stacked with Glufosinate Tolerance Trait in any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

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

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

i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

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

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 23

AAD-1 (v3) Stacked with AHAS Trait in any Crop

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

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

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

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

iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

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

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by AAD-1 (v3) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf. asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 24

AAD-1 (v3) in Rice

24.1—Media Description.

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

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) con-

sisted 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 (½ MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 μl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 μl spermidine (0.1 M) and 50 μl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 μl cold 100% ethanol and 8-9 μl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

24.4—Tolerance Testing.

Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 μl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in Table 35 below.

TABLE 35

Sample Name	140 g ae/ha quizalofop
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

24.5—Tissue Harvesting, DNA Isolation and Quantification.

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

24.6—Southern Blot Analysis.

Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse—GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and

35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

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

TABLE 36

Southern Results

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049 bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

24.7—Western Data

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

TABLE 37

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

EXAMPLE 25

Turf Grass Transformation Procedures

Genetic transformation, with AAD-1 (v3) substituted for the "bar" gene as described below, of creeping bentgrass mediated by *Agrobacterium tumefaciens* could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. See "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo et. al., 2004).

Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60-65%) contained only a single copy of the foreign gene with no apparent rearrangements.

25.1—Seed Preparation for Embryogenic Callus Induction.

Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-o-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120° C. for 20 min).

25.2—Embryogenic Callus Induction.

The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

25.3—*Agrobacterium* Infection and Co-Cultivation.

One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μM acetosyringone. A 10-ul aliquot of *Agrobacterium* (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25° C.

25.4—Resting Stage and *Agrobacterium* Control.

For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

25.5—Selection and Potential Transgenic Colony Identification.

Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

25.6—Regeneration of Transgenic Plants.

For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2-3

weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

25.7—Root Induction and Transfer to Greenhouse.

Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3-5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

25.8—Vernalization and Out-crossing of Transgenic Plants.

Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25° C. under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25° C., and T1 plants were grown in the greenhouse for further analysis.

25.9—Other Target Grasses.

Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra* commutate), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra* rubra), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

EXAMPLE 26

AAD-1 (v3) in Canola

26.1—Canola Transformation.

The AAD-1 (v3) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. *Nexera** 710 with *Agrobacterium*-mediated transformation. The construct contained AAD-1 (v3) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

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

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

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

Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of AAD-1 (v3), whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

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

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse. The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the AAD-1 (v3) gene.

26.2—"Molecular Analysis": Canola Materials and Methods

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

26.2.2—Polmerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR AAD-1 (v3) were (Forward—ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse—CGGGCAGGC-CTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on

a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-1 (v3) events tested positive. Three negative control samples tested negative.

26.3—ELISA.

Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

TABLE 38

Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	–
Control 2	61	2549.60	35.00	13.73	–
Control 3	59	2374.41	22.79	9.60	–

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cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420

ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480

ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540

ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

tgcacccccc ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660

tctattgcca gcgcatcgag ggcatgaccg atgccgaaag caaaccgctg ctgcagttcc 720

tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> SEQ ID NO 5
<211> LENGTH: 919
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 (v3) primary sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(8)
<223> OTHER INFORMATION: additional alanine codon (GCT)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (894)..(919)
<223> OTHER INFORMATION: primer linker

<400> SEQUENCE: 5

ccatggctca tgctgccctc agccctctct cccaacgctt tgagagaata gctgtccagc 60

cactcactgg tgtccttggt gctgagatca ctggagtgga cttgagggaa ccacttgatg 120

acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300

ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420

ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480

aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt 720

tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780

aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca 840

agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta 900

gcttaatcac ctagagctc 919

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<210> SEQ ID NO 6
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rdpA(ncoI) 5' primer

<400> SEQUENCE: 6

cccatggctg ctgcactgtc ccccctctcc 30

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

<400> SEQUENCE: 7

gagctcacta gcgcgccggg cgcacgccac cga 33

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

<400> SEQUENCE: 8

tggtggtgac ccatccgggc agcggctgca agggcc 36

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

<400> SEQUENCE: 9

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 10
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v2 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: Different from v1

<400> SEQUENCE: 10

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 11
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v3 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Different from V1

<400> SEQUENCE: 11

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

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

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

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

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

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

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

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

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

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

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

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

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
                245                 250                 255
```

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```
Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
            260                 265                 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
        275                 280                 285

Val Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> SEQ ID NO 12
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 12

```
atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat     60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac    120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg    180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg    240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag    300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc    360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc    420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc    480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc    540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc    600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc    660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag    720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc    780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg    840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag                  888
```

<210> SEQ ID NO 13
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 13

```
Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                   10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125
```

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```
Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
                245                 250                 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
            260                 265                 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
        275                 280                 285

Val
```

<210> SEQ ID NO 14
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: brjap 5'(speI)

<400> SEQUENCE: 14

```
actagtaaca aagaaggaga tataccatga cgat                                34
```

<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: br jap 3' (xhoI)

<400> SEQUENCE: 15

```
ttctcgagct atcactccgc cgcctgctgc tgc                                 33
```

<210> SEQ ID NO 16
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 forward primer

<400> SEQUENCE: 16

```
gtaaaacgac ggccagt                                                   17
```

<210> SEQ ID NO 17
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 reverse primer

<400> SEQUENCE: 17

-continued

caggaaacag ctatgac 17

<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NcoI of Brady

<400> SEQUENCE: 18

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 19
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SacI of Brady

<400> SEQUENCE: 19

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 20
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
85 90 95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
100 105 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
115 120 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
130 135 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145 150 155 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
165 170 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
180 185 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
195 200 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
210 215 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225 230 235 240

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```
Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525


<210> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine
      183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 21

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                   10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80
```

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```
Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495
```

-continued

```
Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525


<210> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding
      doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added
      sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence
      encoding the EPSPS protien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop
      codons in all six open reading frames, and to introduce a SacI
      restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 22

atggctcaag tctcccgtgt tcacaatctt gctcagtcaa cccaaatctt tggacattca     60

agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttcgcccacg cctttgggga    120

gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt    180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc    240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg    300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc    360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga    420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt    480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat    540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt    600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc    660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga    720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc    780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa    840

atagtggaca agttgatttc tgttccatat gtggaaatga ccctcaaact catggagagg    900

tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag    960

aagtacaaaa gccctggcaa tgcctttgtt gaaggggatg caagctctgc ttcctatctc   1020

ttggctgggg ctgccatcac tggtgggacc atcactgtga atggctgtgg cacctcatcc   1080

cttcaaggtg atgtaaagtt tgcagaggtc ttggagaaaa tgggtgccaa ggtcacctgg   1140

tctgagaaca gtgtaactgt gtctggacct cccagagact tcagtggcag aaaggttctc   1200

cgtggaattg atgtgaacat gaacaagatg ccagatgtgg ccatgaccct cgctgttgta   1260

gccctgtttg caaatggacc aactgcaatc cgtgatgttg cttcatggag ggtgaaggag   1320

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ggaccagatt actgtgtgat aaccccacct gagaagctca atgtgacagc cattgacacc 1440

tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

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agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg 180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt 240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg 300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca 360

gcttcagacc aatcccagcc aagttcagcc tccttttctgc tcgtgttgtg aacccaactg 420

gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

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ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

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20 25 30

Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
35 40 45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
50 55 60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65 70 75 80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
85 90 95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
100 105 110

Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
115 120 125

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Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
    130                 135                 140

Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
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Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
                165                 170                 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
            180                 185                 190

Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
        195                 200                 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
    210                 215                 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
225                 230                 235                 240

Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
                245                 250                 255

Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
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The invention claimed is:

1. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9.

2. The cell of claim **1** wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

3. The cell of claim **2** wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

4. The cell of claim **2** wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

5. A transgenic plant comprising a plurality of cells according to claim **1**, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

6. The plant of claim **5** wherein said herbicide is a phenoxy auxinic herbicide.

7. The plant of claim **5** wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

8. The plant of claim **5** wherein said herbicide is an aryloxyphenoxypropionate.

9. The plant of claim **5** wherein said herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

10. The plant of claim **5** wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

11. The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

12. The plant of claim 11 wherein said second herbicide resistance gene renders said plant resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), and inhibitors of protoporphyrinogen oxidase (PPO).

13. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

14. The method of claim 13 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin.

15. The method of claim 14 wherein said herbicide is selected from the group consisting of R-dichlorprop and R-mecoprop.

16. The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

17. The method of claim 16 wherein said herbicide is selected from the group consisting of 2,4-D and MCPA.

18. The method of claim 13 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

19. The method of claim 18 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

20. The method of claim 13 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

21. The method of claim 20 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

22. The method of claim 13 wherein said method comprises applying a second herbicide.

23. The method of claim 22 wherein said first herbicide and said second herbicide are applied sequentially.

24. The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

25. The method of claim 22 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

26. The method of claim 13 wherein said plant is resistant to glyphosate.

27. The method of claim 14 wherein said R-enantiomer is a component of a racemic mixture.

28. The method of claim 22 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

29. The method of claim 28 wherein said second gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT, PAT (phosphinothricin-N-acetyltransferase), bar, and a dicamba-degrading enzyme.

30. The method of claim 28 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

31. The method of claim 30 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

32. The method of claim 31 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

33. The method of claim 28 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

34. The method of claim 33 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

35. The method of claim 28 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

36. The method of claim 35 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop, and cyhalofop.

37. The method of claim 31 wherein said second herbicide is a sulfonyl urea selected from the group consisting of amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, and triflusulfuron.

38. The method of claim 28 wherein said method further comprises applying a third herbicide.

39. The method of claim 38 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

40. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises the cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate.

41. The method of claim 40 wherein said first herbicide is an acid, an inorganic salt, an organic salt, an ester, an R-enantiospecific isomer, or a component of a racemic mixture.

42. The method of claim 40 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

43. The method of claim 42 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

44. A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein is at least 95% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

45. The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

46. The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

47. An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate, wherein said protein is at least 95% identical with a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

48. The polynucleotide of claim 47 wherein said promoter is a plant promoter.

49. The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

50. A method of selecting for a transformed plant cell wherein said method comprises subject a plurality of plant cells to transformation with the polynucleotide of claim 47 and growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to

grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxyalkanoate.

51. The method of claim **50** wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

52. A seed comprising the plant cell of claim **1**.

53. A plant grown from the seed of claim **52**.

54. A part, progeny, or asexual propagate of the plant of claim **5**, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

55. The method of claim **13** wherein said method is used to treat or prevent herbicide-resistant weeds, wherein said herbicide-resistant weeds are sensitive to a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

56. The plant of claim **5** wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

57. The plant of claim **5** wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

58. A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim **47**, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

59. The method of claim **58** wherein said herbicide is a phenoxy auxin.

60. The method of claim **58** wherein said herbicide is an aryloxy phenoxypropionate.

61. The method of claim **58** wherein said plants are dicots.

62. The method of claim **58** wherein said plants are monocots.

* * * * *

EXHIBIT 13

US009127289B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 9,127,289 B2**
(45) **Date of Patent: *Sep. 8, 2015**

(54) **HERBICIDE RESISTANCE GENES**

(75) Inventors: **Terry R. Wright**, Westfield, IN (US); **Justin M. Lira**, Fishers, IN (US); **Donald J. Merlo**, Carmel, IN (US); **Nicole L. Arnold**, Carmel, IN (US)

(73) Assignee: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1298 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/951,813**

(22) Filed: **Nov. 22, 2010**

(65) **Prior Publication Data**

US 2011/0124503 A1 May 26, 2011

Related U.S. Application Data

(63) Continuation of application No. 11/587,893, filed as application No. PCT/US2005/014737 on Feb. 5, 2005, now Pat. No. 7,838,733.

(60) Provisional application No. 60/567,052, filed on Apr. 30, 2004.

(51) **Int. Cl.**
C12N 15/82 (2006.01)
C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8274*** (2013.01); ***C12N 9/0069*** (2013.01); ***C12N 9/0071*** (2013.01); ***C12N 15/8209*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — David H Kruse
(74) *Attorney, Agent, or Firm* — James Daly, IV; Faegre Baker Daniels LLP

(57) **ABSTRACT**

The subject invention provides novel plants that are not only resistant to 2,4-D and other phenoxy auxin herbicides, but also to aryloxyphenoxypropionate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention alone or "stacked" together with another herbicide resistance gene, preferably a glyphosate resistance gene, so as to provide broader and more robust weed control, increased treatment flexibility, and improved herbicide resistance management options. More specifically, preferred enzymes and genes for use according to the subject invention are referred to herein as AAD (aryloxyalkanoate dioxygenase) genes and proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. This highly novel discovery is the basis of significant herbicide tolerant crop trait opportunities as well as development of selectable marker technology. The subject invention also includes related methods of controlling weeds. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the formation of, and controlling, weeds that are resistant (or naturally more tolerant) to one or more herbicides such as glyphosate.

22 Claims, 24 Drawing Sheets

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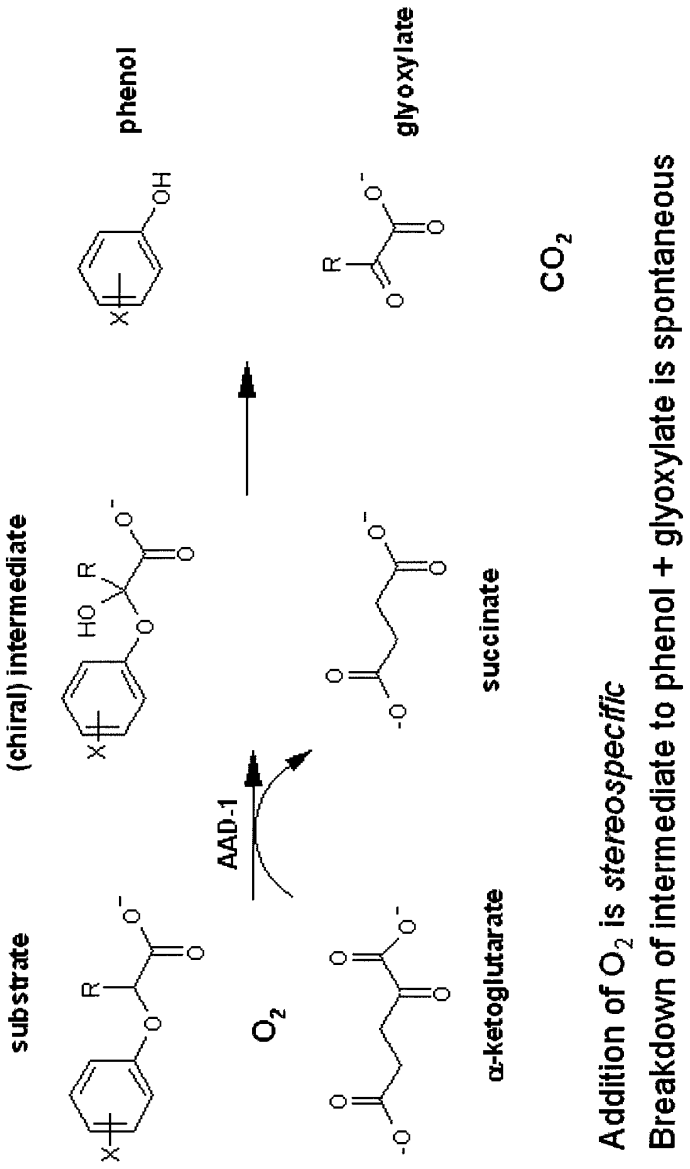
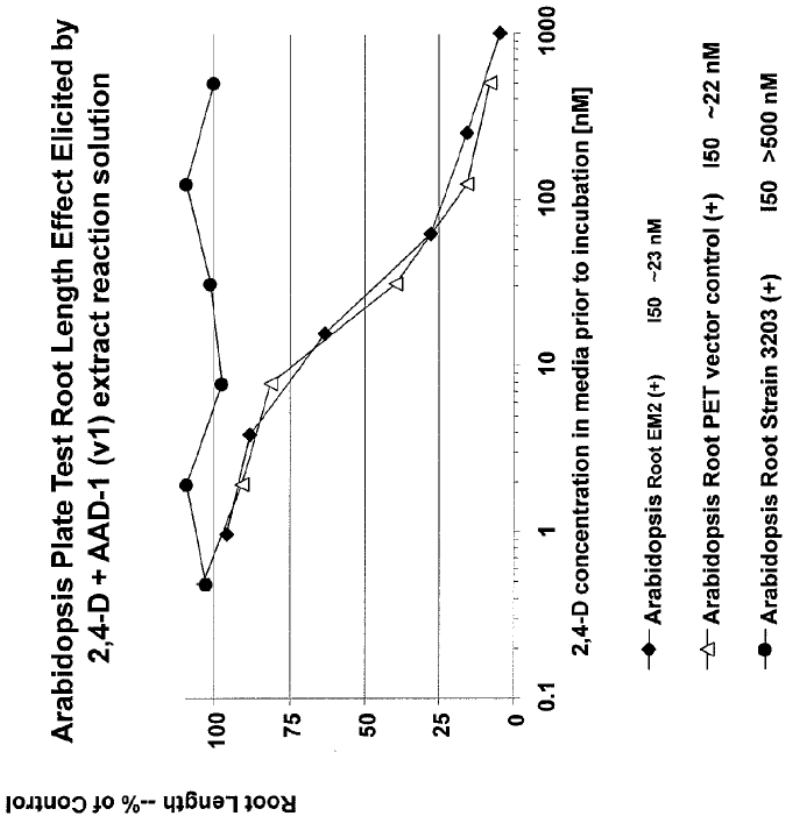

substrate
(chiral) intermediate
phenol
glyoxylate
AAD-1
O_2
α-ketoglutarate
succinate
CO_2
• Addition of O_2 is *stereospecific*
• Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1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Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

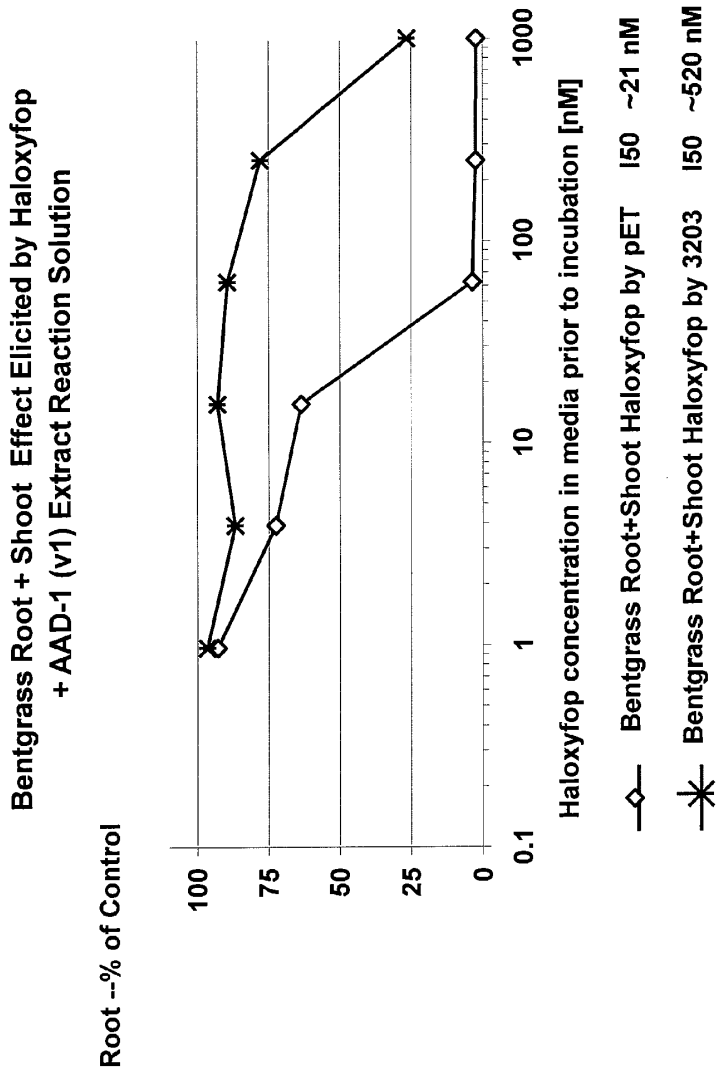

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

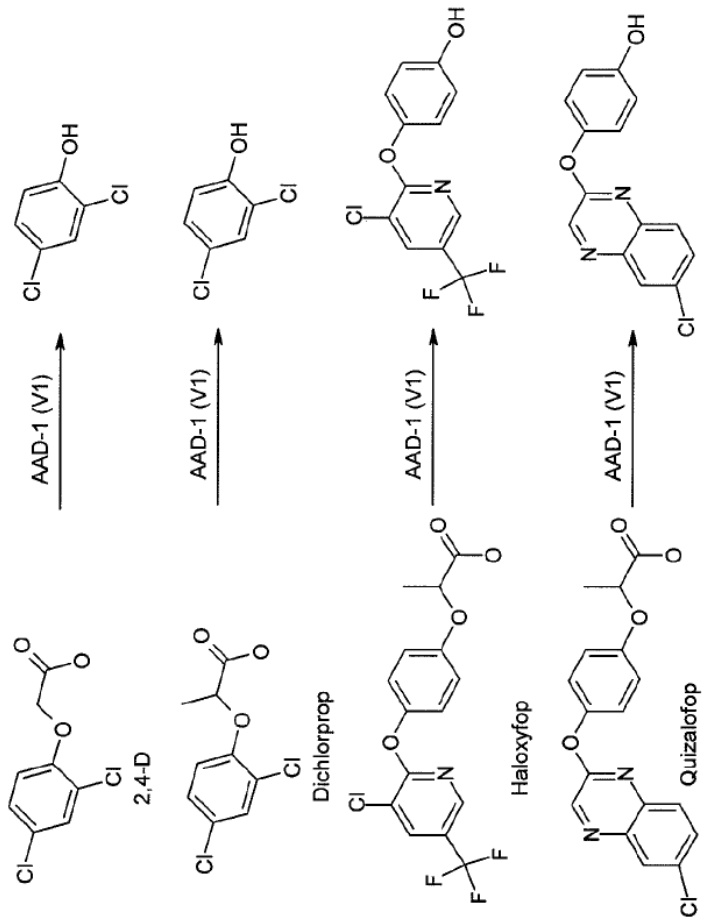

AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
2,4-D
Dichlorprop
Haloxyfop
Quizalofop

Fig. 4

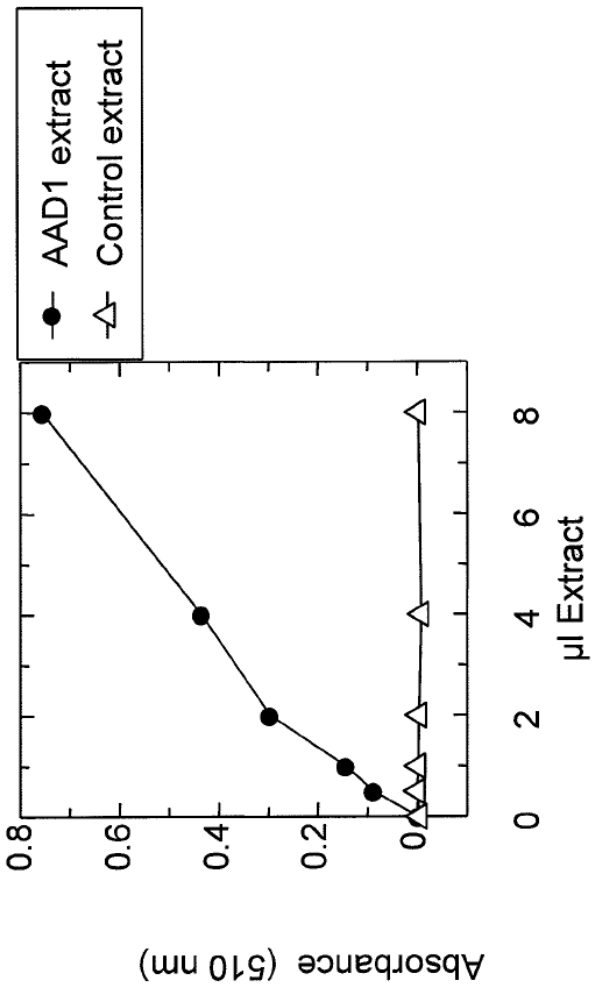

AAD1 extract
Control extract
Absorbance (510 nm)
0.8
0.6
0.4
0.2
0
0
2
4
6
8
µl Extract

Fig. 5

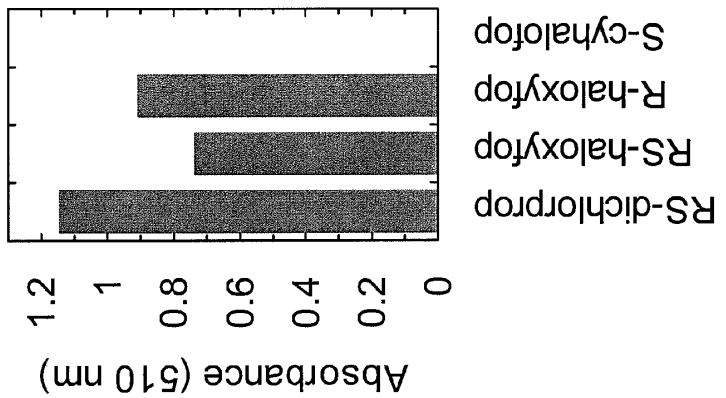

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

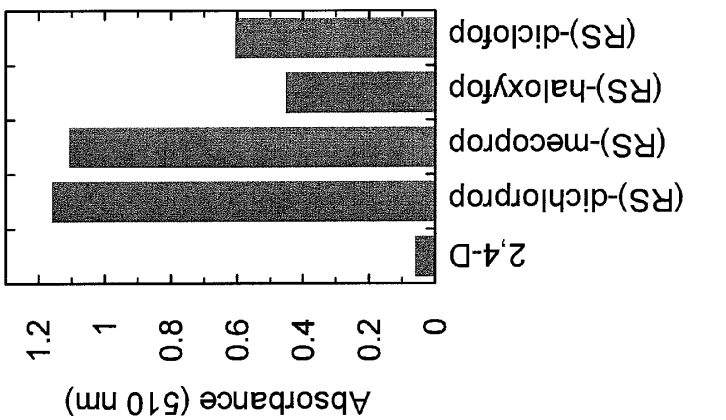

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

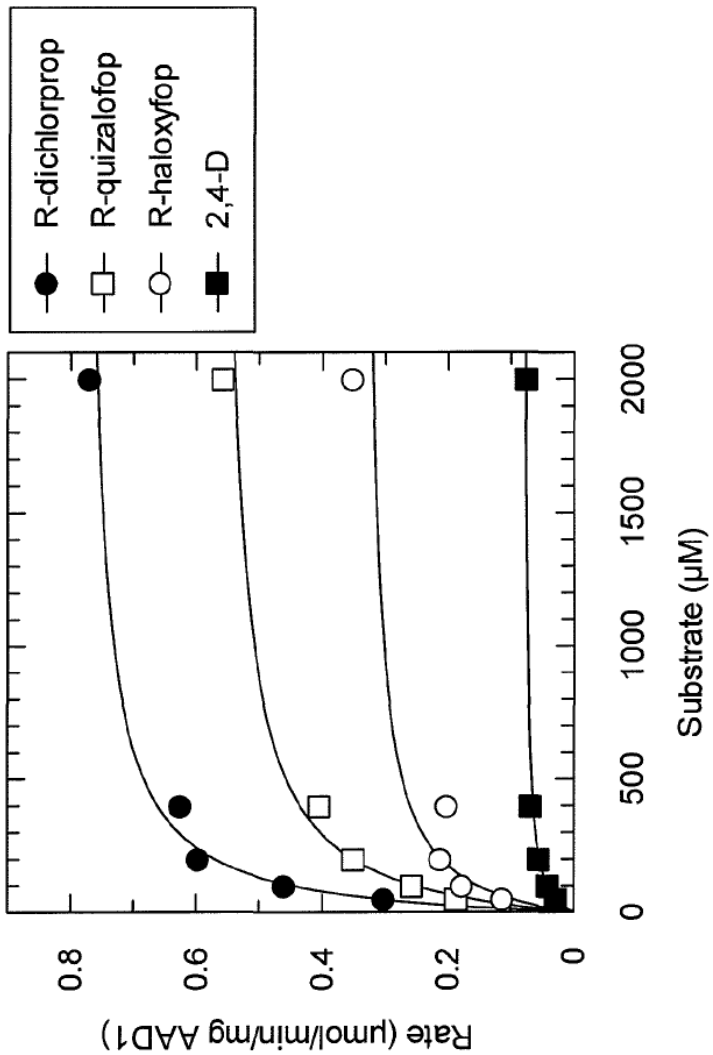

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (μmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (μM)

Fig. 7

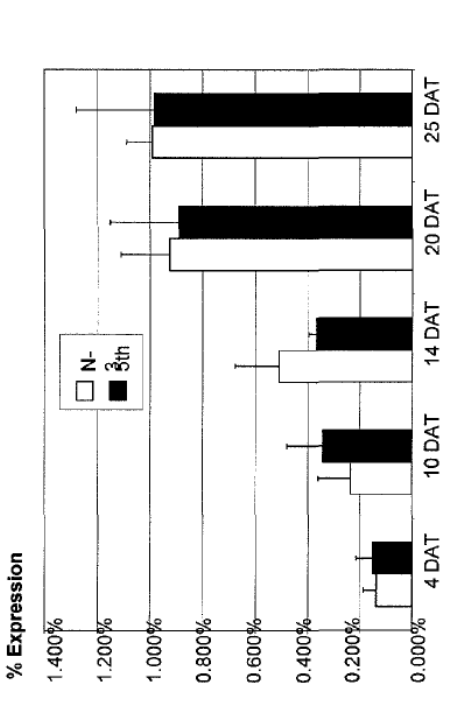

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

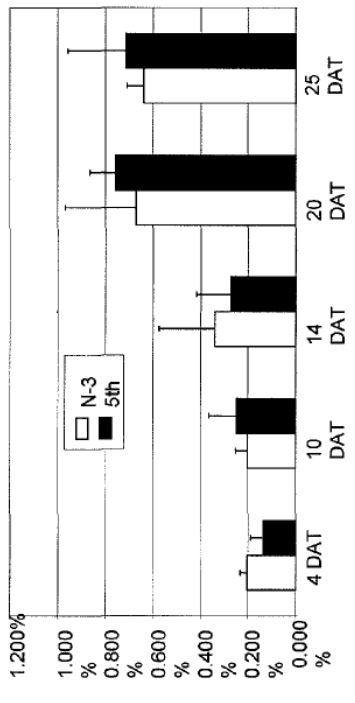

% Expression
1.200%
1.000 %
0.800 %
0.600 %
0.400 %
0.200 %
0.000 %
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

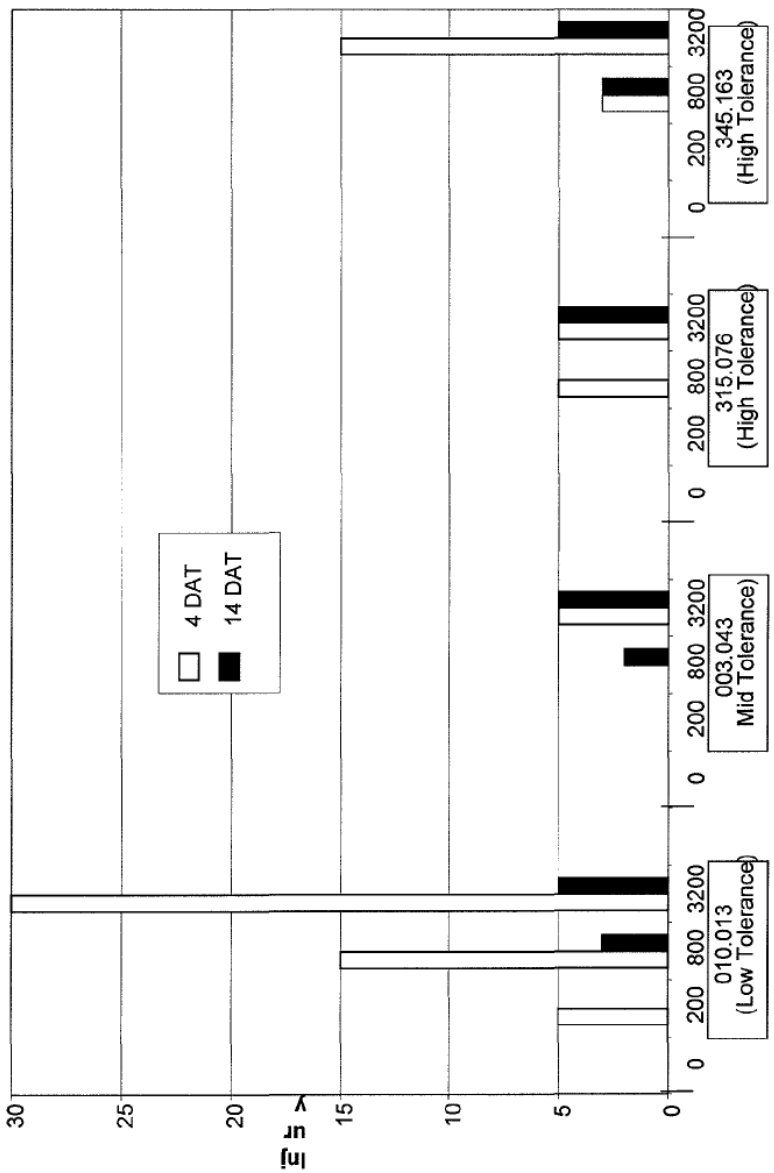

4 DAT
14 DAT
lnj ury
0
5
10
15
20
25
30
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

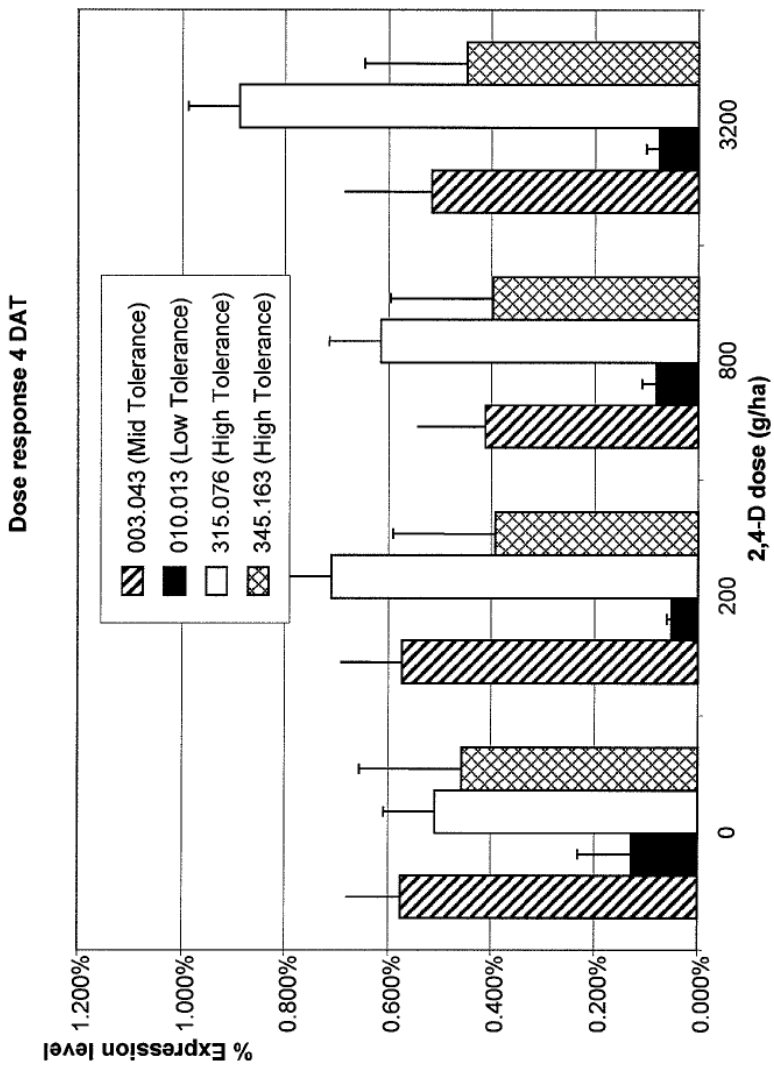

Dose response 4 DAT
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

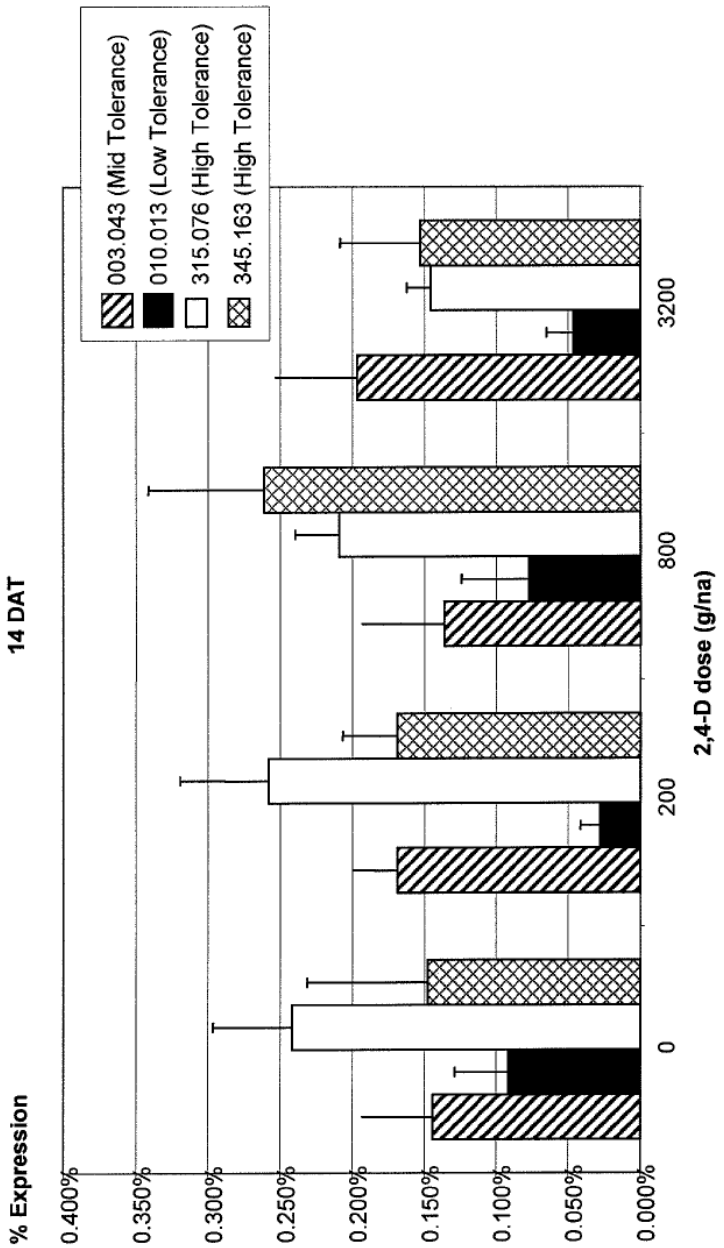

003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
14 DAT
% Expression
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

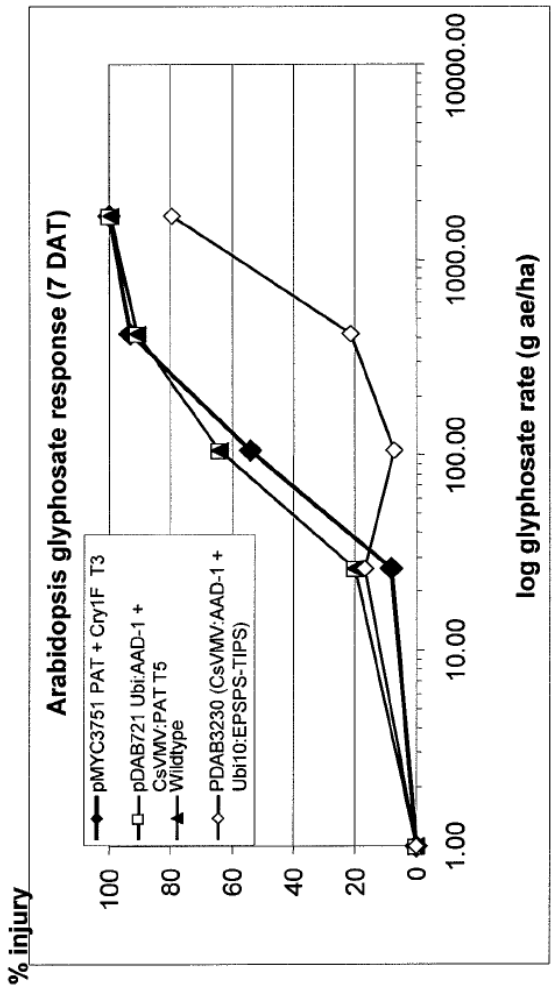

Arabidopsis glyphosate response (7 DAT)
% injury
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)

Fig. 10

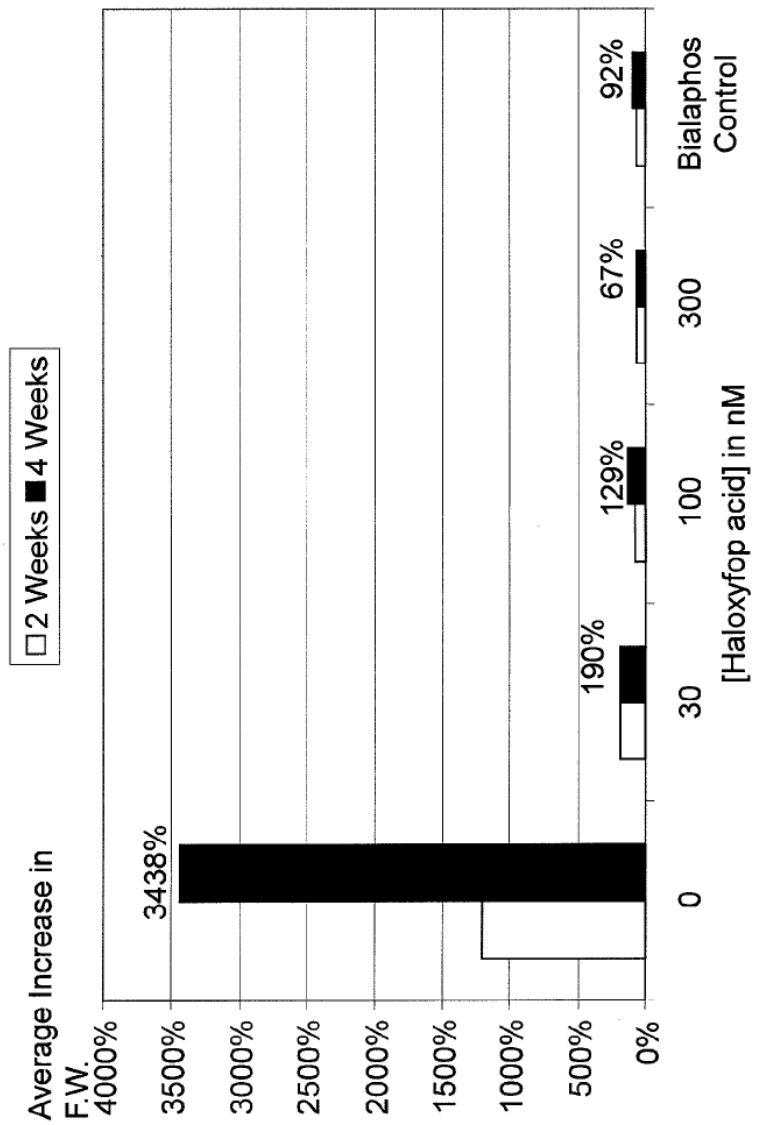

Average Increase in F.W.
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
□2 Weeks ■4 Weeks
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

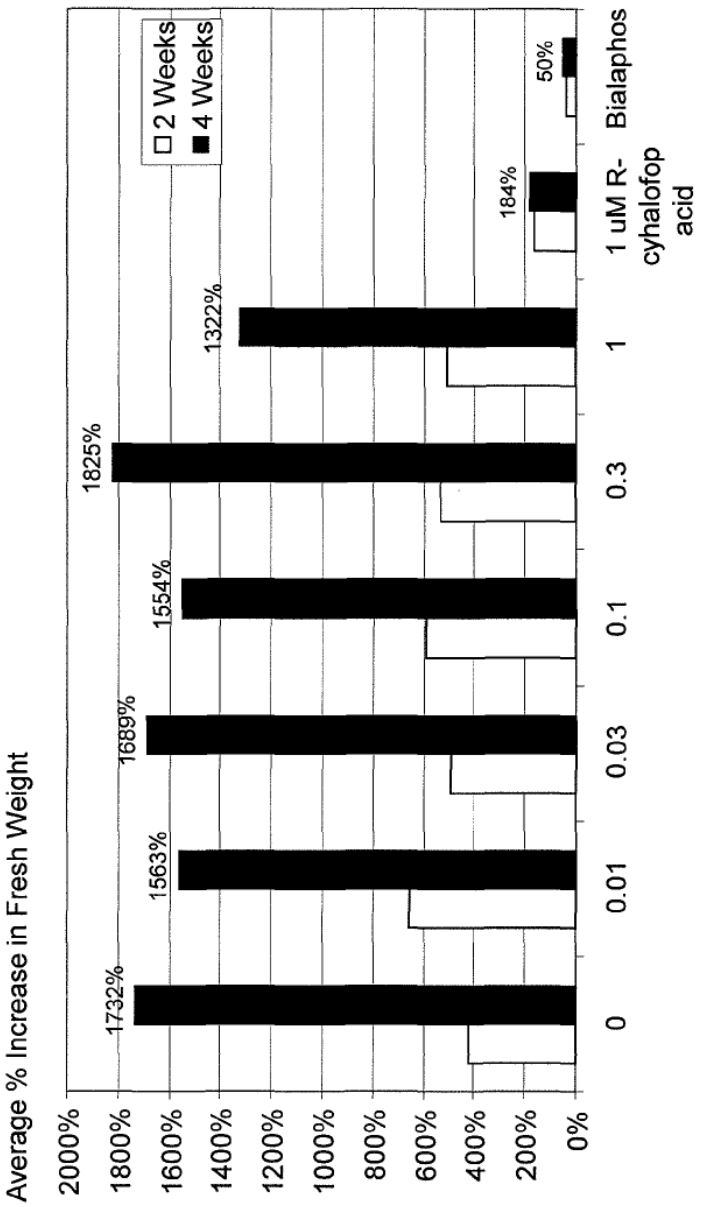

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
2 Weeks
4 Weeks
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

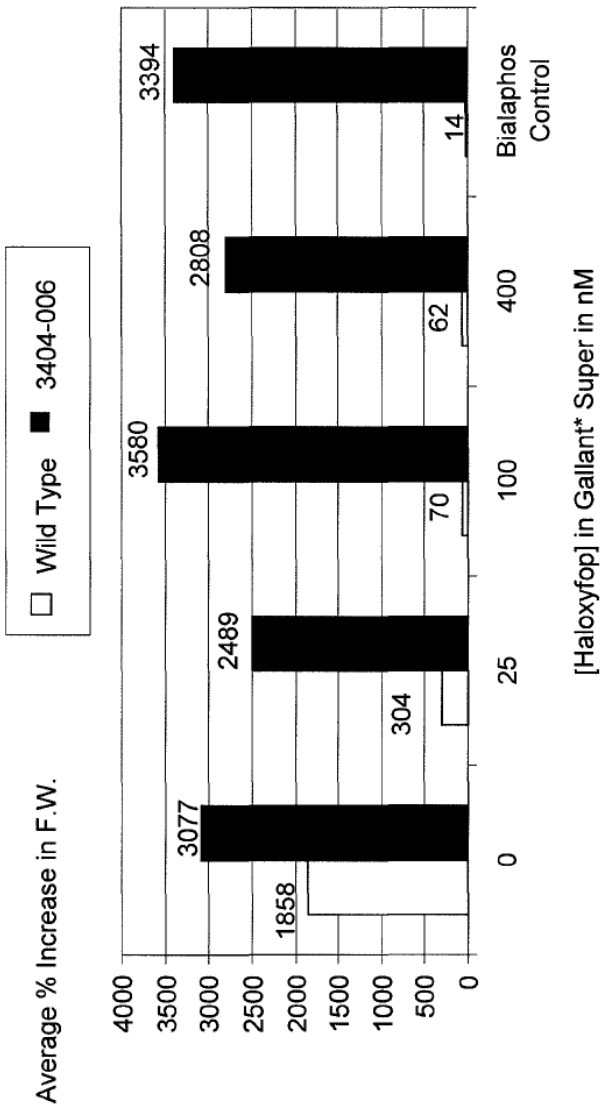

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
3077
1858
2489
304
3580
70
2808
62
3394
14
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

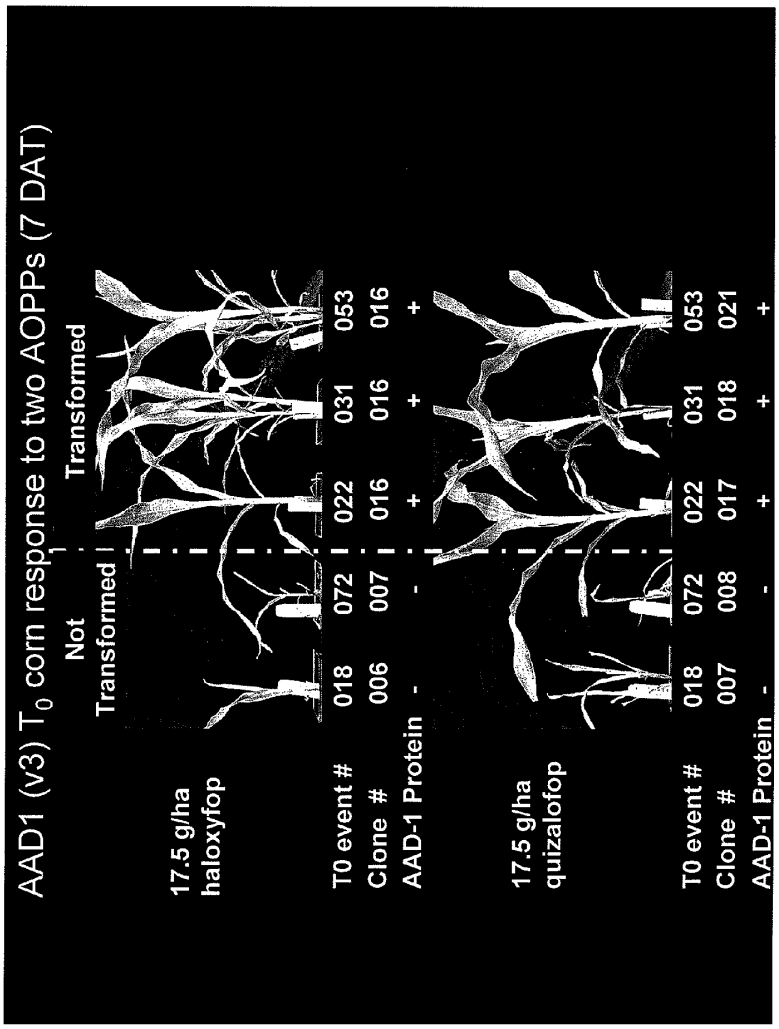

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

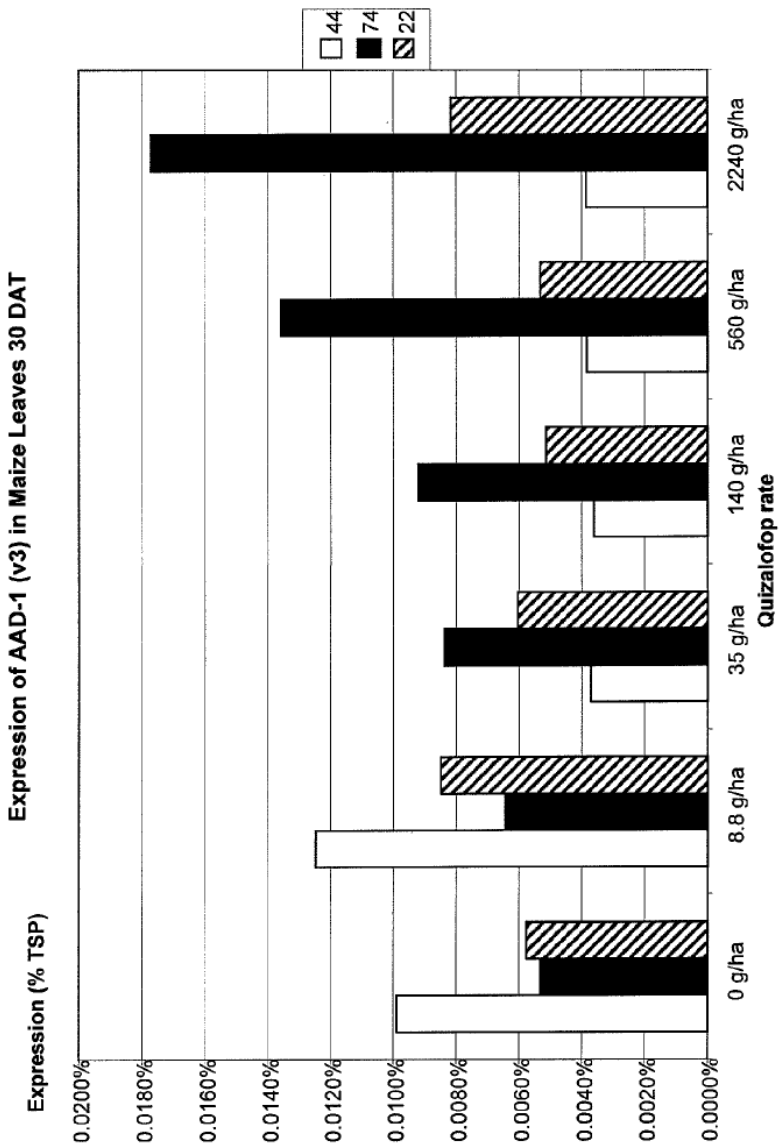

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 28
(AAD 1 transformed)

Fig. 16

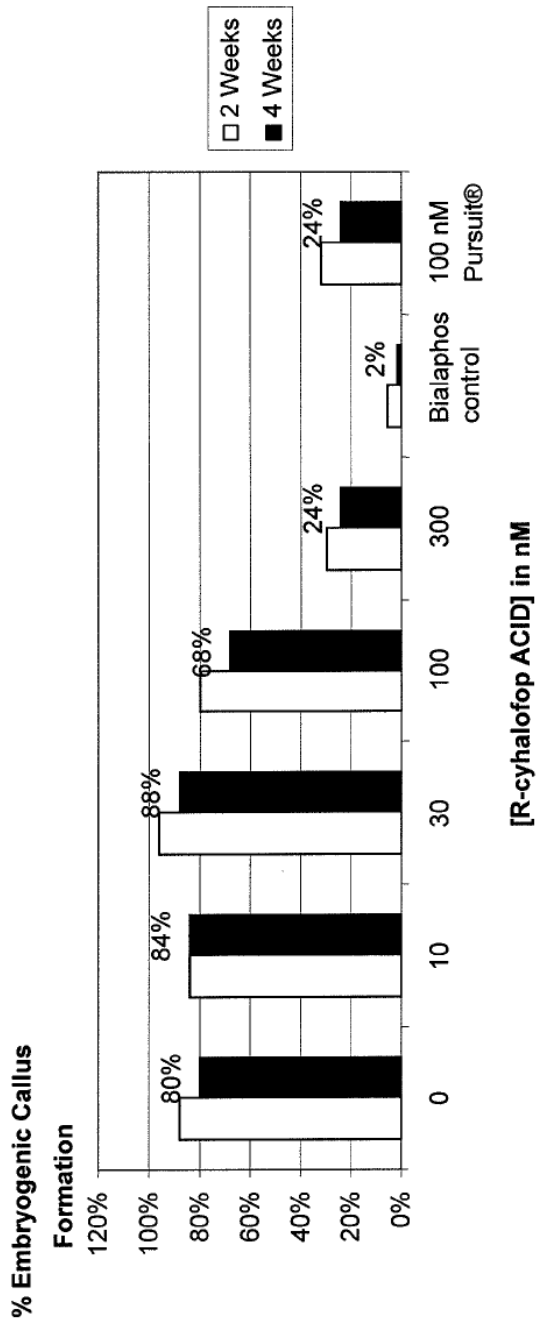
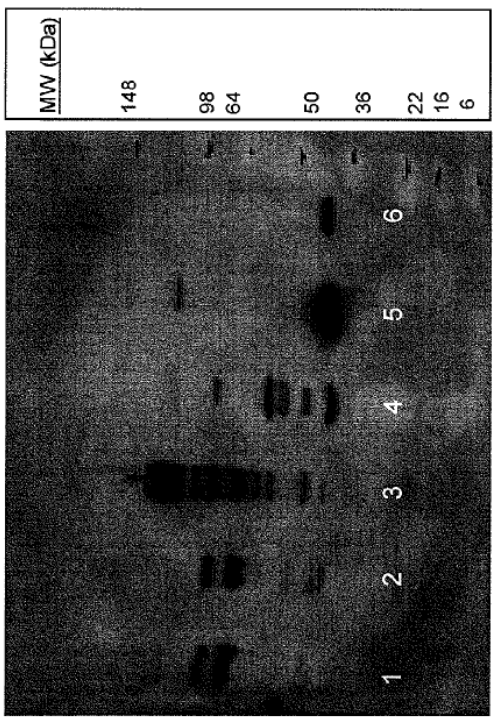

2 Weeks
4 Weeks
% Embryogenic Callus Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
[R-cyhalofop ACID] in nM

Fig. 17



Western

Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

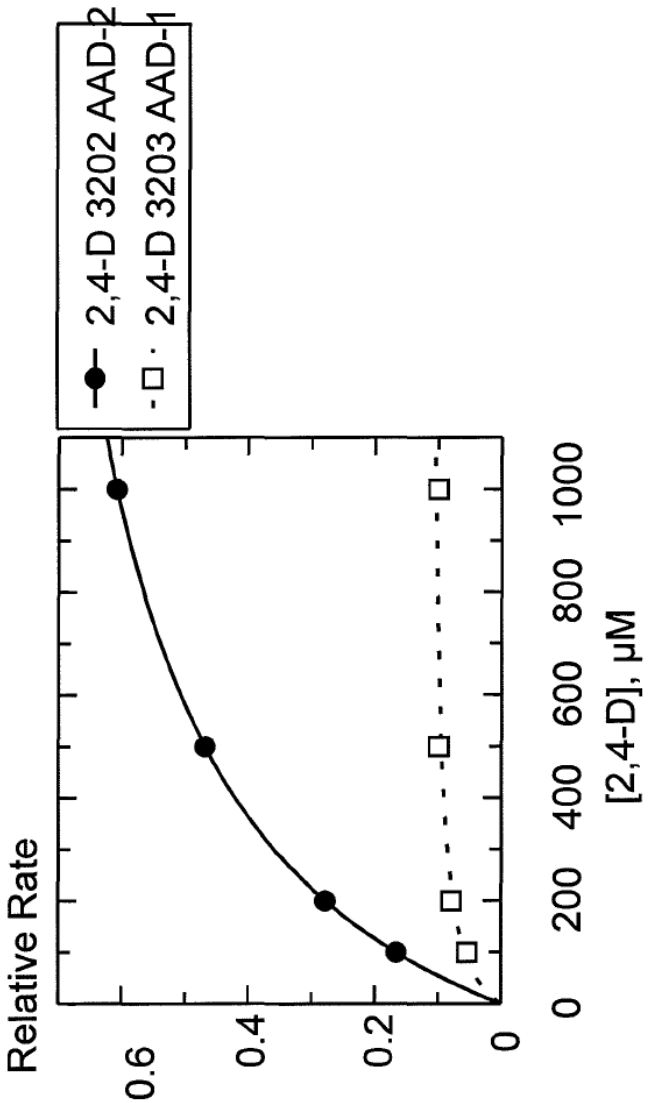

2,4-D 3202 AAD-12
2,4-D 3203 AAD-1
Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

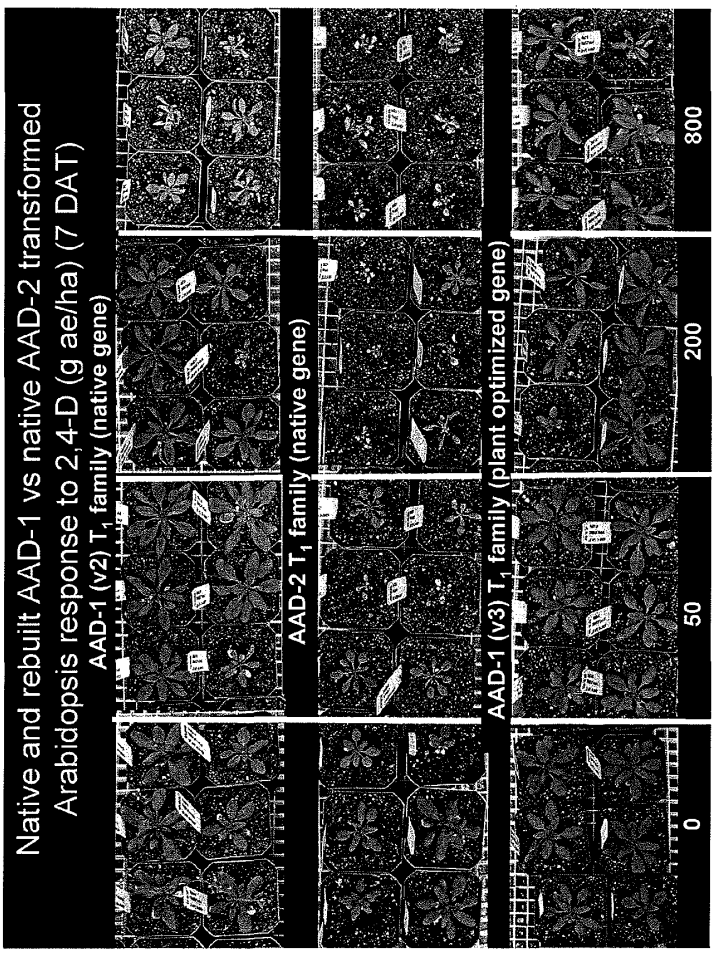

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

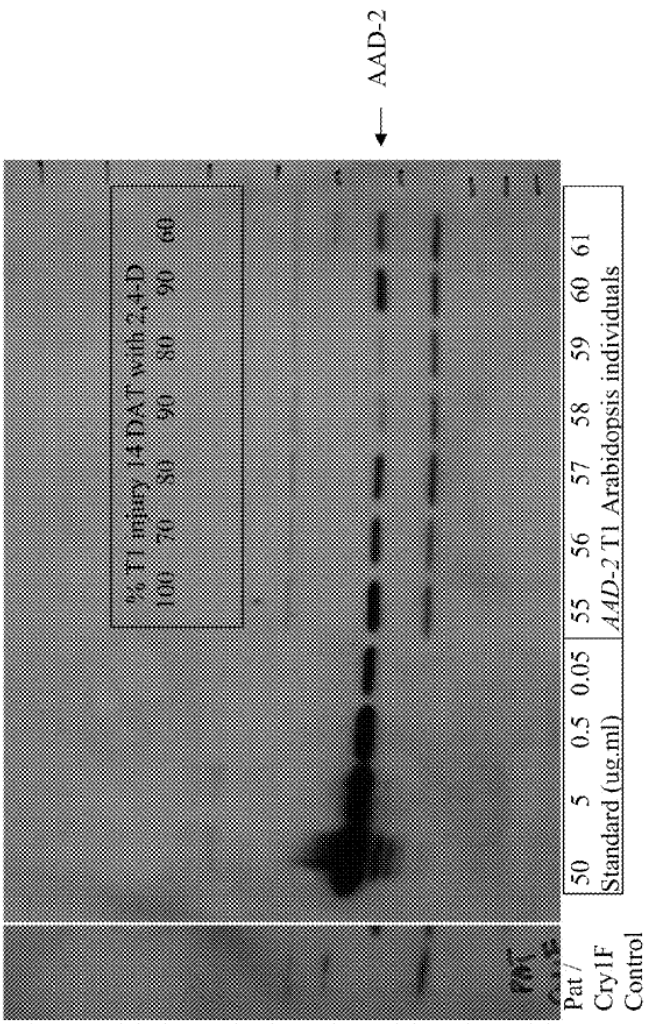

AAD-2
% T1 injury 14 DAT with 2,4-D
100 70 30 90 80 90 90 60
50 5 0.5 0.05
Standard (ug.ml)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
Pat / Cry1F Control

Fig. 21

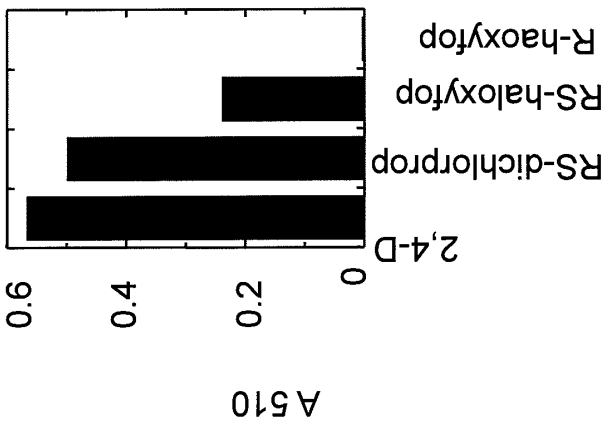


Fig. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of U.S. Ser. No. 11/587,893, filed May 22, 2008, which is based on U.S. National Phase Patent Application based on International Patent Application Serial No. PCT/US2005/014737 filed on May 2, 2005, and claims the benefit of U.S. Provisional Application Ser. No. 60/567,052, filed Apr. 30, 2004, which are hereby incorporated by reference herein in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

BACKGROUND OF THE INVENTION

Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (i.e., weed shifts). (Ng et al., 2003; Simarmata et al., 2003; Lorraine-Colwill et al., 2003; Sfiligoj, 2004; Miller et al., 2003; Heap, 2005; Murphy et al., 2002; Martin et al., 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis*, and *Plantago lanceolata*. Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum*, and *Commelina* species.

In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

2,4-D has different levels of selectivity on certain plants (e.g., dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the

mineralization pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, there are a number of homologues that have a significantly lower identity to tfdA (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

One unique example with low homology to tfdA (28% amino acid identity) is rdpA from *Sphingobium herbicidovorans* (Kohler et al., 1999, Westendorf et al., 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath et al., 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf et al., 2003). Heterologous expression of rdpA in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of tfdA that primarily degrade achiral phenoxyacetic acids (e.g., 2,4-D).

Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluoroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (e.g., glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-1 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

There was no prior motivation to produce plants comprising an AAD-1 gene (preferably an AAD-1 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, et al.). Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an AAD-1 gene. The subject invention also provides methods of using an AAD-1 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-1, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting

transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

FIG. **2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

FIG. **3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

FIG. **4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

FIG. **5** shows 2,4-dichlorophenol production by recombinant AAD-1.

FIGS. **6**A and **6**B show phenol production by recombinant AAD-1 from various herbicide substrates.

FIG. **7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

FIGS. **8**A and **8**B show that AAD-1 (v3) was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean±SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

FIGS. **9**A, **9**B, and **9**C show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean±SEM of five leaves from five different plants received the same treatment.

FIG. **10** illustrates that pDAB3230-transformed *Arabidopsis* (AAD-1+EPSPS) shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

FIG. **11** shows dose response of callused maize suspensions to R-haloxyfop.

FIG. **12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

FIG. **13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

FIG. **14** shows the responses of several AAD-1 (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

FIG. **15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

FIG. **16** shows AAD-1 (v3)-transformed corn tolerant to 8× field rates of quizalofop (Assure II) under field conditions.

FIG. **17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

FIG. **18** shows Western Blotting analysis on soybean calli transformed with AAD-1 (V3) gene indicating that the callus cells are expressing AAD-1 (v3) protein.

FIG. **19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

FIG. **20** shows the response of AAD-1 v3 (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or AAD-2 (v2) (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family.

FIG. **21** shows western blot analysis of individual native AAD-2 (v1)-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native AAD-1 (v2) or plant optimized AAD-1 (v3)-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in AAD-2-transformed and Pat/Cry1F-transformed samples.

FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the sequence of a forward primer used to amplify the rdpA/AAD-1 (v1) gene.

SEQ ID NO:2 is the sequence of a reverse primer used to amplify the rdpA/AAD-1 (v1) gene.

SEQ ID NO:3 is the nucleotide sequence of AAD-1 (v1) from *Sphingobium herbicidovorans*.

SEQ ID NO:4 is the nucleic acid sequence of the native AAD-1 gene with internal NotI restriction site removed. This gene is designated AAD-1 (v2). DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

SEQ ID NO:5 is a "plant-optimized" DNA sequence AAD-1 (v3). This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

SEQ ID NO:6 ("rdpA(ncoI)") and SEQ ID NO:7 ("3' saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

SEQ ID NO:8 is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

SEQ ID NO:9 is the native amino acid sequence encoded by the AAD-1 (v1) gene from *Sphingobium herbicidovorans*.

SEQ ID NO:10 is the amino acid sequence encoded by the AAD-1 (v2) DNA sequence of SEQ ID NO:4.

SEQ ID NO:11 is the amino acid sequence encoded by the AAD-1 (v3) plant-optimized DNA sequence of SEQ ID NO:5.

SEQ ID NO:12 is the DNA sequence of the native AAD-2 (v1) gene.

SEQ ID NO:13 is the amino acid sequence of the AAD-2 (v1) protein.

SEQ ID NO:14 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:15 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

SEQ ID NO:18 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:19 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:20 is the native soybean EPSPS protein.

SEQ ID NO:21 is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

SEQ ID NO:22 is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

SEQ ID NO:23 is primer Pat 5-3.

SEQ ID NO:24 is primer Pat 3-3.

SEQ ID NO:25 is forward primer AAD-1 PTU.

SEQ ID NO:26 is reverse primer AAD-1 PTU.

SEQ ID NO:27 is the forward primer for the Coding Region PCR AAD-1.

SEQ ID NO:28 is the reverse primer for the Coding Region PCR AAD-1.

SEQ ID NO:29 is the AAD-2 (v2) nucleotide (plant optimized).

SEQ ID NO:30 is the translated AAD-2 (v2) protein sequence.

SEQ ID NO:31 is the Southern fragment PCR AAD-1 forward primer.

SEQ ID NO:32 is the Southern fragment PCR AAD-1 reverse primer.

DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-1 gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

Table 1. Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

TABLE 1

Commercially available phenoxy auxins.

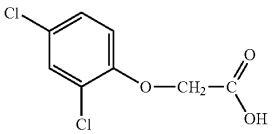
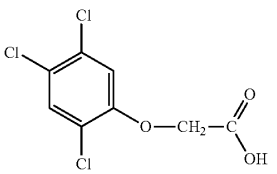
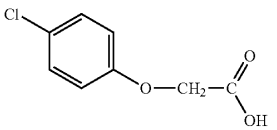
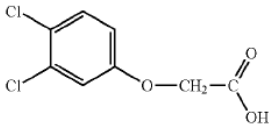
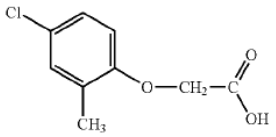
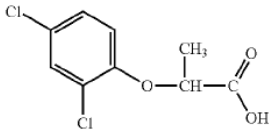
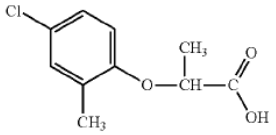
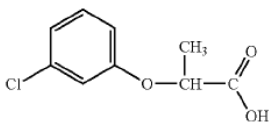
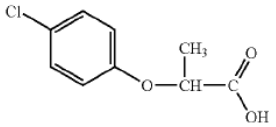
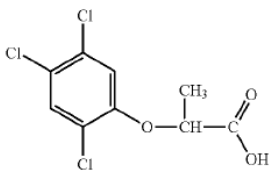
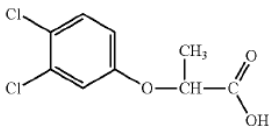
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Dichlorprop	120-36-5	25-12000	100-2250	
Mecoprop	7085-19-0	25-4000	250-3360	
Cloprop	101-10-0	25-4000	25-4000	
4-CPP	3307-39-9	25-4000	25-4000	
Fenoprop	93-72-1	25-4000	25-4000	
3,4-DP	3307-41-3	25-4000	25-4000	

An additional benefit of the AAD-1 gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). See Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing AAD-1, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, sorghum, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most

dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

Table 2. AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

TABLE 2

AOPP graminicidal compounds listed by accepted common names.

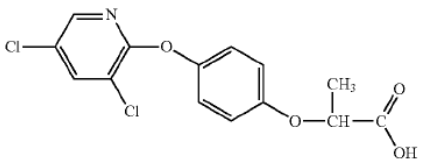
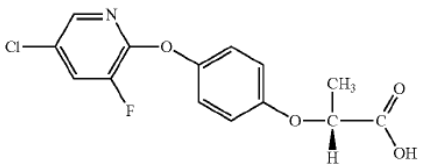
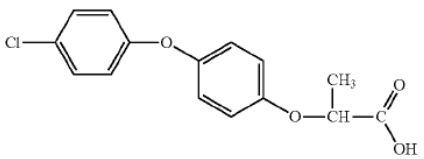
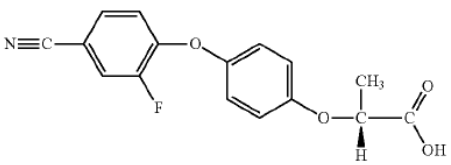
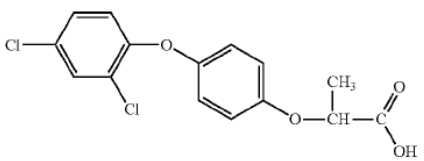
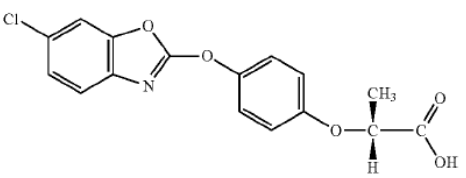
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

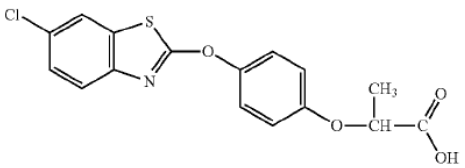
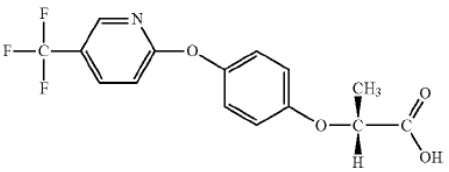
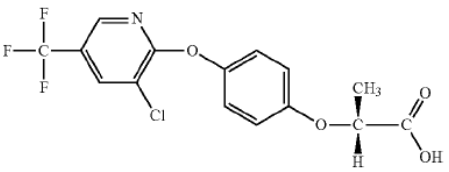
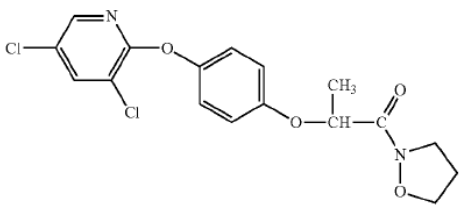
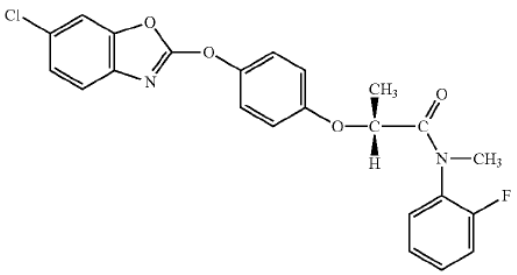
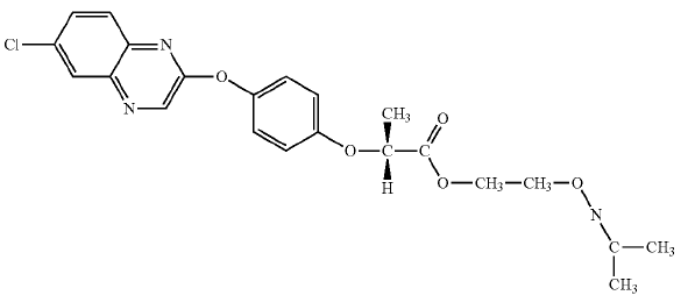
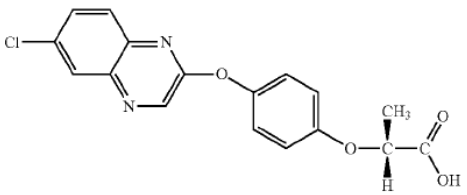
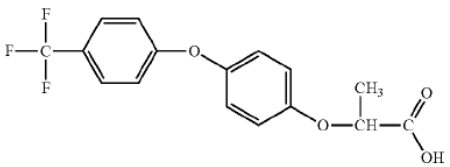
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenthiaprop	95721-12-3	10-2000	25-420	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	
Propaquizafop	111479-05-1	10-2000	30-240	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

A single gene (AAD-1) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, AAD-1 can provide protection in planta to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing AAD-1 alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-1 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and AOPP herbicide(s) (e.g., quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-1 stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, GOX, GAT), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance

(e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries=AHAS, Csr1, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

Additionally, AAD-1 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to tfdA, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes.

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-1 (AryloxyAlkanoate Dioxygenase) genes and proteins.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100% conversion) in vitro. An additional advantage that AAD-1-transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an AAD-1 gene.

In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzyme in the degradation pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. TfdA catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al., 1989; Lyon et al., 1989; Lyon et al., 1993). A large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to tfdA.

RdpA, from *Sphingobium herbicidovorans* (Westendorf et al., 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath et al., 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf et al., 2003). Heterologous expression of rdpA in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of tfdA that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that rdpA or AAD-1 genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

As described in more detail in the Examples below, rdpA was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical

classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf et al., 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. RdpA has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. AAD-1 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (e.g., glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, et al.), and insect resistance traits (Cry1F, Cry1Ab, Cry 34/45, et al.) for example. Additionally, AAD-1 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-1-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (e.g., glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

The transgenic expression of the subject AAD-1 genes is exemplified in, for example, *Arabidopsis*, corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

One gene of the subject invention, referred to herein as AAD-1 (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli*. When this gene is overexpressed (by induction of 1 mM IPTG and culture lysate combined with the following reaction mix: 112.5 μg/ml 2,4-D, 1 mM Ascorbic

acid, 1 mM α-ketoglutarate, 50 μM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay).

Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See Tables 3 and 4).

TABLE 3

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

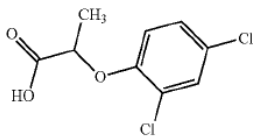
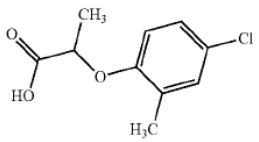
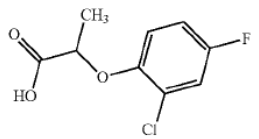
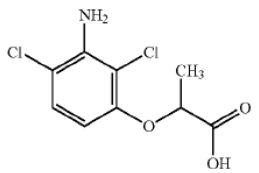
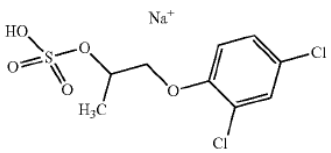
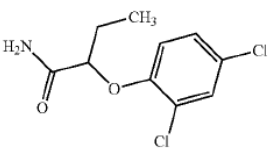
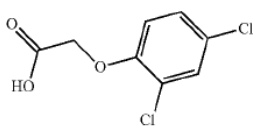
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383

TABLE 3-continued

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

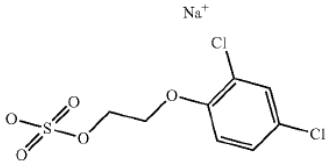
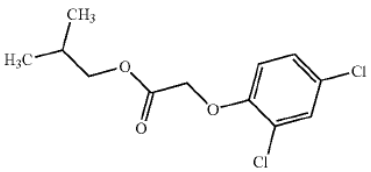
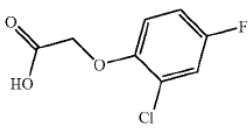
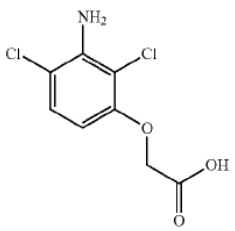
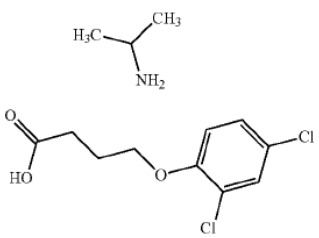
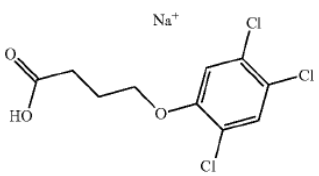
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02

TABLE 3-continued

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.
Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

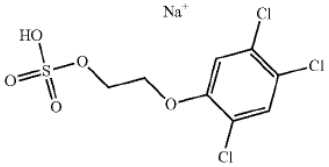
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	188527		0.003	0.036

TABLE 4

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.
Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

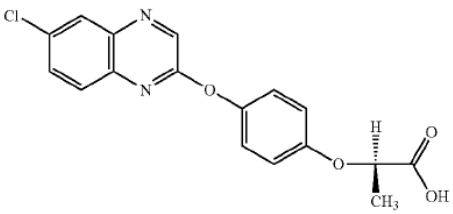
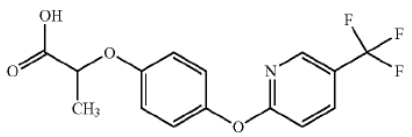
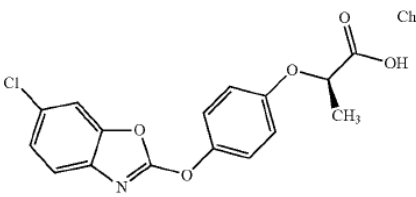
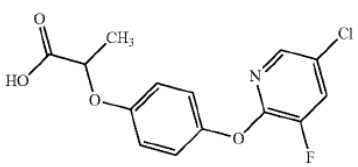
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
 Chiral	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98

TABLE 4-continued

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

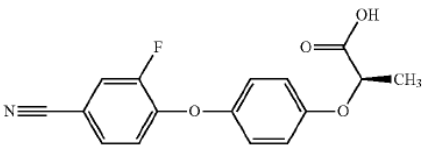
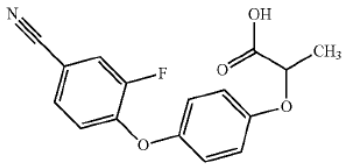
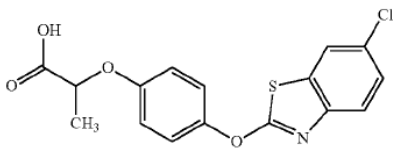
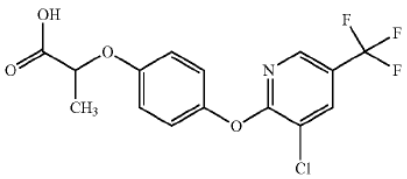
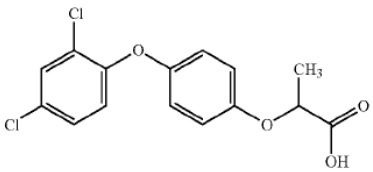
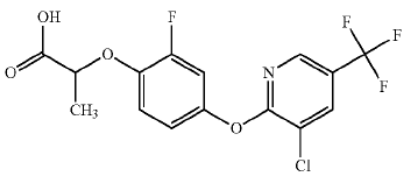
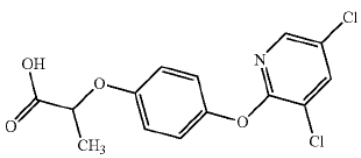
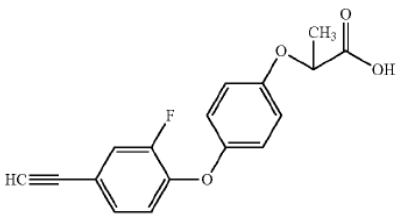
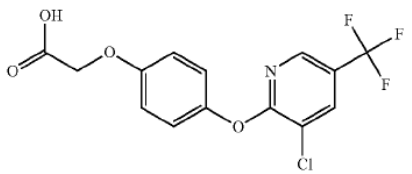
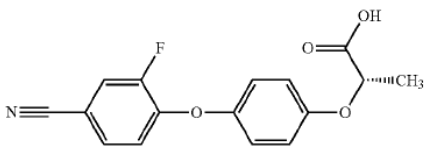
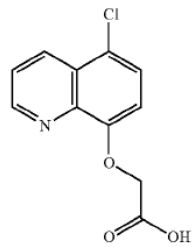
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584

TABLE 4-continued

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize

these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known rdpA's, such as *Sphingobium, Delftia, Rodoferax*, and *Comamonas* for example, can be used as source isolates.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

Polynucleotides and probes. The subject invention further provides nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-1 gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein in vivo, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing

with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G. H., M. M. Manak (1987) *DNA Probes*, Stockton Press, New York, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C. below the melting temperature (Tm) of the DNA hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983):

$$Tm = 81.5° C. + 16.6 \log [Na+] + 0.41(\% G+C) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs.}$$

Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm−20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (Tm) of the hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

$$Tm(° C.) = 2(\text{number } T/A \text{ base pairs}) + 4(\text{number } G/C \text{ base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low: 1 or 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, dele-

tions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

In addition, U.S. Pat. No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

"Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (see, e.g., Höfte et al. (1989), and Adang et al. (1985)). As used herein, the term "protein" can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino

acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12. Gapped BLAST can be used as described in Altschul et al., 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. See NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, Md., U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 5 provides a listing of examples of amino acids belonging to each class.

TABLE 5

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

Vectors comprising an AAD-1 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, 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).

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (PAT), glyphosate (EPSPS), imazethyapyr (AHAS), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. 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. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for

plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimu-

lus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1

Method for Identifying Genes That Impart Resistance to 2,4-D In Planta

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

PCR and cloning of gene into pET. A single gene (rdpA) was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to tfdA from *Ralstonia eutropha*. Percent identity was determined by first translating both the rdpA and tfdA DNA sequences deposited in the database to proteins, then using ClustalW in the Vector NTI software package to perform the multiple sequence alignment.

The strain of *Sphingobium herbicidovorans* containing the rdpA gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days.

A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following conditions: 94° C. 45 sec, 52° C. 1.5 minute, 72° C. 1.5 minute, for 30 cycles, followed by a final cycle of 72° C. 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 4 ml Luria Broth+50 μg/ml Kanamycin (LB K), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency AAD-1 (v1) (AryloxyAlkanoate Dioxygenase).

Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) AAD-1 (v1) was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth+50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between AAD-1 (v1): pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C. for 4 hours with agitation.

Each culture was then spotted onto LB K plates, which were incubated at 37° C. overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

EXAMPLE 2

Expression and Testing

2.1—HPLC Analysis.

Plasmid pDAB 3203 was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C./200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30°

C./200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 μl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

For each reaction, the following were combined: 2,4-D (125 μg/ml), [Ascorbate (1 mM), Ferrous ion (50 μM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 μl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 μl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 μl of Acetonitrile+0.05% TFA.

The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10μ C18(2) 250×4.6 mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN+0.05% TFA: 50% H_2O+0.05% TFA to 100% ACN+0.05% TFA over 5 min.

2.2—Plate Test Bioassays for Herbicide Degradation.

Plant bioassays were used to determine if in vitro enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (i.e., monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. *Pencross* and *Arabidopsis thaliana* var. *Columbia* were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

Arabidopsis seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23° C.) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. Tables 6 and 7 represent herbicidal substrates tested.

TABLE 6

Arabidopsis plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

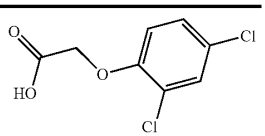
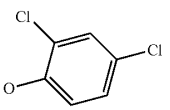
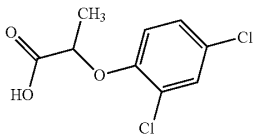
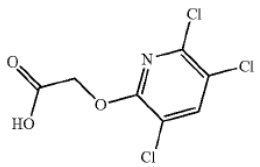
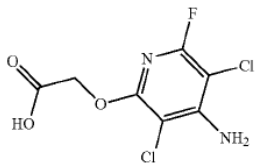
Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR_{50} ratio**	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	

TABLE 6-continued

Arabidopsis plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

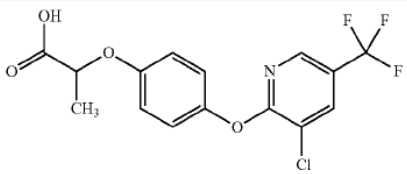
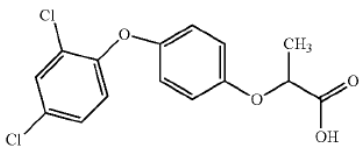
Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR_{50} ratio**	Structure
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

*Blank vector represents of cell lysate treatment where *E. coli* pET vector had no gene insert.

**GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥2 is considered the thresbold for detecting herbicide activity loss with this assay.

TABLE 7

Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR_{50} ratio**	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

*Blank vector represents of cell lysate treatment where *E. coli* pET vector had no gene insert.

**GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥2 is considered the threshold for detecting herbicide activity loss with this assay.

2.3—HPLC Results.

From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see FIG. **1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of

2,4-D herbicidal activity in the *Arabidopsis* plate assay (FIG. **2**). Regardless of the specific conditions of the assay (i.e., longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (i.e., oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluoroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (FIG. **3**) and diclofop. These results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

2.4—Plate Test Bioassays for Herbicide Degradation.

Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (FIG. **2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (Table 6). Often a GR_{50} could not be determined following enzyme treatment; de facto, no detectable herbicide activity remained.

The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (FIG. **3** and Table 7). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

EXAMPLE 3

In vitro Assay of AAD-1 (v1) Activity Via Colorimetric Phenol Detection

3.1—AAD-1 (v1) Assay.

AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily detected. Pyridinols derived from fluoroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C., the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid+3.73 g KCl+44 ml 1 N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 mM, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μA of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2% 4-aminoantipyrine, then adding 10 μl 10.8% potassium ferricyanide.

3.2—Extraction.

Activity of Recombinant AAD-1 (v1) Expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4+1 mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at −70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding AAD-1 (v1) was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without AAD-1 (v1) using 1 mM 2,4-D and is shown in FIG. **5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 μA of the *E. coli* extract per assay with a 15 min assay period. FIG. **6**A shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop=mecoprop>diclofop>haloxyfop>2,4-D. Thus

AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in FIG. **6**B. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

EXAMPLE 4

Substrate Specificity of AAD-1 (v1)

4.1—Additional substrates of AAD-1 (v1).

The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 μg per 160 μl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. Table 3 shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 μg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (Table 8). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7-fold better than 2,4-D as a substrate (Table 3). The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

TABLE 8

Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli*.
Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

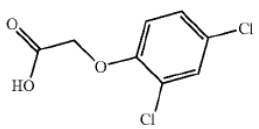
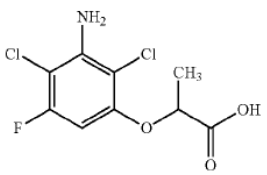
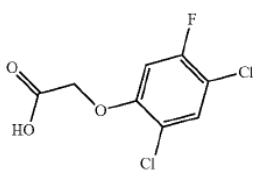
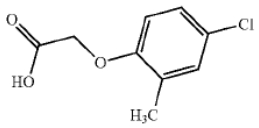
Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125
124987		5-F, 2,4-D	0.056	32

TABLE 8-continued

Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli*. Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
192711		MCPA	0.097	55

4.2—Kinetic Characterization.

The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C.). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in FIG. **7** and Table 9 respectively. The K_m values for the four substrates were fairly similar (75-125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in Table 3 and Table 4 as these were performed at high (saturating) substrate concentrations (1 mM).

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

Kinetic constants were derived from the data in FIG. 7 using Grafit fitting to the Michaelis-Menten equation.

The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf et al. (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

4.3—Additional Substrates for AAD-1 (v1).

Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in Table 8. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate (Table 3). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2, 4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

EXAMPLE 5

Optimization of Sequence for Expression in Plants

5.1—Background.

To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

Table 10 illustrates how high the G+C content is in maize. For the data in Table 10, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelerys, San Diego, Calif.). Intron sequences were ignored in the calculations.

TABLE 10

Compilation of G + C contents of protein coding regions of maize genes

Protein Class.sup.a	Range % G + C	Mean % G + C.sup.b
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+−.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+−.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+−.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+−.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+−.5.2)

.sup.a Number of genes in class given in parentheses.

.sup.b Standard deviations given in parentheses.

.sup.c Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 11

Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding

regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

Codon composition comparison of native AAD-1 (v1) coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
22	GCC	10	45.5	8	35	22	CTC	5	22.7	7	32
	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43		CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
23	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	100	8	100
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53	17	CCC	9	52.9	1	6
19	GAT	4	21.1	9	47		CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0	15	AGT	0	0.0	0	0
1	TAG	1	100.0	0	0		TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69	23	ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29		ACG	5	21.7	0	0
21	GGC	15	71.4	7	33		ACT	1	4.3	8	35
	GGG	3	14.3	2	10	TRP (W)	TGG	5	100	5	100
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (I)	ATA	0	0.0	3	25	28	GTC	11	39.3	8	29
12	ATC	12	100.0	5	42		GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

5.3—Completion of Binary Vectors.

5.3.1—Rebuilt AAD-1 (v3). The plant optimized gene AAD-1 (v3) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-1 (v3) DNA sequence. The AAD-1 (v3) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB720, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-1 (v3): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: AAD-1 (v3): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

5.3.2—Native AAD1 (v1) and modified AAD-1 (v2). The AAD-1 (v1) gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3' saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of AAD-1 (v1). This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC

ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation AAD-1 (v2) (SEQ ID NO:4).

A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and Sad enzymes. The resulting fragment was cloned into the pCR2.1 AAD-1 (v2) construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

The modified AAD-1 (v2) gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI-NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: AAD-1 (v2): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

5.3.3—Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID NO:21.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 13. Columns D and H of Table 13 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 13. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of } C1 = 1/(\%\ C1 + \%\ C2 + \%\ C3 + \text{etc.}) \times \%\ C1 \times 100$$

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 13 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 13. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Ind.).

TABLE 13

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	DNU	9.1
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	DNU*	8.5		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	DNU	9.8
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	DNU	8.2	LYS (K)	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	DNU	6.0	MET (M)	ATG	100.0	100
	CGT	16.9	14.5	PHE (F)	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	PRO (P)	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	DNU	8.3
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	SER (S)	AGC	16.0	15.1
END	TAA	DNU	40.7		AGT	18.2	17.1
	TAG	DNU	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9

TABLE 13-continued

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
GLN (Q)	CAA	55.5	55.5		TCG	DNU	6.1
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	THR (T)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	DNU	8.3
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	TRP (W)	TGG	100.0	100
	GGT	30.4	30.4	TYR (Y)	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	VAL (V)	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

*DNU = Do Not Use

To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston Tex.).

5.3.4—Cloning of additional binary constructs. The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to Gateway Technology: A universal technology to clone DNA sequence for functional analysis and expression in multiple systems, © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, Calif. 92008 (printed—2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis et al., 1982). Table 14 lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

The sacB gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay et al., 1983). The sacB gene was recovered on a Hind III fragment from plasmid pRE112 (Edwards, et al., 1998) and cloned into the unique Hind III site in pDAB3289.

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species* Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	—	—	—
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—

TABLE 14-continued

Binary constructs used in transformation of various plant species.

3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—
3403		Cn, R	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3404		Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3415	1283	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3602	1421	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	—	—

pDAB #	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721	Erythromycin	—	pat	CsVMV	Agro binary
3230	Spectinomycin	—	AAD1 v3	CsVMV	Agro binary
3289	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291	Spectinomycin	—	HptII	AtUbi3	Agro binary
3295	Spectinomycin	—	pat	AtUbi10	Aerosol beam
3297	Spectinomycin	—	pat	AtUbi10	Agro binary
3403	Ampicillin	—	Same as GOI		Whiskers/Gun
3404	Ampicillin	—	pat	OsAct1	Whiskers
3415	Ampicillin	—	AHAS v3	OsAct1	Whiskers
3602	Spectinomycin	—	AHAS v3	OsAct1	Agro Superbinary
3757	Spectinomycin	—	pat	AtUbi11	Agro binary
3705	Erythromycin	—	pat	CsVMV	Agro binary

*A = *Arabidopsis* T = Tobacco S = Soybean Ct = Cotton R = Rice Cn = Corn Ca = Canola
CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
NtOsm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region
(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region
ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

EXAMPLE 6

Transformation into *Arabidopsis* and Selection

6.1—*Arabidopsis thaliana* Growth Conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, Mo.) solution. The suspended seed was stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB+erythromycin). The cultures were incubated overnight at 37° C. with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4 kV, Pulse length: 5 msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C. in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, Mo.) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28° C.

for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP+erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10 µl of water, at 100° C. for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Minis Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5× concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C. for 3 minutes, 2) 94° C. for 45 seconds, 3) 55° C. for 30 seconds, 4) 72° C. for 1 minute, for 29 cycles then 1 cycle of 72° C. for 10 minutes. The reaction was maintained at 4° C. after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

6.3—*Arabidopsis* Transformation.

Arabidopsis was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700×g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 µM benzylamino purine (10 µl/liter of 1 mg/ml stock in DMSO) and 300 µl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

6.4—Selection of Transformed Plants.

Freshly harvested T_1 seed (transformed with native [AAD-1 (v2)] or plant optimized [AAD-1 (v3)] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3) (plant optimized gene) or AAD-1 (v2) (native microbial gene) to provide phenoxy auxin herbicide resistance.

Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, Del.) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200 g ae/ha).

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded in planta.

Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, AAD-1 (v3)-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluoroxypyr.

6.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3) (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were

identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15). A small lot of AAD-1 (v2) (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

TABLE 15

Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	AAD-1 (v2)	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	AAD-1 (v3)	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	AAD-1 (v3)	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	AAD-2 (v1)	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	AAD-2 (v2)	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280 g ai/ha glufosinate applied 5-6 + 10 DAP

[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP

[3]PAT protein expression determined by PAT ELISA strips

[4]nd, not determined

[5]codon bias, n-native microbial gene, p = plant optimized

T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 16 compares the response of native AAD-1 (v2) and plant optimized AAD-1 (v3) genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 16 simply to demonstrate the significant difference between the plants transformed with AAD-1 (v2) or AAD-1 (v3) versus the wildtype or PAT/Cry1F transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence AAD-1 (v3) versus the native sequence AAD-1 (v2) (see Table 16). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6× field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

Table 16. AAD-1 v3 (plant optimized), or AAD-1 v2 (native), or AAD-2 (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 16

AAD-1 v3 (plant optimized), or AAD-1 v2 (native), or AAD-2 (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury			% Injury	
					Std
Averages	<20%	20-40%	>40%	Ave	Dev
Native AAD-1 (v2) gene					
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2
Native AAD-2 gene					
Untreated control-buffer	4	1	1	25.0	21.7
50 g ae/ha 2,4-D	1	2	11	68.2	30.2
200 g ae/ha 2,4-D	0	3	11	82.7	28.8
800 g ae/ha 2,4-D	0	0	14	99.8	0.8
Rebuilt AAD-1 (v3) gene					
Untreated control-buffer	9	0	0	0.0	0.0
50 g ae/ha 2,4-D	10	1	5	24.3	35.9
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
Wildtype					
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
PAT/Cry1F (transformed control)					
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.7	4.2
200 g ae/ha 2,4-D	0	0	15	97.2	1.7
800 g ae/ha 2,4-D	0	0	15	100.0	0.0

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some AAD1 (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first in planta data that AAD-1 (v3) will provide AOPP resistance. No resistance was observed with fluoroxypyr (a pyridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with in vitro work using heterologously expressed enzyme.

Table 17. T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 17

T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100
PAT/Cry1F						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

Table 18. T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 18

T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

	% Injury			% Injury		
					Std	
Averages	<20%	20-40%	>40%	Ave	Dev	Range
AAD-1 v3						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100
Wildtype						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

6.6—AAD-1 (v3) as a Selectable Marker.

The ability to use AAD-1 (v3) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed transformed with PAT and AAD1 (v3) (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50 g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active PAT gene.

Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, PAT. The low

number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (Table 15). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with AAD-1 (v3) or PAT genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

6.7—Heritability.

A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis (P>0.05).

Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show AAD1 (v3) is stably integrated and inherited in a Mendelian fashion to at least three generations.

6.8—Additional Herbicide Resistance Attributable to AAD-1 in *Arabidopsis*.

The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified in vitro plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id=PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C.) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1% (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, N.J.). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25° C. under continual fluorescent lighting (75 $\mu E\, m^{-2}\, s^{-1}$). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio>2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluoroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

In vitro plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

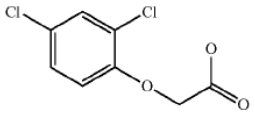
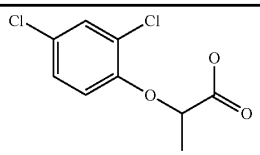
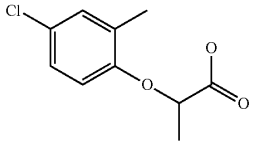
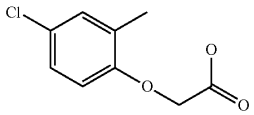
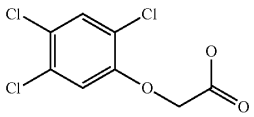
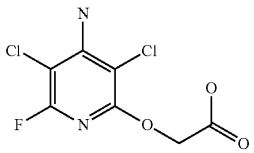
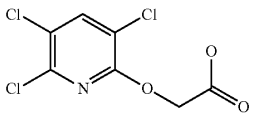
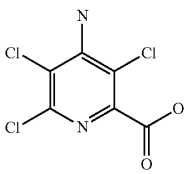
Compound	Structure	wt ARBTH shoot 150 ppm	PAAD1.315.064 T3 ARBTH shoot 150 ppm	wt ARBTH root 150 ppm	PAAD1.315.064 ARBTH root 150 ppm	shoot ratio	root ratio
Phenoxy auxins							
2,4-D		0.2	10	<0.01	0.04	50	>4

TABLE 19-continued

In vitro plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot 150	PAAD1.315.064 T3 ARBTH shoot 150	wt ARBTH root 150	PAAD1.315.064 ARBTH root 150	shoot ratio	root ratio
		ppm					
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

6.9—Foliar applications herbicide resistance in AAD-1 *Arabidopsis*.

The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for AAD-1 (v3) (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyac-

etates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluoroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

These results (see Table 20) confirm that AAD-1 (v3) in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates the in vitro enzyme and whole plate substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis*.

TABLE 20

Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Ave % Injury 14DAT	
Herbicide Treatment	AAD1.01.315.076.T_4 homozygous AAD1 plants	PatCry1f - Control
Phenoxypropionic auxins		
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100
Pyridyloxyacetic auxins		
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
Inactive DCP metabolite		
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

6.10—Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, AAD-1 (v3) T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae/ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. FIGS. **8**A and **8**B showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (FIG. **9**A), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

6.11—Molecular Analysis of AAD-1 (v3) *Arabidopsis*.

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-1 (v3) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-1 (v3). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling r×n (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using

the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

Results showed all 2,4-D resistant plants assayed contained PAT (and thus by inference, AAD-1 (v3)). Copy number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

6.12—*Arabidopsis* Transformed with Molecular Stack of AAD-1 (V3) and Glyphosate resistance gene.

T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (AAD-1 (v3)+EPSPS) coding for a putative glyphosate resistance trait. T_1 transformants were selected using AAD-1 (v3) as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-1 (v3)+PAT T_5 homozygous lines (pDAB721-transformed), and PAT+Cry1F homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by EPSPS molecularly stacked with AAD-1 (v3) (see FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

EXAMPLE 7

WHISKERS-Mediated Transformation Into Maize, and Use of AAD-1 (v3) as a Selectable Marker

7.1—Cloning of AAD-1 (v3).

The AAD-1 (v3) fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and Sad and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5a cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbi1/po-aad1/ZmPer5::OsAct1/PAT/ZmLip.

7.2—Callus/Suspension Initiation.

To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu et al., 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter et al., 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

7.3—Cryopreservation and Thawing of Suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C. for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C. in a controlled rate freezer, then allowed to freeze at a rate of −0.5° C./minute until reaching a final temperature of −40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Form a Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100×25 mm Petri dishes. Liquid was allowed to absorb into the filters for several min-

utes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

7.4—Dose Response of Non-Transformed Tissue to Haloxyfop Acid.

Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60×20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in FIG. **11**.

Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see FIG. **12**) shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 μg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60×20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C. in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

After 1 week, filter papers were transferred to 60×20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L bialaphos, 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C.) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100×15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C. in dark boxes.

Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60×20 mm plates. If sustained growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu m^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150×25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 μg of pDAB3403 and 85 μg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop

acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

7.7—Results. Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with AAD-1 (v3), unequivocally demonstrate stable integration of AAD-1 (v3) following Whiskers-mediated transformation coupled with "fop" selection.

7.8—Quantitative Demonstration of In Vitro Tolerance from AAD-1 (v3) Expressing Callus Events from Bialaphos Selection

Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and AAD-1 (v3) PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in Table 21 below. At least 15 T_0 plants were regenerated from each of these events and sent for spray testing and seed production.

TABLE 21

Event	PAT Copy #	PCR for AAD-1 (v3) PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in FIG. **13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

The ZmUbi1/AAD-1 (v3)/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the AAD-1 (v3) and AHAS Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 μM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression—a subset of the data is shown below. For the events listed in Table 22, the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

TABLE 22

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
1283[1]-001	2	–
1283[1]-002	206	+++
1283[1]-003	1	–
1283[1]-004	90	+++
1283[1]-005	1	–
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	–
1283[1]-011	4	–
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	–
1283[1]-015	194	+++
1283[1]-016	4	–
1283[1]-017	196	+++
1283[1]-018	3	–
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	–
1283[1]-027	1006	++
Neg Control	3	–
Neg Control	3	–
Standard (0.5 μg/mL)		++
Standard (5 μg/mL)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

7.10.1—Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

7.10.2—Invader assay analysis. The DNA samples are diluted to 20 ng/μl then denatured by incubation in a ther-

mocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma) The plates are then incubated at 63° C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

7.10.3—Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-1 (v3) PTU were (Forward-ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse-CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. Primers for Coding Region PCR AAD-1 (v3) were (Forward-ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse-CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

7.10.4—Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling r×n (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

EXAMPLE 8

In Vivo Tolerance and Field Tolerance Data Generated from PAT-Selected (pDAB3404) AAD-1 (v3) events

8.1—Tolerance of T_0 Corn Plants to AOPP Herbicides.

If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1× lethal dose (~⅛× field dose) up to 8× field doses (64× lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each AAD-1 (v3)-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8× field dose (64× lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 in vitro.

FIG. **14** shows the responses of several AAD-1 (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

Table 23 shows data for the responses of selected AAD-1 (v3)-transformed T_0 corn events to three AOPP herbicides applied postemergence.

TABLE 23

Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super[#] + 1% COC (v/v)
**Clincher[#] + 1% COC (v/v)
***Assure II + 1% COC (v/v)
[#]Trademark of Dow AgroSciences, LLC

8.2—Field Tolerance of pDAB3404 T_1 Corn Plants to Quizalofop 2,4-D, and Glufosinate Herbicides.

Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II×5×H571 is of the same parentage as the AAD-1 (v3) event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the subplot was AAD-1 (v3) event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

Glufosinate at 560 g ai/ha was applied to AAD-1 (v3) plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

8.2.1—Results.

AAD-1 (v3) event response to the highest rates tested of quizalofop and 2,4-D are shown in Table 24. These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All AAD-1 (v3) events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the AAD-1 (v3) events except with the events noted above.

TABLE 24

Event or Hybrid	Treatment (rate) = Quizalofop (140 g ae/ha) Evaluation = % Injury 3 WAA (0-100 scale) IN	HI	2,4-D amine (1120 g ae/ha) % Leaning 2 DAA (0-100 scale) IN	HI	2,4-D amine (1120 g ae/ha) Braceroot Deformation 3-4 WAA (0-10 scale) IN	HI	Analysis Results AAD-1 copy number leaf (Southern analysis)	AAD-1 Leaf Western Blot T_0	AAD-1 Leaf ELISA T_0	Resistance Segregation Ratios following Liberty Spray T_1 Population Average IN	HI	T_2 Population Average HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/−	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51%	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/−	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493 HI-II X 5XH571 CROPLAN 585SR	100	100	20	23	8	9						
	100	100	13	34	7	9						
	80	96	11	33	9	9						

*Fits single locus dominant trait segregation as determined by chi square analysis ($P > 0.05$)

2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all AAD-1 (v3) events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all AAD-1 (v3) events except one lineage of 3404.001.

Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

These results indicate that most AAD-1 (v3) transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. See also FIG. **16**.

8.2.2—Expected Mendelian segregation ratios on three T_2 events. Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see Table 24 above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all AAD-1 (v3) lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see Table 24). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to AAD-1 (v3) Invader assay description for corn). Hemizygous plants were removed and homozygous AAD-1 (v3) plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, AAD-1 (v3), and glufosinate resistance.

8.3—Stacking of AAD-1 (v3) and Pat with Glyphosate Resistance Genes in Corn.

Homozygous T_2 AAD-1 (v3)/PAT corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing AAD-1 (v3), PAT, and glyphosate resistance genes as described in the previous example.

F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha,

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50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in Table 25.

TABLE 25

		% Injury at 14 DAT	
	Field Rate	Hi II X 5XH751 Ave	RR/PAT/AAD1 Ave
Untreated control	—	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate + 1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X + 4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X + 2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

This study demonstrated that the AAD-1 (v3) gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

8.3.1—Resistance of AAD-1 (v3) corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

8.3.2—Tolerance of (pDAB3403) T_0 corn plants to quizalofop herbicide. A target of approximately eight T_0 plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1× field rate, 4× lethal dose) to 3-leaf, greenhouse-adapted T_0 corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more T_0 clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4× lethal dose of quizalofop at the whole plant level.

8.3.3 Expression of AAD-1 (v3) with Respect to Quizalofop Tolerance.

Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see FIG. **15**.). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× and higher field rates. This data

concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1× field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

EXAMPLE 9

Agrobacterium-Mediated Transformation of Maize with AAD-1 (v3)

9.1—Plant Material.

Seeds of a "High II" (i.e., Parent A and B) F_1 cross (Armstrong et al., 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

9.2—Tissue Source.

For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

9.3—Preparation of a Superbinary Vector.

Construction of an *Agrobacterium* construct, pDAB2272, containing the AAD-1 (v3) gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/AAD-1 (v3)/ZmPer5 v2 and inserting it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/AAD-1 (v3)/ZmPer5 v2 and the OsAct1 v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~1×10^9 cfu/ml) and 100 μM acetosyringone added to the solution.

9.5—Infection and Cocultivation.

The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$, 1, 100 μM acetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

9.6—Dose Response Using Immature Embryos.

Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously. Once treated, embryos were allowed to co-cultivate for 5 days at 25° C. and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50-100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in FIG. **17**.

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

9.8—Regeneration and Seed Production.

For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 μM cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

9.9—Event Recovery and Analysis Particulars; Whole Plant Screening of T_0 Corn Lineages Containing AAD-1 (v3) and AHAS (pDAS1421).

Seventy-two *Agrobacterium*-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid in vitro. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of AAD-1 (v3) into the genome as described previously. Ten single cony events, as indicated in Table 26, were chosen to be regenerated.

TABLE 26

Corn event	In vitro Selection agent (nM)	Southern Copy # (callus) AAD-1	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0

Events with more than 1 copy were not taken to the greenhouse

A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the T_0 generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (AHAS) on the function of the AAD-1 (v3).

EXAMPLE 10

Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000 g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

EXAMPLE 11

Recombinant AAD1 Purification and Antibody Production

Plasmid pDAB3203 containing the AAD-1 (v1) gene was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C. with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C. with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000×g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

All AAD-1 (v1) purifications were conducted at 4° C. as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

11.1—Extracting AAD-1 (v3) from Plant Leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1% Triton X-100 and 10 mM DTT). The tubes were shaken for 4 mM with a bead beater at maximum speed followed by centrifu-

gation for 10 min at 5,000×g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 μg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

11.3—Enzyme Linked Immuno-Sorbent Assay (ELISA).

The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C. for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL, 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

11.4—Western Blotting Analysis.

Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

EXAMPLE 12

Tobacco Transformation

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C. and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, AAD-1 (v3)+PAT), grown overnight in a flask on a shaker set at 250 rpm at 28° C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Fla.) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C. in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C. greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C., 16 hour day, 8 hour night, minimum natural+supplemental light=500 $\mu E/m^2s^1$).

Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-1 (v3) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

The DNA samples were diluted to 9 ng/μl, then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl2 (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma). The plates were then incubated at 63° C. for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (Table 27).

TABLE 27

Tobacco T0 events transformed with pDAB721 (AAD-1(v3) + PAT).

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (µg AAD-1/ml plant extract)	Relative tolerance T0 spray with 2,4-D*
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:

Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
Low	>50% injury
Medium	20-50% injury
High	<20% injury
Variable	inconsistent

Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

All events were also assayed for the presence of the AAD-1 (v3) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 64° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (Table 27). Protein expression varied across events.

T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5× field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6× field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (Table 27).

Verification of High 2,4-D Tolerance.

Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two AAD-1 (v3) tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (Table 28). No injury was observed at rates below IX field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by AAD-1 (v3), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

TABLE 28

Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

Herbicide	KY160 - Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

A 100 plant progeny test was also conducted on each of the two AAD-1 (v3) lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with AAD-1 (v3)-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

EXAMPLE 13

Soybean Transformation

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette et al., 1995), amino acid modification (Falco et al., 1995), and insect resistance (Parrott et al., 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe et al., 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee et al., 1988) or zygotic embryos (Chee et al., 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchee et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-1 (v3) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-1 (v3).

13.1—Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that

have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This de novo meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee et al., 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang et al., 1999; Zeng et al., 2004).

13.1.1—*Agrobacterium* preparation. The plasmid, pDAB721, contains the AAD-1 (v3) gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the PAT gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101 S by electroporation.

Agrobacterium cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

13.1.2—Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C., with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg et al. (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100×25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N)HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee et al., 1988).

Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from 10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

13.1.3—Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-1 (v3) and PAT genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of AAD-1 (v3) will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

13.2—Transformation Method 2: "No-Shake" *Agrobacterium* Mediated Trans-Formation of Non-Regenerable Soybean Suspension Cells.

The DAS Soybean cell suspensions were cultured on a 3d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were then transferred to orbital shakers at 140 rpm.

Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100×25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 μL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

13.2.1—Selection of soybean suspension cells and isolation of transformed colonies. After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100×15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L

level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (Table 29) and Western Blotting (FIG. **18**) analysis on two separate soybean calli transformed with AAD-1 (v3) gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

TABLE 29

PCR and ELISA data for transgenic soybean events.

Event	AAD-1 Coding region PCR	PAT Coding region PCR	T SP (µg/mL)	AAD-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	−	−	2074.63	−1.22	−0.0001%

13.3—Transformation Method 3: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Pat. No. 6,809,232 (Held et al.).

Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

Variations of media referred to in Table 30 were tested, e.g., B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	B3	B5G
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8 g	8 g	
Gelrite			2 g
pH	5.8	5.8	5.8

EXAMPLE 14

AAD-1 (v3) Enablement in Cotton

14.1—Cotton Transformation Protocol.

Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 31) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 µE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 31) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 31) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 31). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

Agrobacterium preparation. Inoculate 35 ml of Y media (Table 31) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C., while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 31) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using

M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (Table 31), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 31) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

Larger, well-developed embryos are isolated and transferred to DK media (Table 31) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 31

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

14.2—Experiment Specifics.

For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

14.3—Callus Analysis Results.

The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (Table 32). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (Table 32). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (Table 32). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

TABLE 32

Analysis of cotton callus

Line	Copy #	PTU	Western
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*
standard	AAD1	5 ug/ml	*****
standard	AAD1	0.5 ug/ml	**

14.4—Plant Regeneration.

Two AAD-1 (v3) cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the AAD-1 (v3) gene provides resistance to 2,4-D in cotton, both the AAD-1 (v3) cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the AAD-1 (v3) plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the AAD-1 (v3) plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

This study demonstrates that the AAD-1 (v3) gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

EXAMPLE 15

Agrobacterium Transformation of Other Crops

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchee et al., 1988. For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao et al., 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay et al., 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng et al., 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei et al., 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-1 (v3), for example, into these and other plants, including but not limited to Maize (Gramineae *Zea mays*), Wheat (Pooideae *Triticum* spp.), Rice (Gramineae *Oryza* spp. and *Zizania* spp.), Barley (Pooideae *Hordeum* spp.), Cotton (Abroma Dicotyledoneae *Abroma augusta*, and Malvaceae *Gossypium* spp.), Soybean (Soya Leguminosae *Glycine max*), Sugar beet (Chenopodiaceae *Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (Solanaceae *Lycopersicon esculentum* and other spp., *Physalis ixocarpa, Solanum incanum* and other spp., and Cyphomandra betacea), Potato, Sweet potato, Rye (Pooideae *Secale* spp.), Peppers (Solanaceae *Capsicum annuum, sinense*, and *frutescens*), Lettuce (Compositae *Lactuca sativa, perennis*, and *pulchella*), Cabbage, Celery (Umbelliferae *Apium graveolens*), Eggplant (Solanaceae *Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (Leguminosae *Medicago sativum*), Carrot (Umbelliferae *Daucus carota sativa*), Beans (Leguminosae *Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (Leguminosae *Pisum, Vigna*, and *Tetragonolobus* spp.), Sunflower (Compositae *Helianthus annuus*), Squash (Dicotyledoneae *Cucurbita* spp.), Cucumber (Dicotyledoneae genera), Tobacco (Solanaceae *Nicotiana* spp.), *Arabidopsis* (Cruciferae *Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis*, and other families), and Clover (Leguminosae). Such plants, with AAD-1 (v3) genes, for example, are included in the subject invention.

EXAMPLE 16

Stacking AAD-1 (v3) with AHAS Herbicide Resistance Gene

Stacking AAD-1 (v3) with an AHAS herbicide resistance gene is described in Example 7.9.

EXAMPLE 17

Further Evidence of Surprising Results: AAD-1 vs. AAD-2

17.1—AAD-2 (v1) Initial Cloning.

Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the Vector NTI software package to perform the multiple sequence alignment.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 μl, ea. primer 1 μl (50 ng/μl), gDNA 1 μl (200 ng/μl), H_2O 21 μl, Taq polymerase 1 μl (2.5 units/μl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C. 3.0 minutes heat denature cycle; 95° C. 1.0 minute, 50° C. 1.0 minute, 72° C. 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C. 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 3 μl Luria Broth+1000 μg/ml Ampicillin (LB Amp), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

17.2—Completion of AAD-2 (v1) Binary Vector.

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium.*

17.3—Comparison of Substrate Specificities of AAD-2 (v1) and AAD-1 (v1)

The activity of an extract from *E. coli* expressing AAD-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 μl (42 μg) of *E. coli* extract per assay with a 15 min assay period. FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. Table 33 shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

TABLE 33

Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

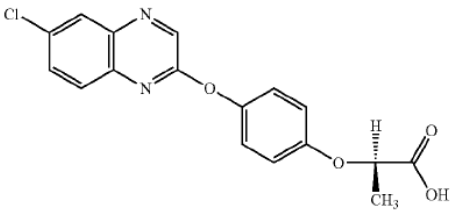
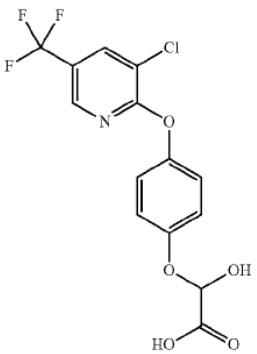
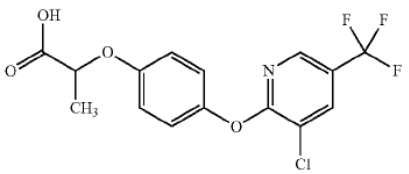
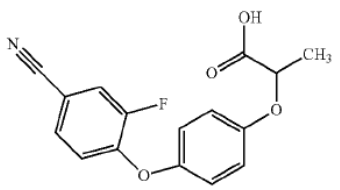
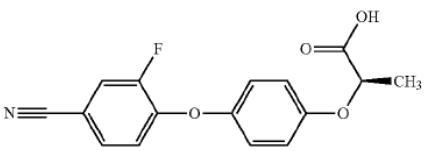
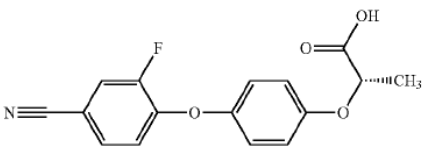
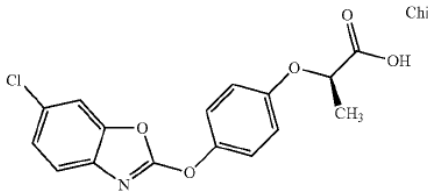
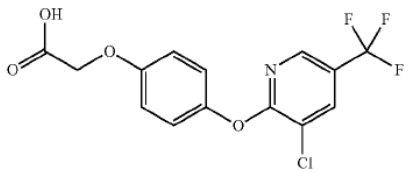
				A510	
STRUCTURE	Reg ID	Compound	Enantiomer	AAD1	AAD2
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

STRUCTURE		Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	Chiral	11044492	fenoxaprop	R	0.14	0
		43865	haloxyfop-acetate	—	0	0.22

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **19**. The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (Table 34). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D in vitro than AAD-1 (v1). This is in surprising contrast to the in planta findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

TABLE 34

Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ± SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes:

Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

17.4—Evaluation of Transformed *Arabidopsis*.

Freshly harvested T_1 seed transformed with a native [AAD-1 (v2)], plant optimized [AAD-1 (v3)], or native AAD-2 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1) to provide phenoxy auxin herbicide resistance.

Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, Del.) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0).

17.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and

493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15 in Example 6.5 above). A small lot of native AAD-1 (v2) transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native AAD-2(v1) gene provided a very low transformation frequency when selected for glufosinate tolerance (PAT selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% (see Table 15). Transformation frequency for native AAD-2 (v1) was only 6% of that of native AAD-1 (v2). The native AAD2 (v1) gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into *Arabidopsis* using methods previously described (see Example 5). The plant optimized AAD2 (v2) (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (see Table 15).

T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 16 (in Example 6.5 above) compares the response of AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) and AAD-1(v2) genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 16 simply to demonstrate the significant difference between the plants transformed with AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) or AAD-2 (v2) versus the wildtype or PAT/Cry1F transformed controls.

Surprisingly, AAD-2 (v1) transformants were far less resistant to 2,4-D than either AAD-1 (v2) or AAD-1 (v3) genes (Table 16) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v1) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) AAD-1 (v2)-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average=34%), and >73% (11 of 15) AAD-1 (v3) T_1 plants were uninjured (population injury average=14%). See FIG. **20**. Tolerance improved slightly for plant-optimized AAD2 (v2) versus the native gene; however, comparison of both AAD-1 and -2 plant optimized genes indicates a significant advantage for AAD-1 (v3) in planta (see Table 16).

These results are unexpected given that in vitro comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. See FIG. **21**. No large difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) in planta for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected.

EXAMPLE 18

Preplant Burndown Applications

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With AAD-1 (v3) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed.

EXAMPLE 19

In-Crop Use of Phenoxy Auxins Herbicides in AAD-1 (v3) Only Transformed Soybeans, Cotton, and Other Dicot Crops

AAD-1 (v3) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D, dichlorprop, MCPA, et al.) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3)-transformed dicot crop (i.e., soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of AAD-1 (v3)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 20

In-Crop Use of Phenoxy Auxins and AOPP Herbicides in AAD-1 (v3) Only Transformed Corn, Rice, and Other Monocot Species

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-1 (v3) would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. AAD-1 (v3)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, et al. could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of AAD-1 (v3)-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3) transformed monocot crop (e.g., corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and

reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the AAD-1 (v3) gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 21

AAD-1 (v3) Stacked with Glyphosate Tolerance Trait in any Crop

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-1 (v3) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD–1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-1 (v3)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 22

AAD-1 (v3) Stacked with Glufosinate Tolerance Trait in any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

By stacking AAD-1 (v3) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel

transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
   i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 23

AAD-1 (v3) Stacked with Ahas Trait in any Crop

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking AAD-1 (v3) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.
   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by AAD-1 (v3) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

EXAMPLE 24

AAD-1 (v3) in Rice

24.1—Media Description.

Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryo-

genic calli were cultured in 100×20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C., and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (½MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 μl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 μl spermidine (0.1 M) and 50 μl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 μl cold 100% ethanol and 8-9 μl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

24.4—Tolerance Testing.

Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 μl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in Table 35 below.

TABLE 35

Sample Name	140 g ae/ha quizalofop
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

24.5—Tissue Harvesting, DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

24.6—Southern Blot Analysis.

Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane

was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse—GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in Table 36.

TABLE 36

Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049 bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

24.7—Western Data

Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in Table 37.

TABLE 37

Lane	Sample Name	TSP (μg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	–
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	–
7	AAD1	Standard (0.5 μg/mL)			+++
8	AAD1	Standard (5.0 μg/mL)			+++++

EXAMPLE 25

Turf Grass Transformation Procedures

Genetic transformation, with AAD-1 (v3) substituted for the "bar" gene as described below, of creeping bentgrass mediated by *Agrobacterium tumefaciens* could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. See "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo et. al., 2004).

Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60-65%) contained only a single copy of the foreign gene with no apparent rearrangements.

25.1—Seed Preparation for Embryogenic Callus Induction.

Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-o-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120° C. for 20 min).

25.2—Embryogenic Callus Induction.

The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

25.3—*Agrobacterium* Infection and Co-Cultivation.

One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μM acetosyringone. A 10-ul aliquot of *Agrobacterium* (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25° C.

25.4—Resting Stage and *Agrobacterium* Control.

For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

25.5—Selection and Potential Transgenic Colony Identification.

Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

25.6—Regeneration of Transgenic Plants.

For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2-3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

25.7—Root Induction and Transfer to Greenhouse.

Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3-5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

25.8—Vernalization and Out-Crossing of Transgenic Plants.

Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25° C. under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25° C., and T1 plants were grown in the greenhouse for further analysis.

25.9—Other Target Grasses.

Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/cheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

EXAMPLE 26

AAD-1 (v3) in Canola

26.1—Canola Transformation.

The AAD-1 (v3) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. *Nexera** 710 with *Agrobacterium*-mediated transformation. The construct contained AAD-1 (v3) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C., and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z7075 or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of AAD-1 (v3), whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

The transformed TO plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the AAD-1 (v3) gene.

26.2—"Molecular Analysis": Canola Materials and Methods

26.2.1—Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular

Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/ul.

26.2.2—Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR AAD-1 (v3) were (Forward—ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse—CGGGCAGGC-CTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-1 (v3) events tested positive. Three negative control samples tested negative.

26.3—ELISA.

Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

TABLE 38

Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	–
Control 2	61	2549.60	35.00	13.73	–
Control 3	59	2374.41	22.79	9.60	–

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Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145 150 155 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln

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               165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 10
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v2 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: Different from v1

<400> SEQUENCE: 10

Met Ala Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1               5                   10                  15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65                  70                  75                  80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190
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Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 11
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v3 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Different from V1

<400> SEQUENCE: 11

Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1               5                   10                  15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
            20                  25                  30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
        35                  40                  45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
    50                  55                  60

Asn Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro
65                  70                  75                  80

Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
        115                 120                 125

Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
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225                 230                 235                 240

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
                245                 250                 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
            260                 265                 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
        275                 280                 285

Val Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 12
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 12

atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat     60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac    120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg    180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg    240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag    300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc    360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc    420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc    480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc    540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc    600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc    660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag    720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc    780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg    840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag                 888


<210> SEQ ID NO 13
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 13

Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                   10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
```

```
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
                245                 250                 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
            260                 265                 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
        275                 280                 285

Val


<210> SEQ ID NO 14
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: brjap 5'(speI)

<400> SEQUENCE: 14

actagtaaca aagaaggaga tataccatga cgat                                34


<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: br jap 3' (xhoI)

<400> SEQUENCE: 15

ttctcgagct atcactccgc cgcctgctgc tgc                                 33


<210> SEQ ID NO 16
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 forward primer

<400> SEQUENCE: 16

gtaaaacgac ggccagt                                                   17


<210> SEQ ID NO 17
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: M13 reverse primer

<400> SEQUENCE: 17

caggaaacag ctatgac 17

<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NcoI of Brady

<400> SEQUENCE: 18

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 19
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SacI of Brady

<400> SEQUENCE: 19

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 20
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
85 90 95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
100 105 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
115 120 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
130 135 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145 150 155 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
165 170 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
180 185 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
195 200 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
210 215 220

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Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525


<210> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine
      183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 21

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                   10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
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    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480
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Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525


<210> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding doubly-
      mutated EPSPS disclosed in SEQ ID NO:21, with added sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence
      encoding the EPSPS protien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop
      codons in all six open reading frames, and to introduce a SacI
      restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 22

atggctcaag tctcccgtgt tcacaatctt gctcagtcaa cccaaatctt tggacattca     60

agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttcgcccacg cctttgggga    120

gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt    180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc    240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg    300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc    360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga    420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt    480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat    540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt    600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc    660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga    720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc    780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa    840

atagtggaca agttgatttc tgttccatat gtggaaatga ccctcaaact catggagagg    900

tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag    960

aagtacaaaa gccctggcaa tgcctttgtt gaaggggatg caagctctgc ttcctatctc   1020

ttggctgggg ctgccatcac tggtgggacc atcactgtga atggctgtgg cacctcatcc   1080

cttcaaggtg atgtaaagtt tgcagaggtc ttggagaaaa tgggtgccaa ggtcacctgg   1140

tctgagaaca gtgtaactgt gtctggacct cccagagact tcagtggcag aaaggttctc   1200

cgtggaattg atgtgaacat gaacaagatg ccagatgtgg ccatgaccct cgctgttgta   1260

gccctgtttg caaatggacc aactgcaatc cgtgatgttg cttcatggag ggtgaaggag   1320
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acagagagga tgattgccat ttgcacagaa ctccgcaaac ttggtgcaac agttgaagag 1380

ggaccagatt actgtgtgat aaccccacct gagaagctca atgtgacagc cattgacacc 1440

tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 5-3

<400> SEQUENCE: 23

agataccctt ggttggttgc 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 3-3

<400> SEQUENCE: 24

cagatggatc gtttggaagg 20

<210> SEQ ID NO 25
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU forward primer

<400> SEQUENCE: 25

ataatgccag cctgttaaac gcc 23

<210> SEQ ID NO 26
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU reverse primer

<400> SEQUENCE: 26

ctcaagcata tgaatgacct cga 23

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for Coding Region PCR AAD-1
(RdpAcodF)

<400> SEQUENCE: 27

atggctcatg ctgccctcag cc 22

<210> SEQ ID NO 28
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for Coding Region PCR AAD-1

-continued

(RdpAcodR)

<400> SEQUENCE: 28

cgggcaggcc taactccacc aa 22

<210> SEQ ID NO 29
<211> LENGTH: 932
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-2 v2 plant-optimized nucleotide

<400> SEQUENCE: 29

ccatggctac catagcaatc agacagctcc agacccactt tgtgggtcaa gtttctggat 60

tggacctcag aaagccactc actcctggag aagccagaga agttgaatca gctatggaca 120

agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg 180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt 240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg 300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca 360

gcttcagacc aatcccagcc aagttcagcc tccttttctgc tcgtgttgtg aacccaactg 420

gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

ttgccagcat gtccgtccct gaagggaggc tccttttgag ggatttgaat gaacatgcta 720

ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

gtgcaacagt tgctgggacc gagcccacag tgcaacagca agcagcagag tgagtagtta 900

gcttaatcac ctagagctcg gtcaccagat ct 932

<210> SEQ ID NO 30
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Translated AAD-2 v2 protein

<400> SEQUENCE: 30

Met Ala Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln
1 5 10 15

Val Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg
20 25 30

Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
35 40 45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
50 55 60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65 70 75 80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
85 90 95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
100 105 110

-continued

```
Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
        115                 120                 125

Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
    130                 135                 140

Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
145                 150                 155                 160

Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
                165                 170                 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
            180                 185                 190

Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
        195                 200                 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
    210                 215                 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
225                 230                 235                 240

Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
                245                 250                 255

Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
            260                 265                 270

Ser Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro
        275                 280                 285

Thr Val Gln Gln Gln Ala Ala Glu
    290                 295


<210> SEQ ID NO 31
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 forward primer

<400> SEQUENCE: 31

atggctcatg ctgccctcag cc                                              22


<210> SEQ ID NO 32
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 reverse primer

<400> SEQUENCE: 32

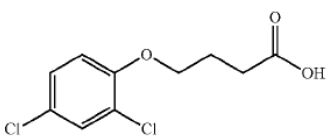
gggcaggcct aactccacca a                                               21
```

The invention claimed is:

1. A method of controlling at least one weed in an area, wherein said area comprises at least one transgenic plant, wherein said method comprises applying a phenoxy auxin herbicide to said area, said transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9, wherein expression of said polynucleotide renders said transgenic plant tolerant to said phenoxy auxin herbicide.

2. The method of claim **1** wherein said method includes applying said phenoxy auxin herbicide to said plant.

3. The method of claim **1** wherein said phenoxy auxin herbicide comprises the following chemical structure



or a salt thereof.

4. A transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9

wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9, wherein expression of said polynucleotide renders said transgenic plant tolerant to a phenoxy auxin herbicide, said transgenic plant further comprising one or more other herbicide resistance genes.

5. The method of claim **1**, said transgenic plant further comprising one or more other herbicide resistance genes.

6. The transgenic plant of claim **4** wherein said transgenic plant comprises a glyphosate tolerance trait.

7. The method of claim **1**, said transgenic plant further comprising a glyphosate tolerance trait, and said method comprising applying 2,4-D and glyphosate to said area.

8. The transgenic plant of claim **4**, said transgenic plant further comprising a glyphosate tolerance trait and a glufosinate tolerance trait.

9. The method of claim **1**, said transgenic plant further comprising a glyphosate tolerance trait and a glufosinate tolerance trait, and said method comprising applying 2,4-D, glyphosate, and glufosinate to said area.

10. The method of claim **1**, said transgenic plant further comprising a gene for tolerance to dicamba, and said method comprising applying 2,4-D and dicamba to said area.

11. The transgenic plant of claim **4**, said transgenic plant comprising a gene for tolerance to dicamba.

12. The method of claim **1**, said transgenic plant further comprising a gene for tolerance to a herbicide that is an acetolactate synthase inhibitor.

13. The transgenic plant of claim **4**, said transgenic plant comprising a gene for tolerance to a herbicide that is an acetolactate synthase inhibitor.

14. The method of claim **1**, said transgenic plant further comprising a gene for tolerance to glufosinate.

15. The transgenic plant of claim **4**, said transgenic plant comprising a gene for tolerance to glufosinate.

16. The method of claim **1**, said transgenic plant further comprising a gene for tolerance to a sulfonyl urea herbicide.

17. The transgenic plant of claim **4**, said transgenic plant comprising a gene for tolerance to a sulfonyl urea herbicide.

18. The method of claim **1** wherein said transgenic plant is a corn plant.

19. The transgenic plant of claim **4** wherein said transgenic plant is a corn plant.

20. The method of claim **1** wherein said herbicide is applied pre-plant, at plant, pre-emergence, and/or post-emergence of said plant.

21. The method of claim **5** wherein said method comprises applying one or more additional herbicides to said area.

22. The method of claim **21** wherein said one or more additional herbicides are applied together and/or sequentially.

* * * * *

EXHIBIT 14

US010174337B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 10,174,337 B2**
(45) **Date of Patent: *Jan. 8, 2019**

(54) **HERBICIDE RESISTANCE GENES**

(71) Applicant: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(72) Inventors: **Terry Wright**, Westfield, IN (US); **Justin Lira**, Fishers, IN (US); **Donald Merlo**, Carmel, IN (US); **Nicole Arnold**, Indianapolis, IN (US)

(73) Assignee: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 554 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/820,893**

(22) Filed: **Aug. 7, 2015**

(65) **Prior Publication Data**

US 2015/0344903 A1 Dec. 3, 2015

Related U.S. Application Data

(63) Continuation of application No. 12/951,813, filed on Nov. 22, 2010, now Pat. No. 9,127,289, which is a continuation of application No. 11/587,893, filed as application No. PCT/US2005/014737 on May 2, 2005, now Pat. No. 7,838,733.

(60) Provisional application No. 60/567,052, filed on Apr. 30, 2004.

(51) **Int. Cl.**
C12N 15/82 (2006.01)
C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8274*** (2013.01); ***C12N 9/0069*** (2013.01); ***C12N 15/8209*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

7,838,733	B2 *	11/2010	Wright	C12N 9/0069 800/300
9,127,289	B2 *	9/2015	Wright	C12N 9/0069 800/300
2003/0041357	A1 *	2/2003	Jepson	C07K 14/415 800/300

OTHER PUBLICATIONS

Spencer et al 1992, Plant Molecular Biology 18: 201-210.*
Kohler Oct. 1, 2002, Genbank Accession No. Q8KSC8.*

* cited by examiner

Primary Examiner — David H Kruse
(74) *Attorney, Agent, or Firm* — Barnes & Thornburg LLP

(57) **ABSTRACT**

The subject invention provides novel plants that are not only resistant to 2,4-D and other phenoxy auxin herbicides, but also to aryloxyphenoxypropionate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention alone or "stacked" together with another herbicide resistance gene, preferably a glyphosate resistance gene, so as to provide broader and more robust weed control, increased treatment flexibility, and improved herbicide resistance management options. More specifically, preferred enzymes and genes for use according to the subject invention are referred to herein as AAD (aryloxyalkanoate dioxygenase) genes and proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. This highly novel discovery is the basis of significant herbicide tolerant crop trait opportunities as well as development of selectable marker technology. The subject invention also includes related methods of controlling weeds. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the formation of, and controlling, weeds that are resistant (or naturally more tolerant) to one or more herbicides such as glyphosate.

13 Claims, 24 Drawing Sheets

Specification includes a Sequence Listing.

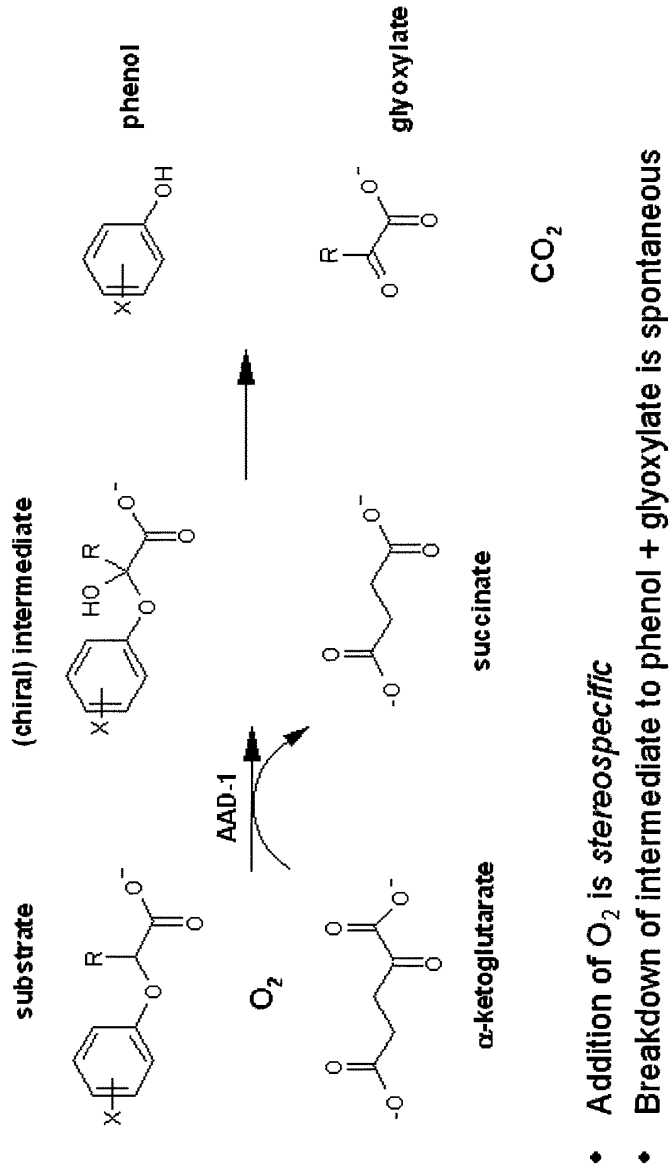

substrate
(chiral) intermediate
phenol
glyoxylate
AAD-1
O_2
α-ketoglutarate
succinate
CO_2
• Addition of O_2 is *stereospecific*
• Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

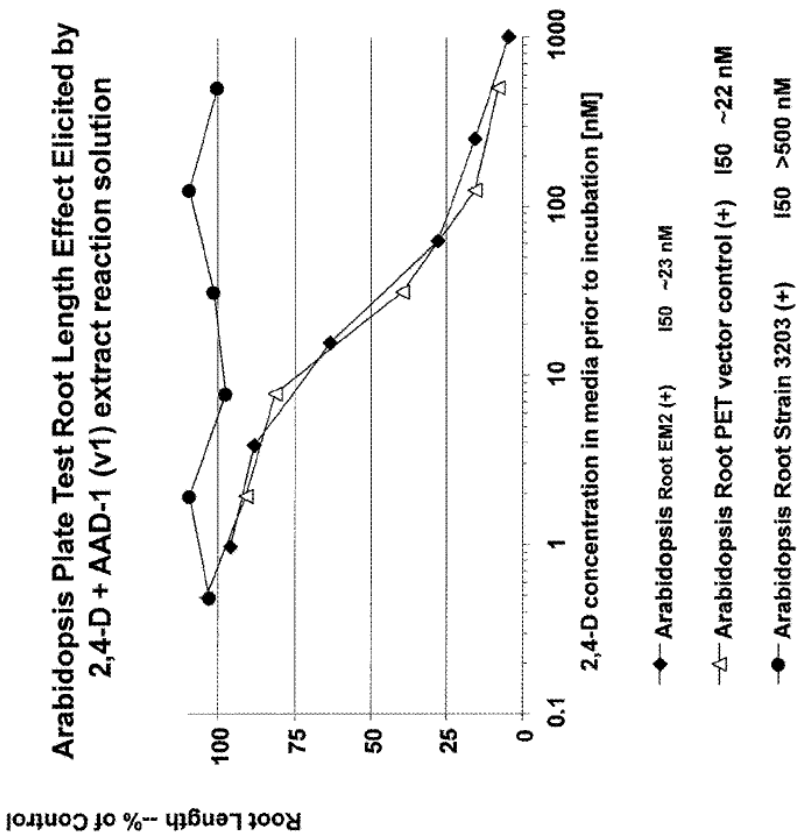

Arabidopsis Plate Test Root Length Effect Elicited by 2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

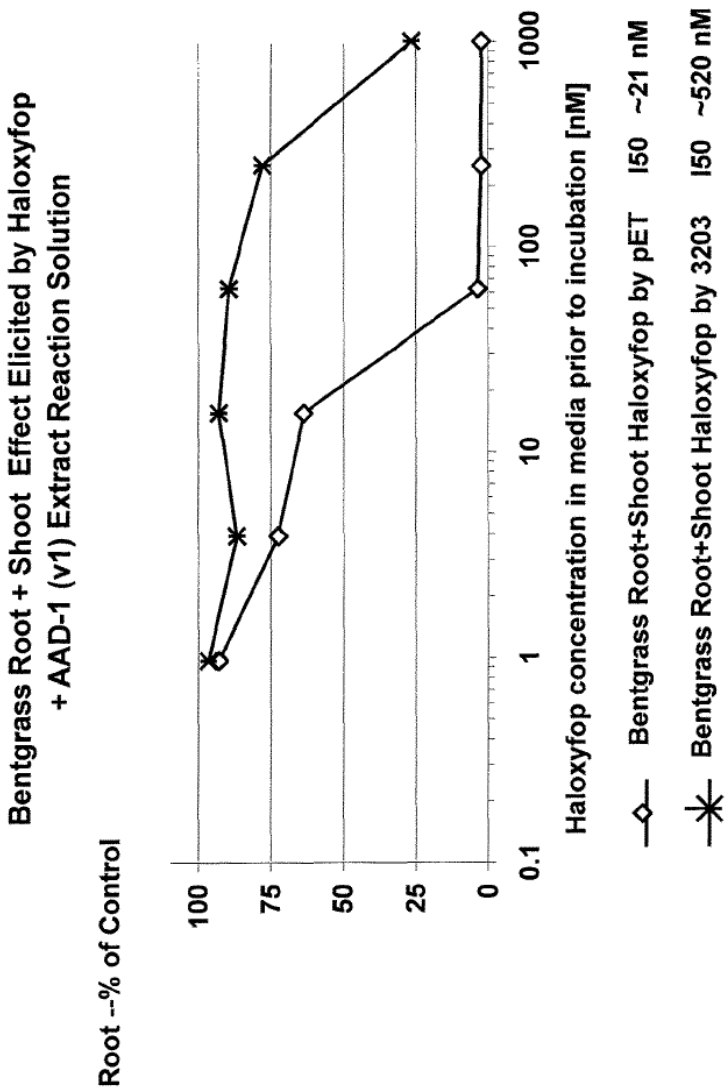

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

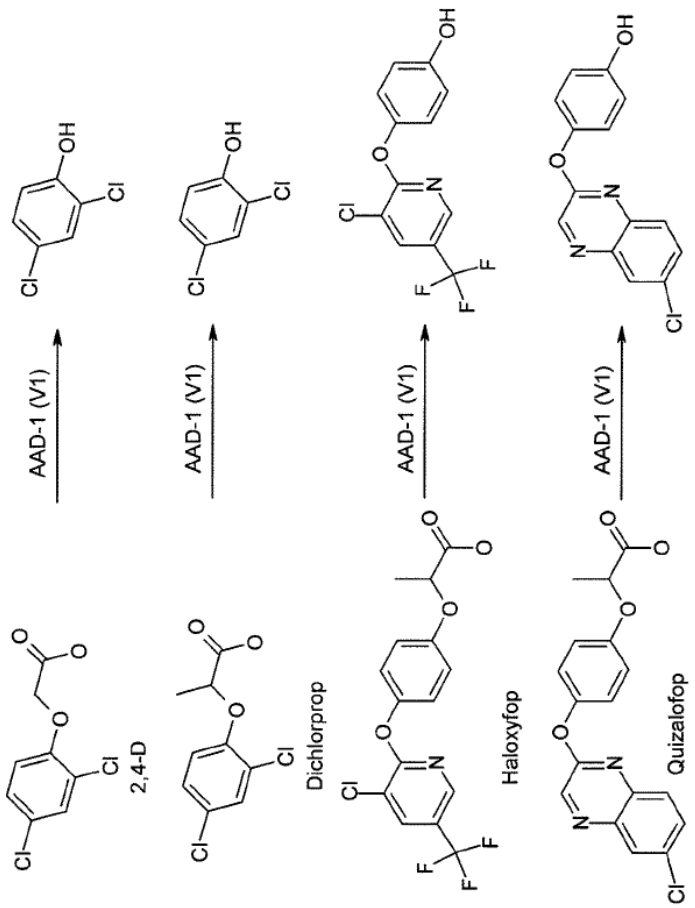

AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
2,4-D
Dichlorprop
Haloxyfop
Quizalofop

Fig. 4

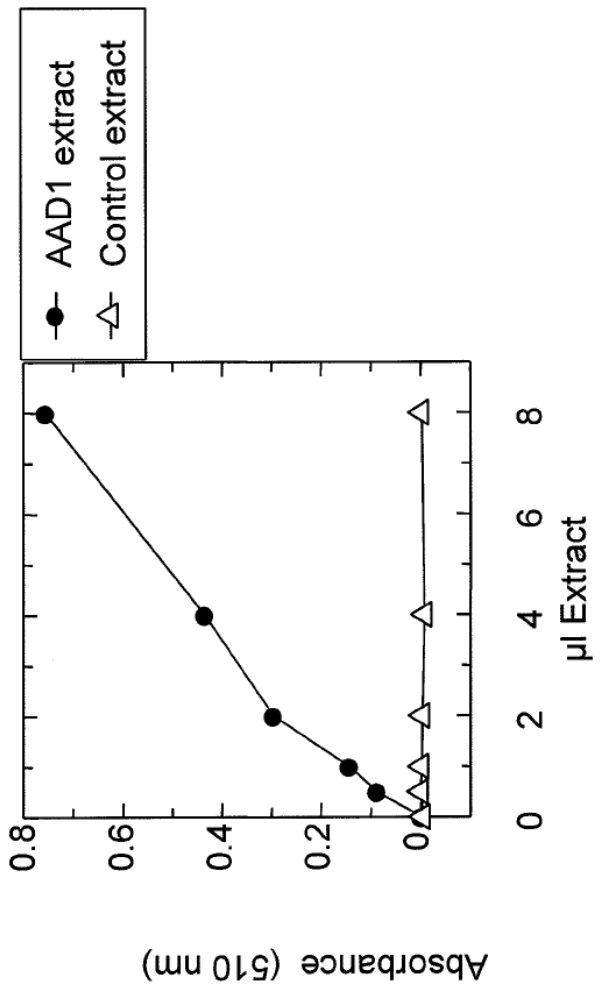

AAD1 extract
Control extract
Absorbance (510 nm)
0.8
0.6
0.4
0.2
0
0
2
4
6
8
µl Extract

Fig. 5

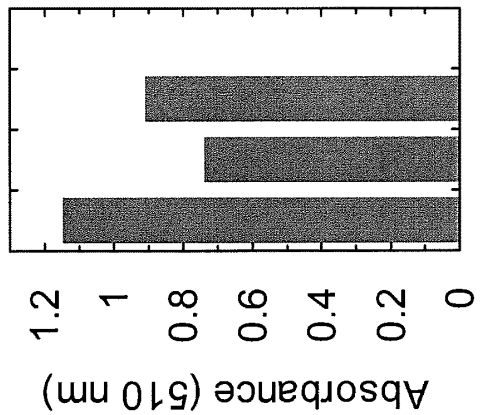

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

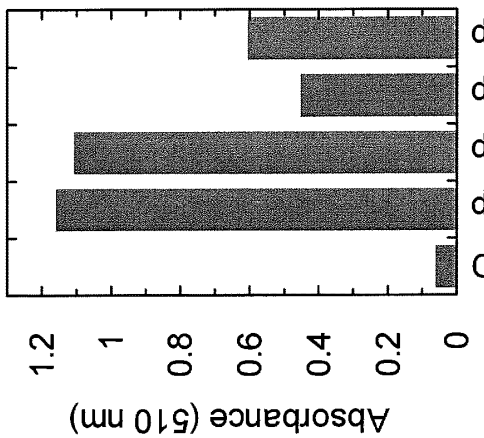

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

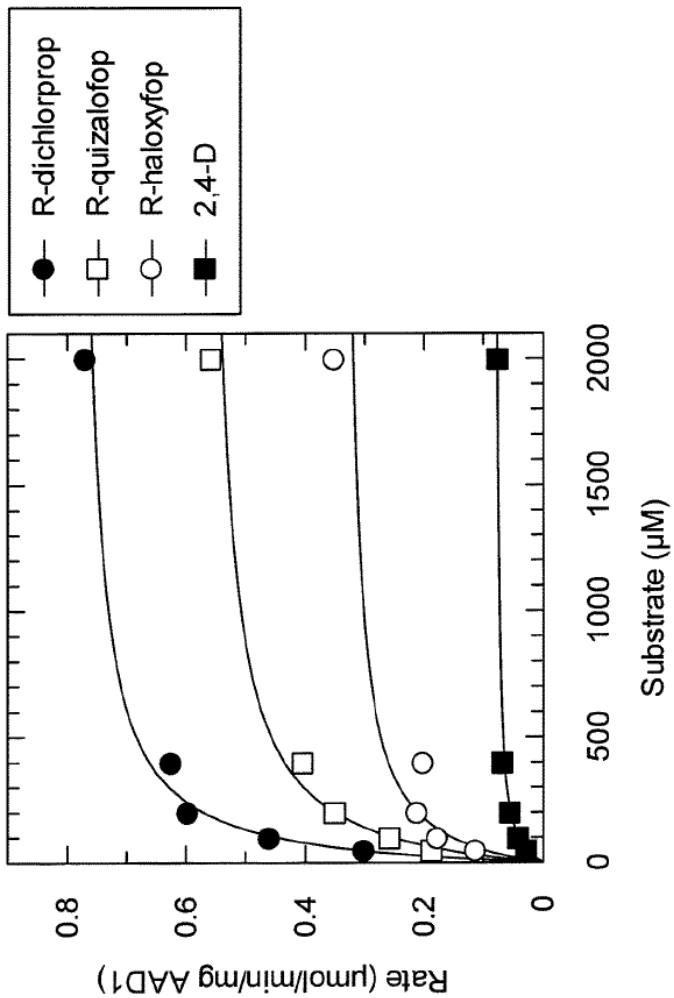

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

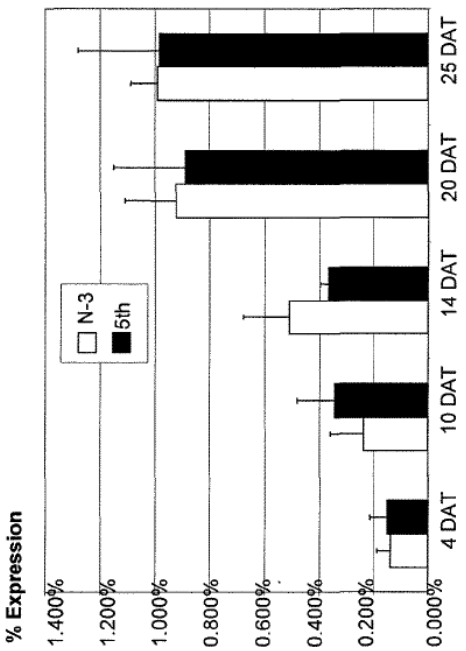

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

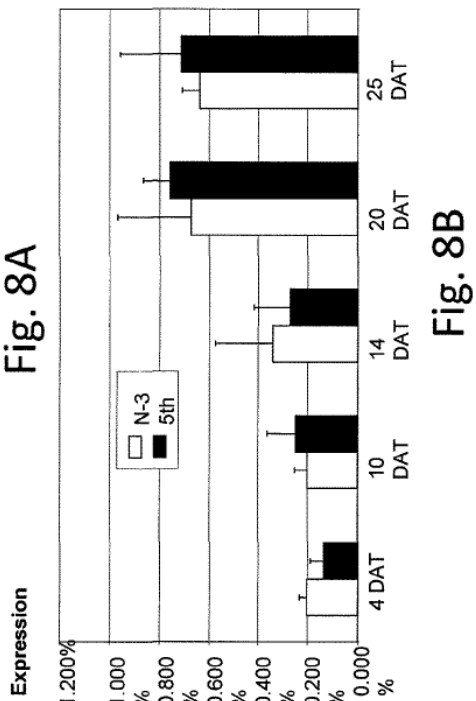

% Expression
1.200%
1.000
%
0.800
%
0.600
%
0.400
%
0.200
%
0.000
%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

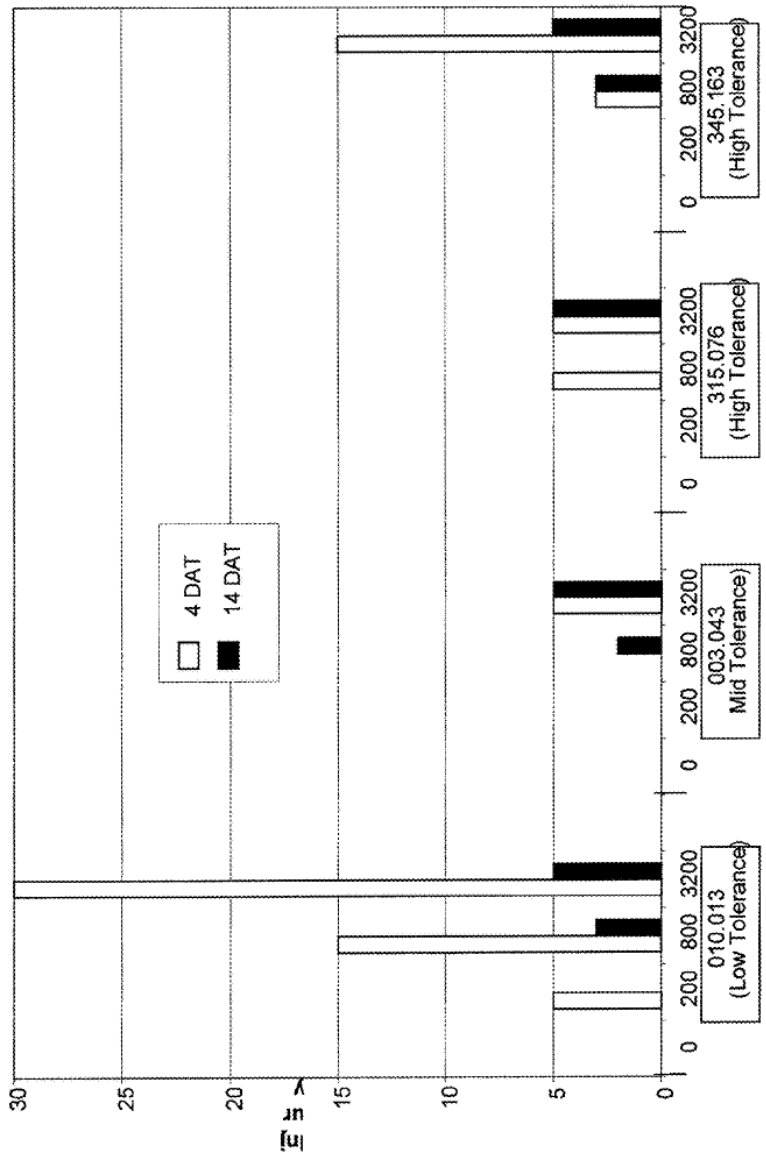

4 DAT
14 DAT
30
25
20
15
10
5
0
0
200
800
3200
010.013
(Low Tolerance)
003.043
Mid Tolerance)
315.076
(High Tolerance)
345.163
(High Tolerance)

Fig. 9A

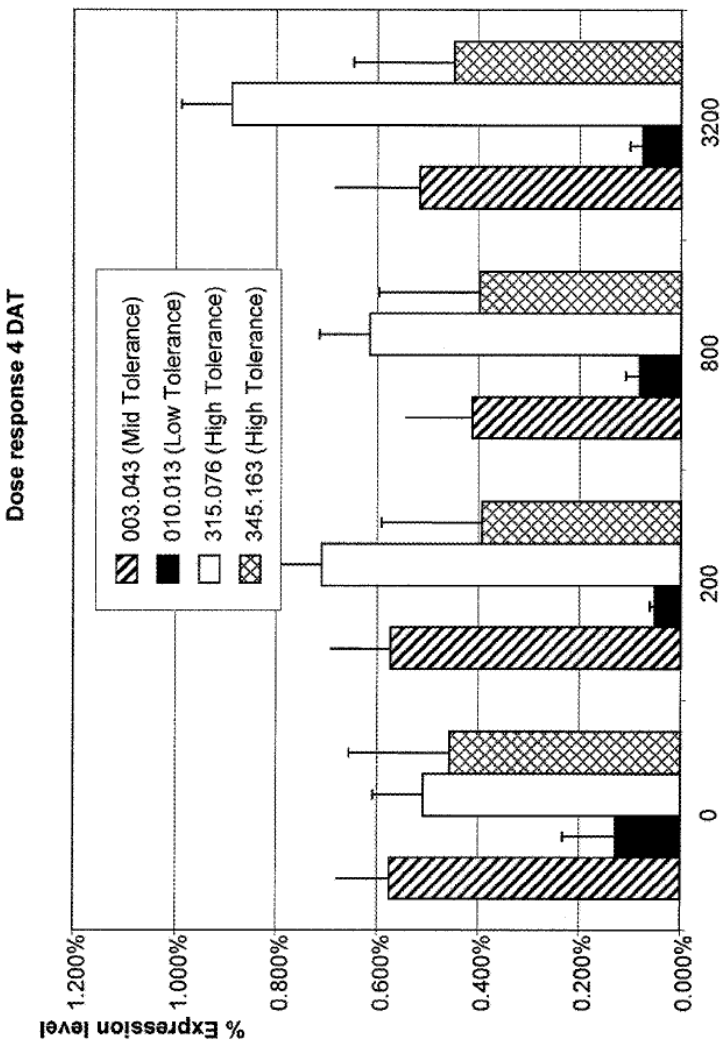

Dose response 4 DAT
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

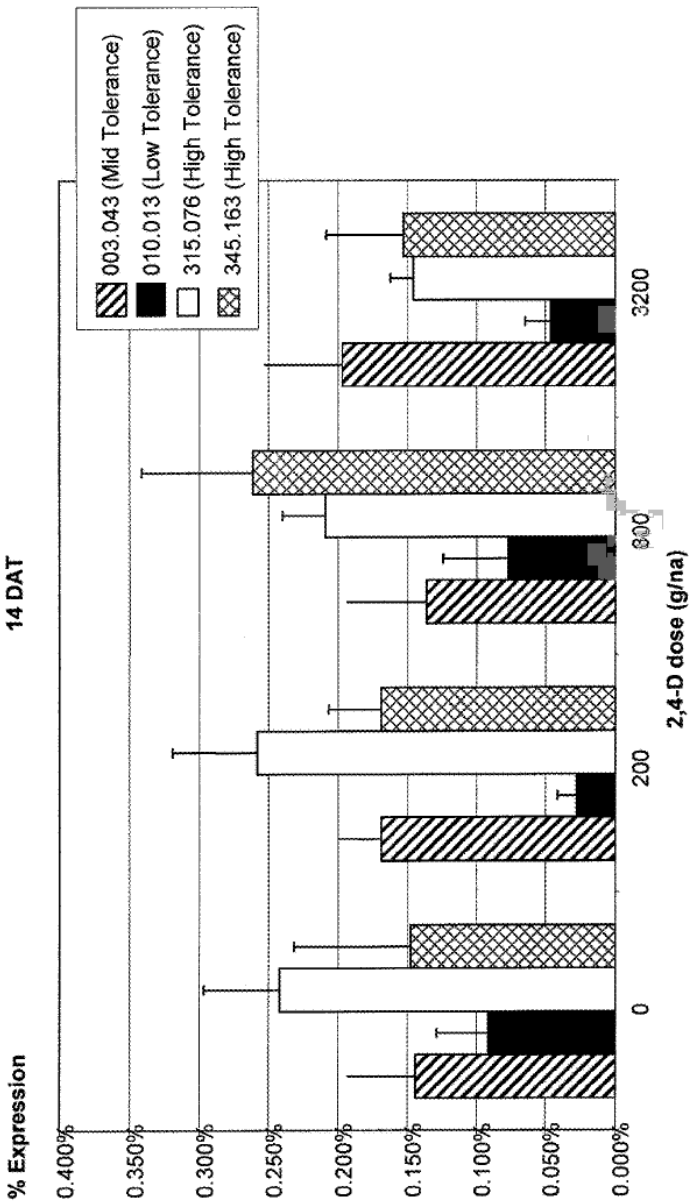

003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
14 DAT
% Expression
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

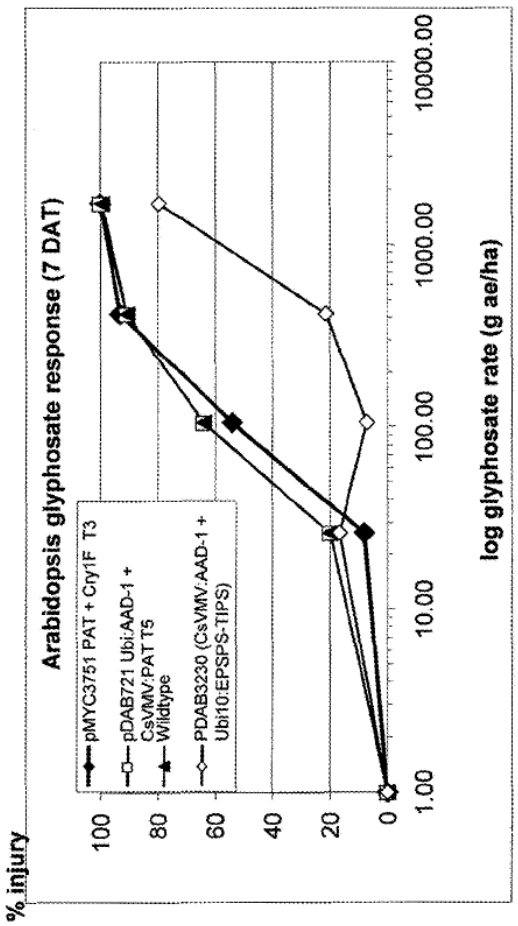

Arabidopsis glyphosate response (7 DAT)
% injury
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)

Fig. 10

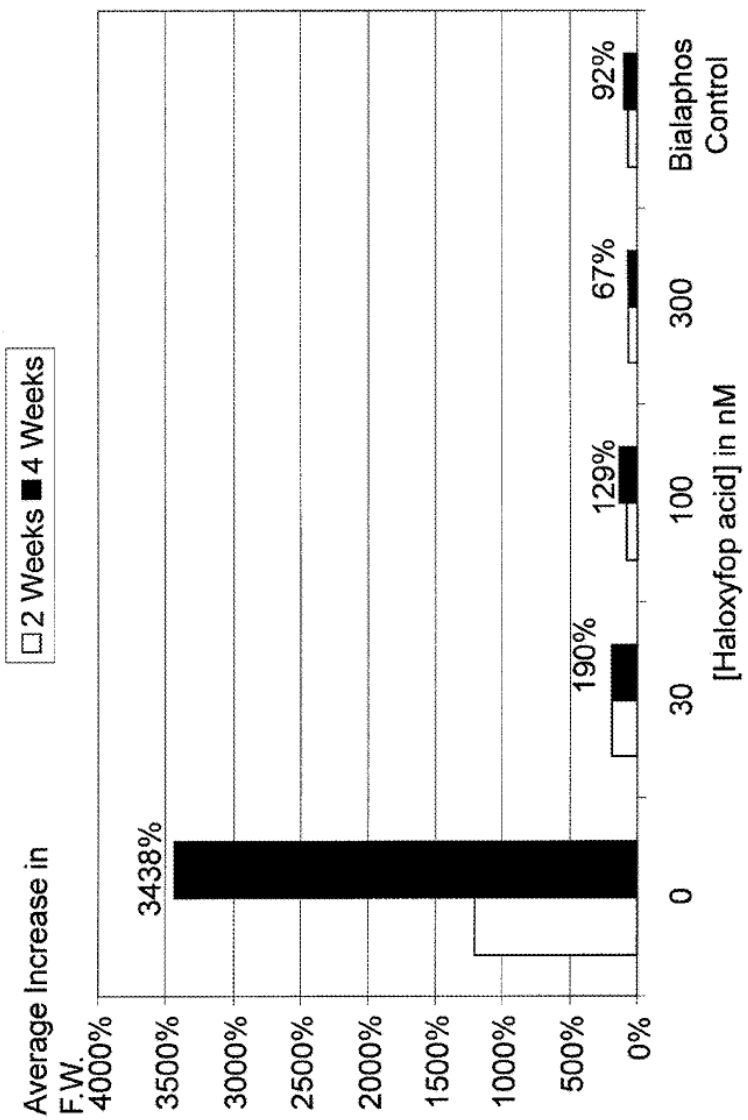

Average Increase in F.W.
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
2 Weeks
4 Weeks
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

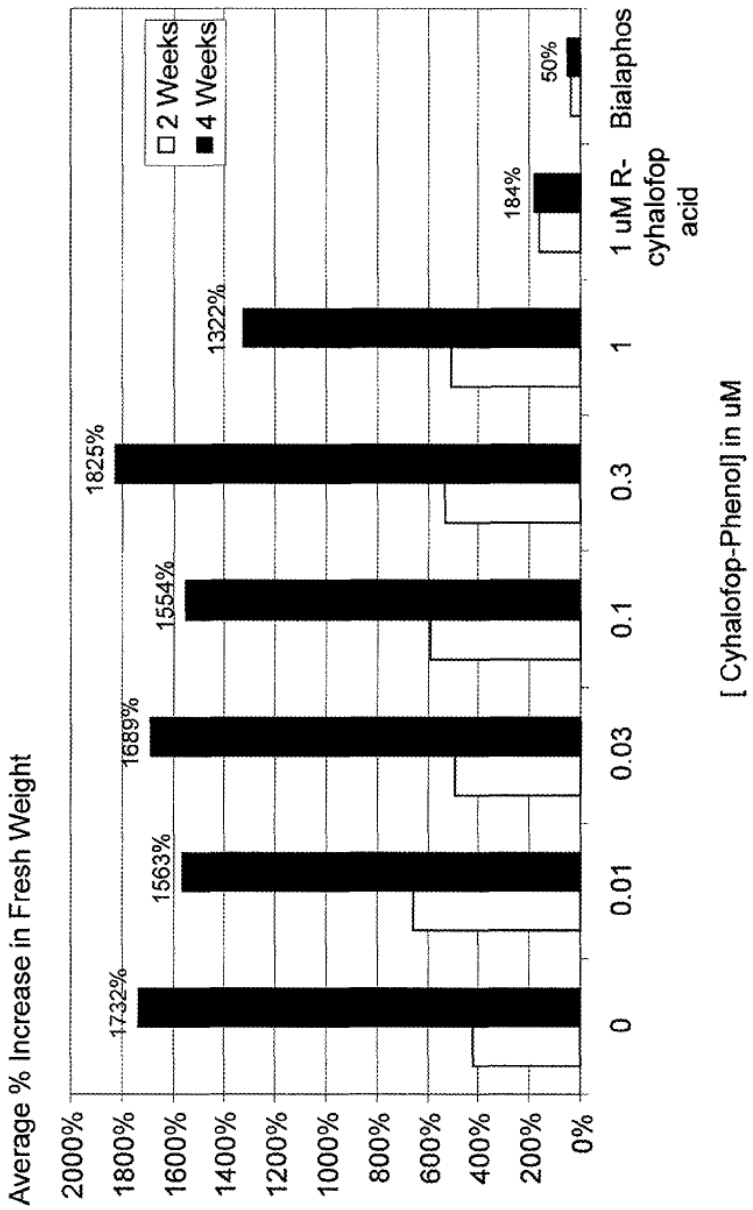

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
2 Weeks
4 Weeks
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

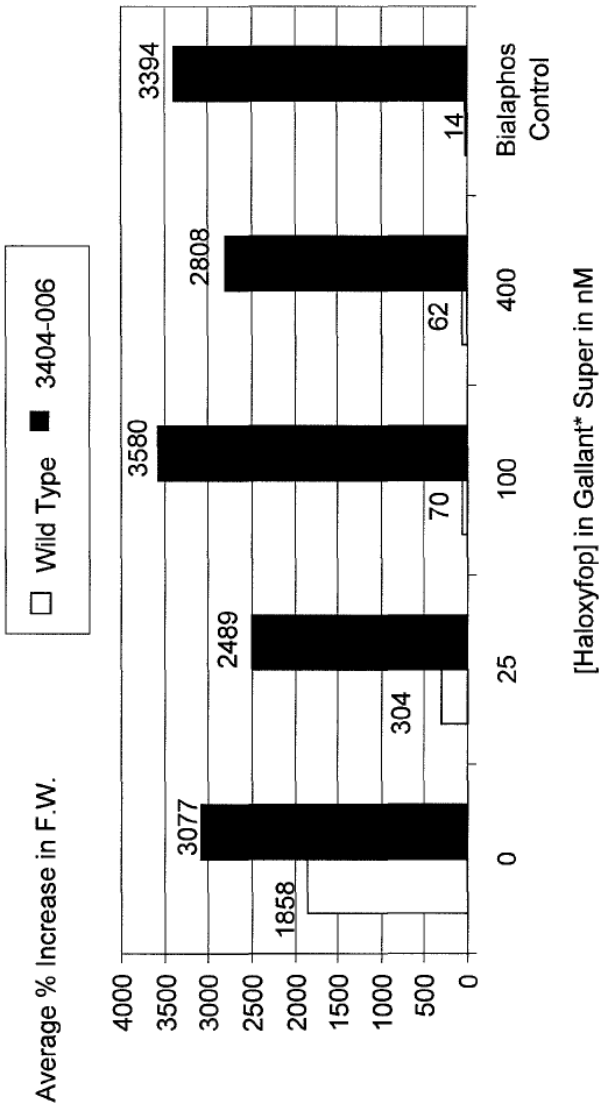

Wild Type
3404-006
Average % Increase in F.W.
4000
3500
3000
2500
2000
1500
1000
500
0
3077
1858
2489
304
3580
70
2808
62
3394
14
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

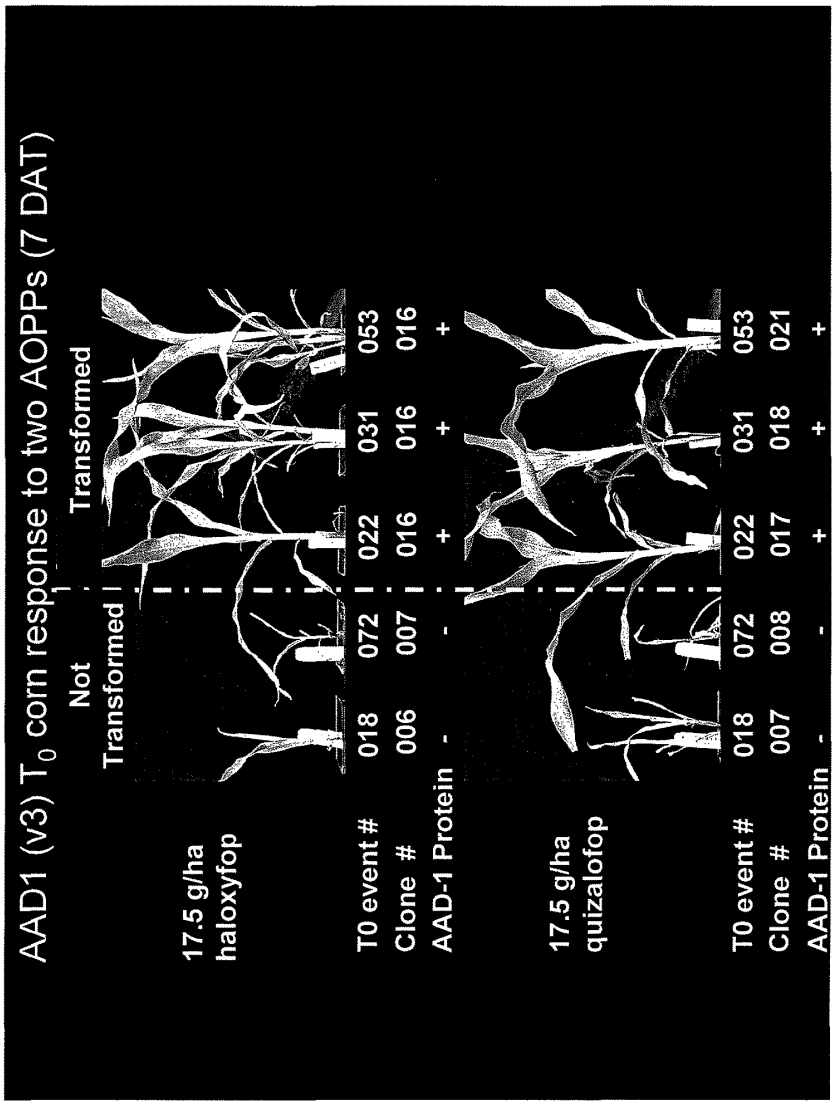

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

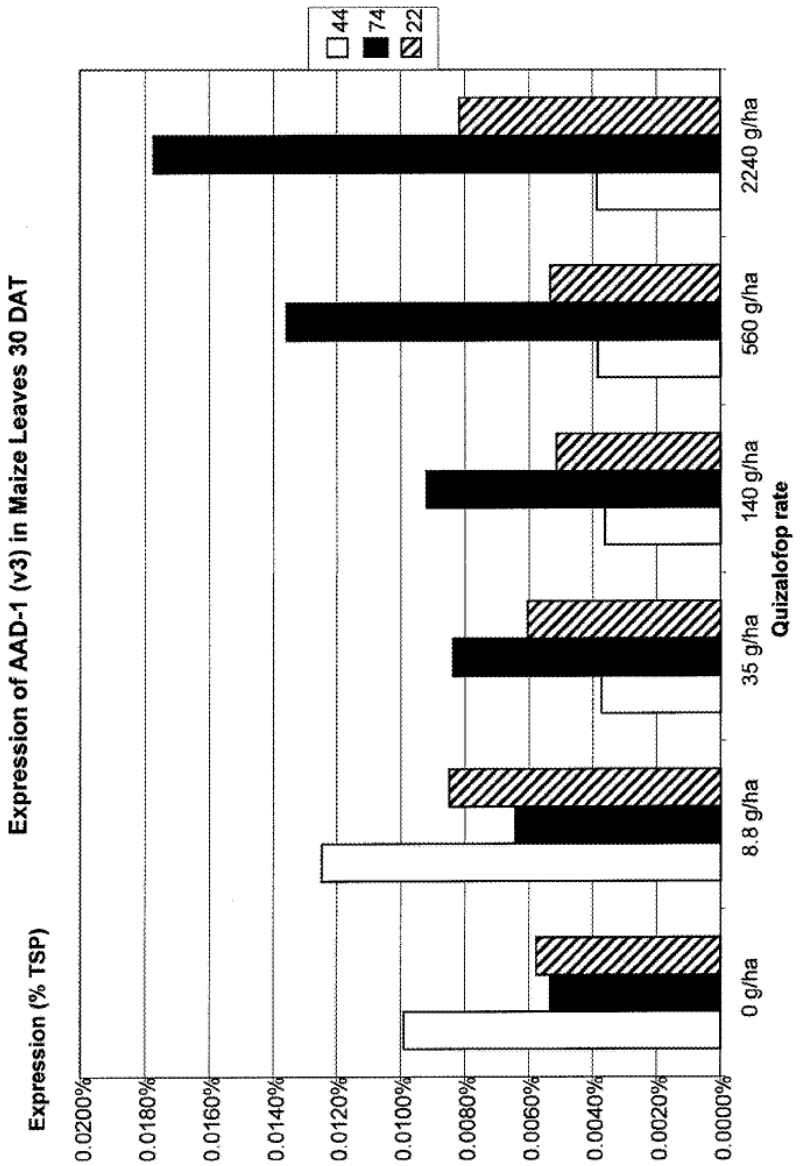

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

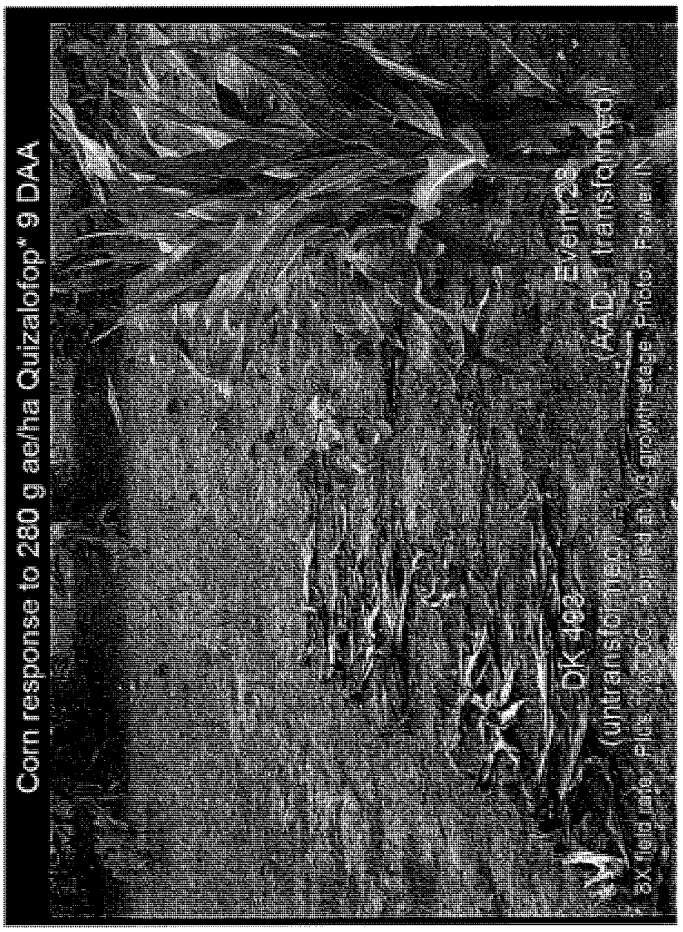

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 28
(AAD-1 transformed)

Fig. 16

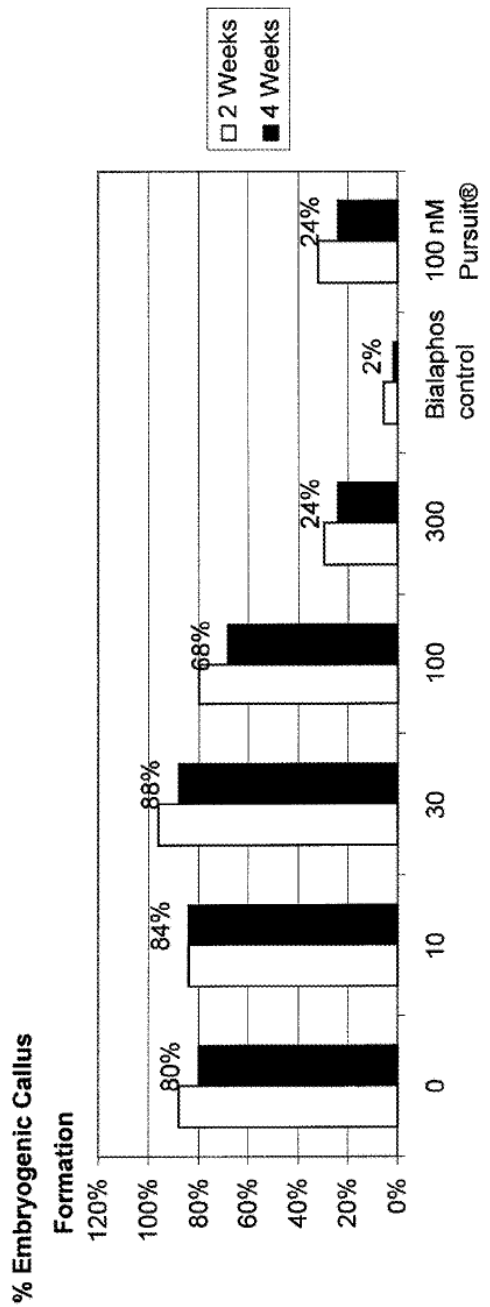
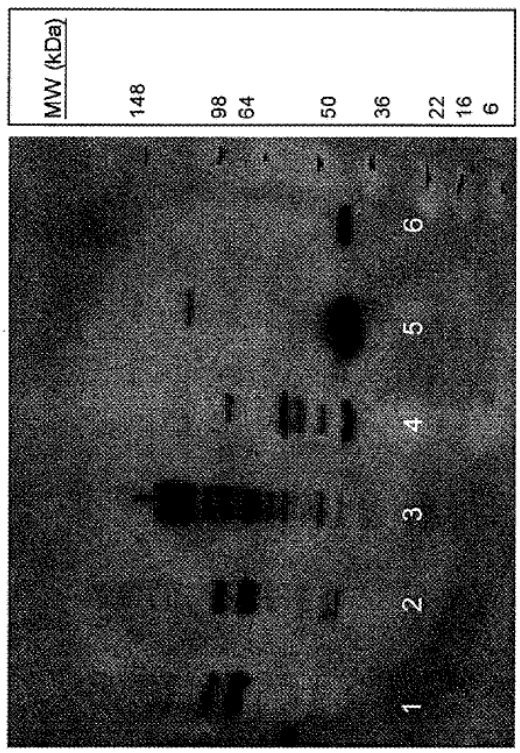

% Embryogenic Callus Formation
120%
100%
80%
60%
40%
20%
0%
2 Weeks
4 Weeks
80%
84%
88%
68%
24%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
[R-cyhalofop ACID] in nM

Fig. 17



Western

Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

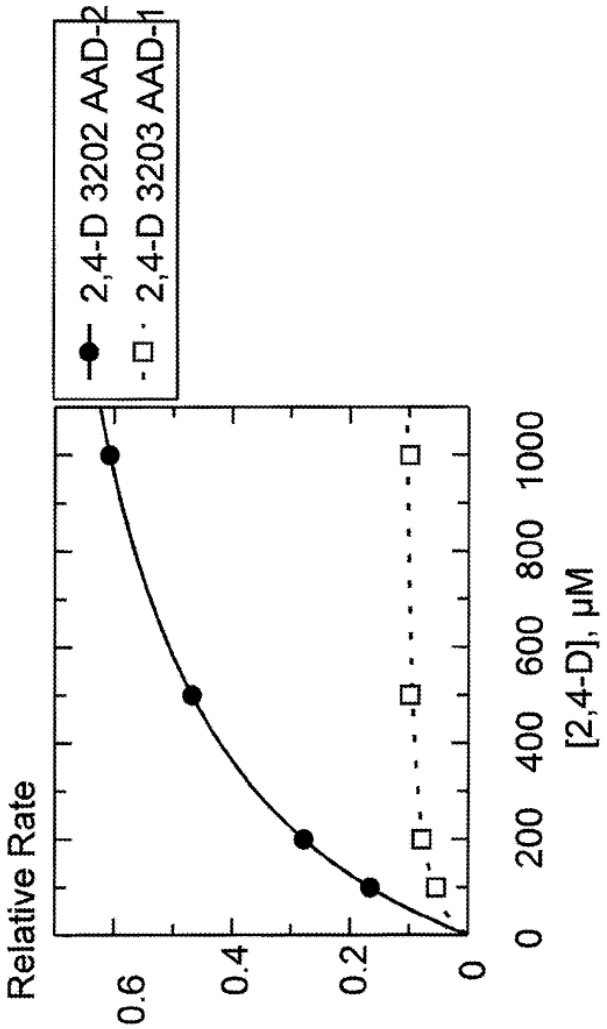

2,4-D 3202 AAD-2
2,4-D 3203 AAD-1
Relative Rate
0
0.2
0.4
0.6
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

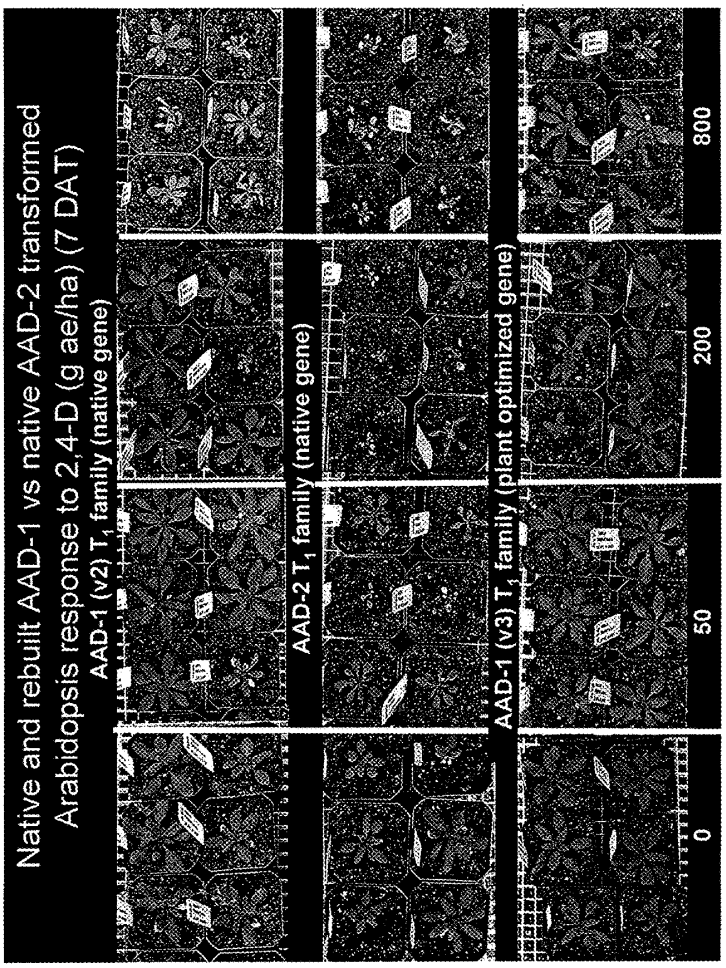

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

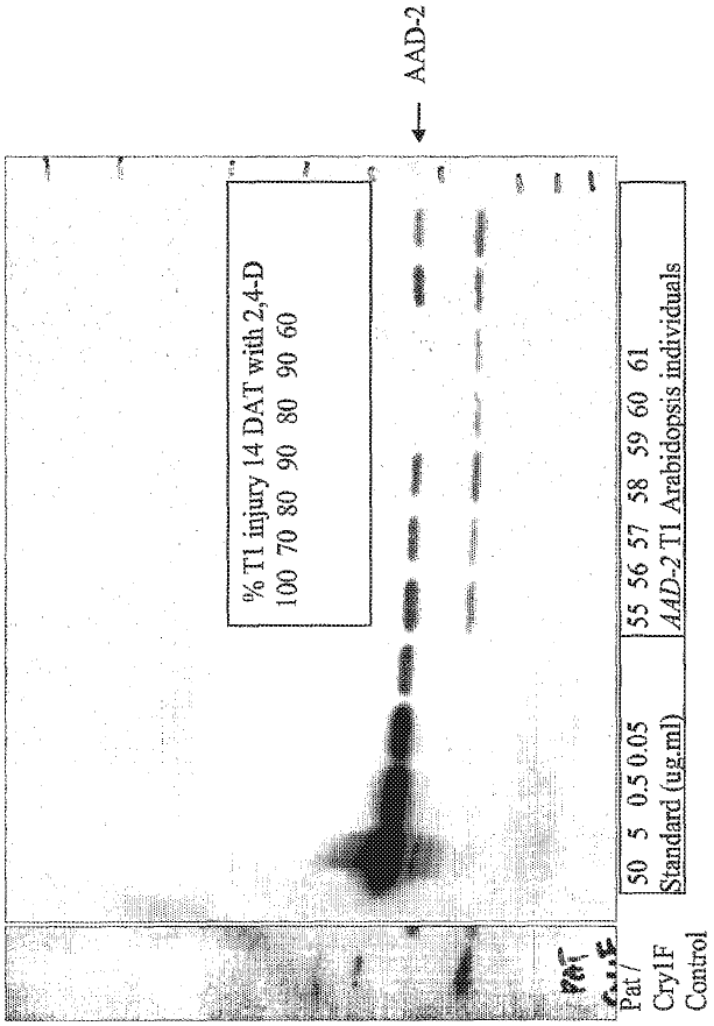

AAD-2
% T1 injury 14 DAT with 2,4-D
100 70 80 90 80 90 60
50 5 0.5 0.05
Standard (ug.ml)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
Pat / Cry1F Control

Fig. 21

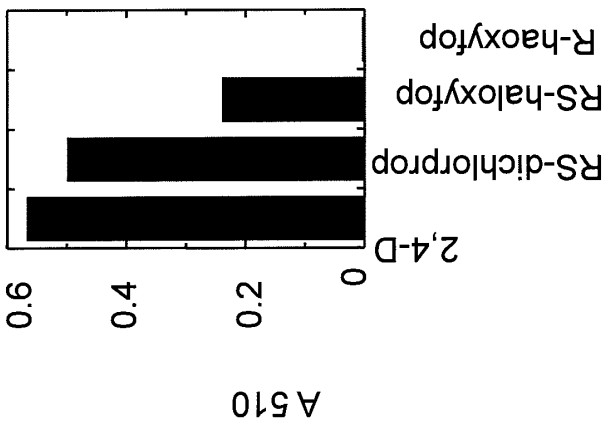


Fig. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation application of U.S. Ser. No. 12/951,813, filed Nov. 22, 2010, which is a divisional of U.S. Ser. No. 11/587,893, filed May 22, 2008, now patented as U.S. Pat. No. 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Ser. No. 60/567,052, filed Apr. 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

BACKGROUND OF THE INVENTION

Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (i.e., weed shifts). (Ng et al., 2003; Simarmata et al., 2003; Lorraine-Colwill et al., 2003; Sfiligoj, 2004; Miller et al., 2003; Heap, 2005; Murphy et al., 2002; Martin et al., 2002.) Although glyphosate has been widely used globally

for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis*, and *Plantago lanceolata*. Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum*, and *Commelina* species.

In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

2,4-D has different levels of selectivity on certain plants (e.g., dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the mineralization pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, there are a number of homologues that have a significantly lower identity to tfdA (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

One unique example with low homology to tfdA (28% amino acid identity) is rdpA from *Sphingobium herbicidovorans* (Kohler et al., 1999, Westendorf et al., 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath et al., 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf et al., 2003). Heterologous expression of rdpA in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of tfdA that primarily degrade achiral phenoxyacetic acids (e.g., 2,4-D).

Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (e.g., glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-1 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

There was no prior motivation to produce plants comprising an AAD-1 gene (preferably an AAD-1 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, et al.). Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an AAD-1 gene. The subject invention

also provides methods of using an AAD-1 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-1, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

FIG. **2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

FIG. **3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

FIG. **4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

FIG. **5** shows 2,4-dichlorophenol production by recombinant AAD-1.

FIGS. **6**A and **6**B show phenol production by recombinant AAD-1 from various herbicide substrates.

FIG. **7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

FIGS. **8**A and **8**B show that AAD-1 (v3) was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean±SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

FIGS. **9**A, **9**B, and **9**C show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean±SEM of five leaves from five different plants received the same treatment.

FIG. **10** illustrates that pDAB3230-transformed *Arabidopsis* (AAD-1+EPSPS) shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

FIG. **11** shows dose response of callused maize suspensions to R-haloxyfop.

FIG. **12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

FIG. **13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

FIG. **14** shows the responses of several AAD-1 (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

FIG. **15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

FIG. **16** shows AAD-1 (v3)-transformed corn tolerant to 8× field rates of quizalofop (Assure II) under field conditions.

FIG. **17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

FIG. **18** shows Western Blotting analysis on soybean calli transformed with AAD-1 (V3) gene indicating that the callus cells are expressing AAD-1 (v3) protein.

FIG. **19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

FIG. **20** shows the response of AAD-1 v3 (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or AAD-2 (v2) (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family.

FIG. **21** shows western blot analysis of individual native AAD-2 (v1)-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native AAD-1 (v2) or plant optimized AAD-1 (v3)-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in AAD-2-transformed and Pat/Cry1F-transformed samples.

FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop >(R,S)-haloxyfop>>(R)-haloxyfop.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the sequence of a forward primer used to amplify the rdpA/AAD-1 (v1) gene.

SEQ ID NO:2 is the sequence of a reverse primer used to amplify the rdpA/AAD-1 (v1) gene.

SEQ ID NO:3 is the nucleotide sequence of AAD-1 (v1) from Sphingobium *herbicidovorans*.

SEQ ID NO:4 is the nucleic acid sequence of the native AAD-1 gene with internal NotI restriction site removed. This gene is designated AAD-1 (v2). DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

SEQ ID NO:5 is a "plant-optimized" DNA sequence AAD-1 (v3). This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

SEQ ID NO:6 ("rdpA(ncoI)") and SEQ ID NO:7 ("3'saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

SEQ ID NO:8 is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

SEQ ID NO:9 is the native amino acid sequence encoded by the AAD-1 (v1) gene from *Sphingobium herbicidovorans*.

SEQ ID NO:10 is the amino acid sequence encoded by the AAD-1 (v2) DNA sequence of SEQ ID NO:4.

SEQ ID NO:11 is the amino acid sequence encoded by the AAD-1 (v3) plant-optimized DNA sequence of SEQ ID NO:5.

SEQ ID NO:12 is the DNA sequence of the native AAD-2 (v1) gene.

SEQ ID NO:13 is the amino acid sequence of the AAD-2 (v1) protein.

SEQ ID NO:14 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:15 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

SEQ ID NO:18 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:19 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:20 is the native soybean EPSPS protein.

SEQ ID NO:21 is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

SEQ ID NO:22 is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

SEQ ID NO:23 is primer Pat 5-3.

SEQ ID NO:24 is primer Pat 3-3.

SEQ ID NO:25 is forward primer AAD-1 PTU.

SEQ ID NO:26 is reverse primer AAD-1 PTU.

SEQ ID NO:27 is the forward primer for the Coding Region PCR AAD-1.

SEQ ID NO:28 is the reverse primer for the Coding Region PCR AAD-1.

SEQ ID NO:29 is the AAD-2 (v2) nucleotide (plant optimized).

SEQ ID NO:30 is the translated AAD-2 (v2) protein sequence.

SEQ ID NO:31 is the Southern fragment PCR AAD-1 forward primer.

SEQ ID NO:32 is the Southern fragment PCR AAD-1 reverse primer.

DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-1 gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

Table 1.

Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

TABLE 1

Commercially available phenoxy auxins.

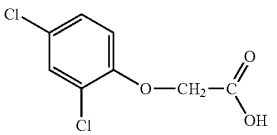
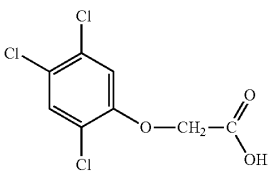
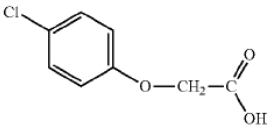
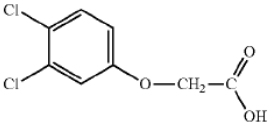
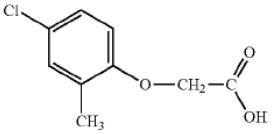
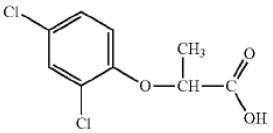
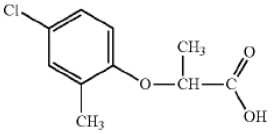
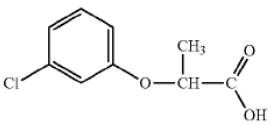
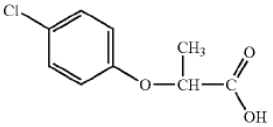
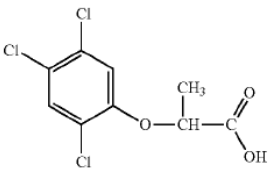
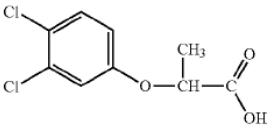
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Dichlorprop	120-36-5	25-12000	100-2240	
Mecoprop	7085-19-0	25-4000	250-3360	
Cloprop	101-10-0	25-4000	25-4000	
4-CPP	3307-39-9	25-4000	25-4000	
Fenoprop	93-72-1	25-4000	25-4000	
3,4-DP	3307-41-3	25-4000	25-4000	

An additional benefit of the AAD-1 gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). See Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing AAD-1, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, *sorghum*, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

Table 2.

AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

TABLE 2

AOPP graminicidal compounds listed by accepted common names.

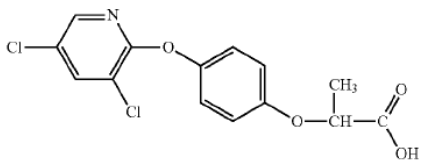
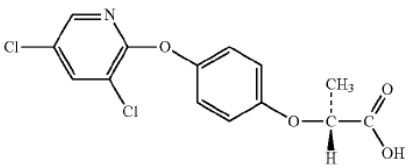
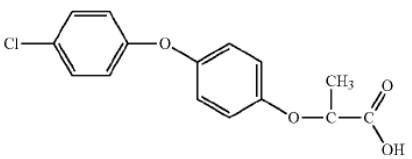
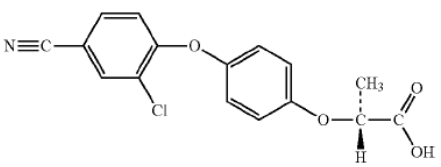
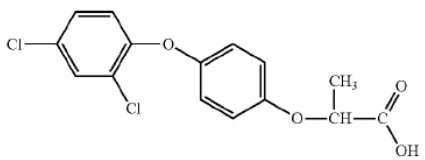
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

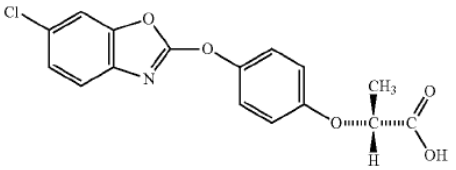
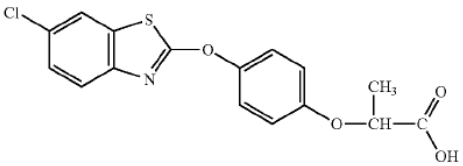
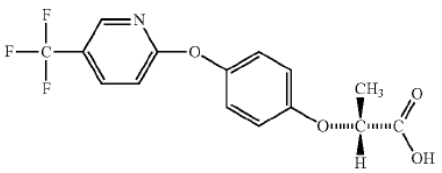
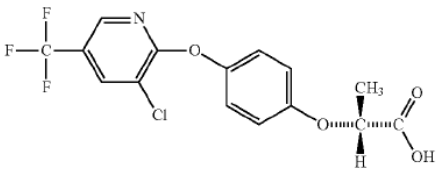
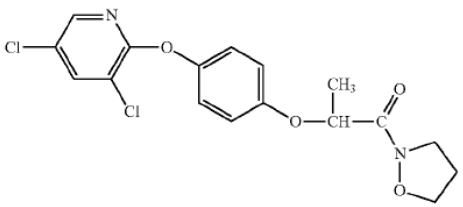
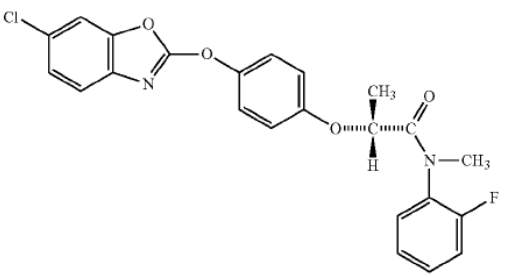
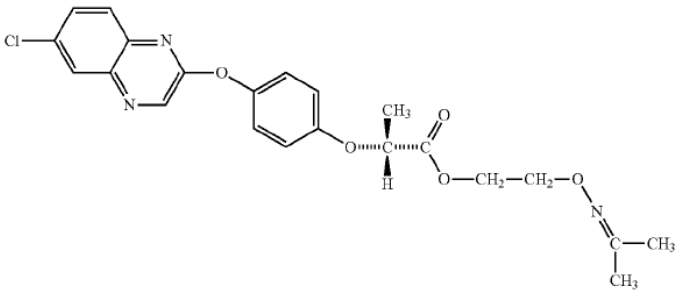
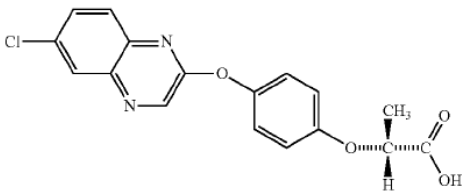
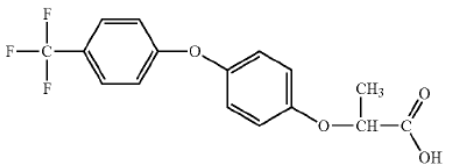
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

A single gene (AAD-1) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, AAD-1 can provide protection in planta to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing AAD-1 alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-1 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and AOPP herbicide(s) (e.g., quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-1 stack

could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, GOX, GA7), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries=AHAS, Csr1, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

Additionally, AAD-1 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to tfdA, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes.

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-1 (AryloxyAlkanoate Dioxygenase) genes and proteins.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100% conversion) in vitro. An additional advantage that AAD-1-transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an AAD-1 gene.

In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzyme in the degradation pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. TfdA catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al., 1989; Lyon et al., 1989; Lyon et al., 1993). A large number of tfdA-type genes that

encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to tfdA.

RdpA, from *Sphingobium herbicidovorans* (Westendorf et al., 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath et al., 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf et al., 2003). Heterologous expression of rdpA in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of tfdA that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that rdpA or AAD-1 genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

As described in more detail in the Examples below, rdpA was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf et al., 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. RdpA has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. AAD-1 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (e.g., glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, et al.), and insect resistance traits (Cry1F, Cry1Ab, Cry 34/45, et al.) for example. Additionally, AAD-1 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-1-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (e.g., glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

The transgenic expression of the subject AAD-1 genes is exemplified in, for example, *Arabidopsis*, corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf

crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

One gene of the subject invention, referred to herein as AAD-1 (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli*. When this gene is overexpressed (by induction of 1 mM IPTG and culture lysate combined with the following reaction mix: 112.5 μg/ml 2,4-D, 1 mM Ascorbic acid, 1 mM α-ketoglutarate, 50 μM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See Tables 3 and 4).

Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10×) purified AAD-1 (v1) per 0.16 ml assay.

TABLE 3

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

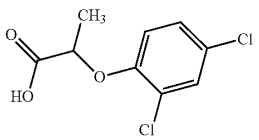
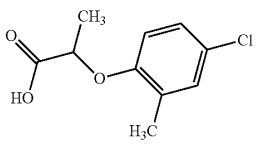
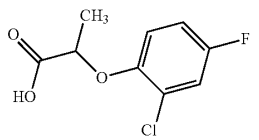
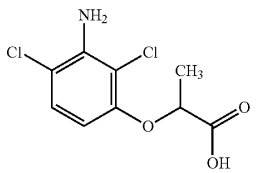
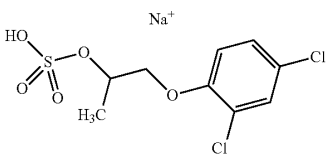
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687

TABLE 3-continued

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

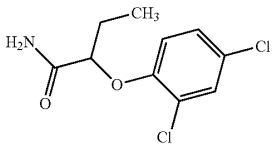
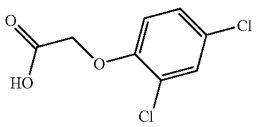
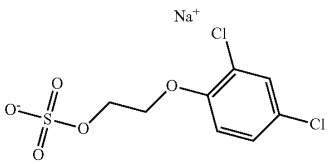
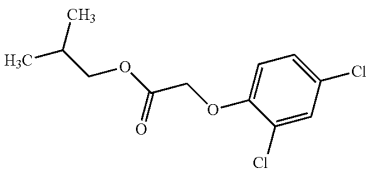
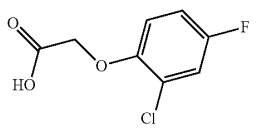
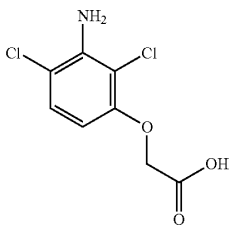
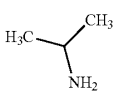
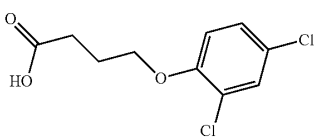
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
				

TABLE 3-continued

Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

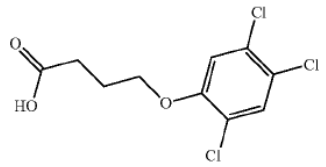
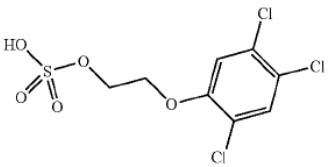
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
Na^+ 	178587		0.003	0.02
Na^+ 	188527		0.003	0.036

Table 4: Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10×) purified AAD-1 (v1) per 0.16 ml assay.

TABLE 4

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

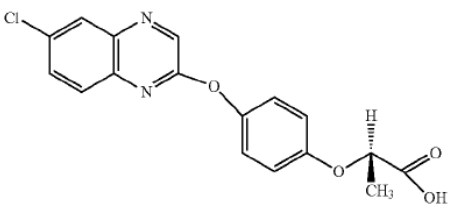
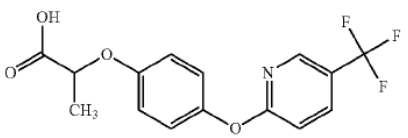
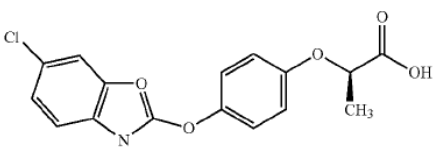
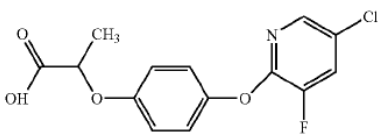
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
Chiral 	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98

TABLE 4-continued

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

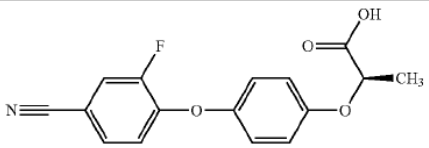
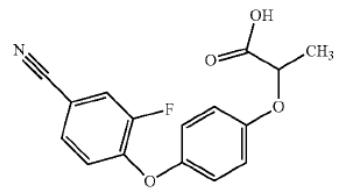
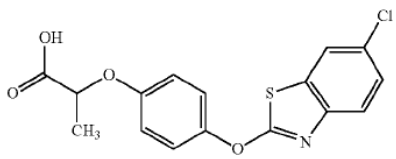
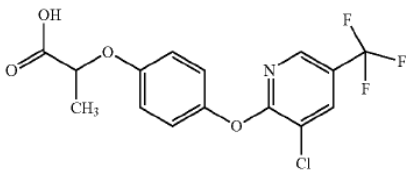
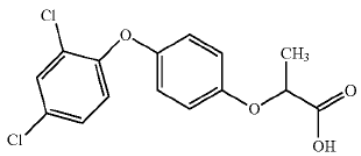
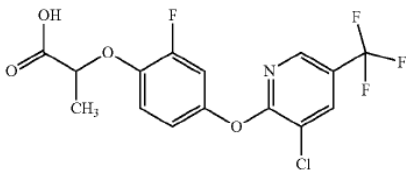
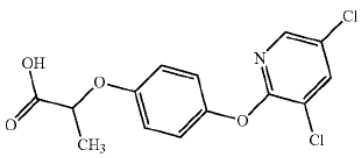
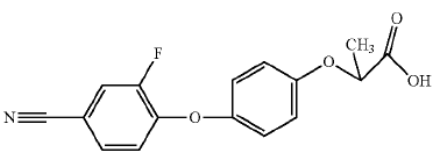
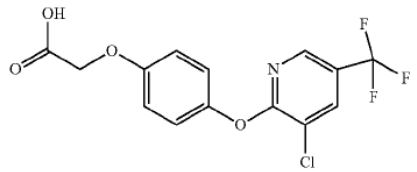
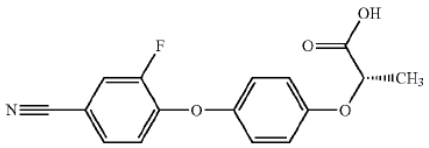
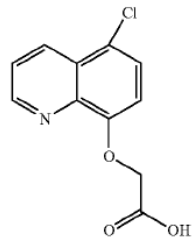
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26

TABLE 4-continued

Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

Proteins (and Source Isolates) of the Subject Invention.

The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject

invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known rdpA's, such as *Sphingobium, Delftia, Rodoferax*, and *Comamonas* for example, can be used as source isolates.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

Polynucleotides and Probes.

The subject invention further provides nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-1 gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein in vivo, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G. H., M. M. Manak (1987) *DNA Probes*, Stockton Press, New

York, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C. below the melting temperature (Tm) of the DNA hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983):

Tm=81.5° C.+16.6 Log [Na+]+0.41(% *G*+*C*)−0.61(% formamide)−600/length of duplex in base pairs.

Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (Tm) of the hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm(° C.)=2(number *T/A* base pairs)+4(number *G/C* base pairs)

(Suggs et al., 1981).

Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low: 1 or 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

PCR Technology.

Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Modification of Genes and Proteins.

The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length

sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

In addition, U.S. Pat. No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

"Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (see, e.g., Hofte et al. (1989), and Adang et al. (1985)). As used herein, the term "protein" can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity

can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12. Gapped BLAST can be used as described in Altschul et al., 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. See NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, Md., U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 5 provides a listing of examples of amino acids belonging to each class.

TABLE 5

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

Optimization of Sequence for Expression in Plants.

To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

Transgenic Hosts.

The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

Insertion of Genes to Form Transgenic Hosts.

One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

Vectors comprising an AAD-1 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors com-

prise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (PA7), glyphosate (EPSPS), imazethyapyr (AHAS), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Pat. No. 5,177,010 to University of Toledo; U.S. Pat. No. 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. See U.S. Pat. Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transfor-

mation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for

expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—Method for Identifying Genes that Impart Resistance to 2,4-D in Planta

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e., α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

PCR and Cloning of Gene into pET.

A single gene (rdpA) was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to tfdA from *Ralstonia eutropha*. Percent identity was determined by first translating both the rdpA and tfdA DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

The strain of *Sphingobium herbicidovorans* containing the rdpA gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days.

A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCTAGAAGGAGATATACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTCGAGTTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following conditions: 94° C. 45 sec, 52° C. 1.5 minute, 72° C. 1.5 minute, for 30 cycles, followed by a final cycle of 72° C. 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 4 ml Luria Broth+50 μg/ml Kanamycin (LB K), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency AAD-1 (v1) (AryloxyAlkanoate Dioxygenase).

Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) AAD-1 (v1) was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth+50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between AAD-1 (v1): pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C. for 4 hours with agitation.

Each culture was then spotted onto LB K plates, which were incubated at 37° C. overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2—Expression and Testing

2.1—HPLC Analysis.

Plasmid pDAB 3203 was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C./200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C./200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 μl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

For each reaction, the following were combined: 2,4-D (125 μg/ml), [Ascorbate (1 mM), Ferrous ion (50 μM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 μl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 μl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 μl of Acetonitrile+0.05% TFA.

The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10μ C18(2) 250×4.6 mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN+0.05% TFA: 50% H_2O+0.05% TFA to 100% ACN+0.05% TFA over 5 min.

2.2—Plate Test Bioassays for Herbicide Degradation.

Plant bioassays were used to determine if in vitro enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (i.e., monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis thaliana* var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

Arabidopsis seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2$ s^1, 23° C.) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. Tables 6 and 7 represent herbicidal substrates tested.

TABLE 6

Arabidopsis plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

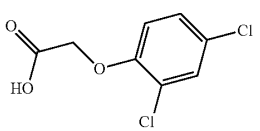
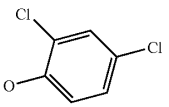
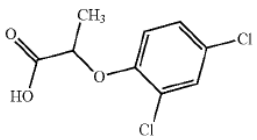
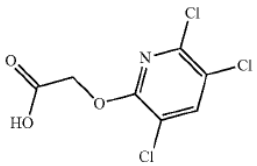
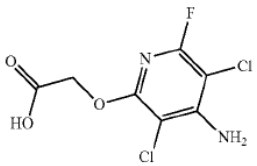
	GR_{50} (nM)				
Chemical tested	Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	

TABLE 6-continued

Arabidopsis plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

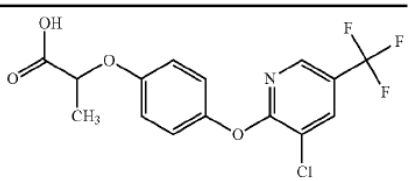
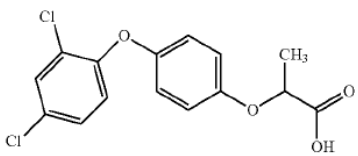
Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 vl	GR_{50} ratio **	Structure
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* Blank vector represents of cell lysate treatment where *E. coli* pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number

TABLE 7

Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 vl	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

* Blank vector represents of cell lysate treatment where *E. coli* pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number

2.3—HPLC Results.

From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see FIG. **1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based

on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (FIG. **2**). Regardless of the specific conditions of the assay (i.e., longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (i.e., oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (FIG. **3**) and diclofop. These results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

2.4—Plate Test Bioassays for Herbicide Degradation.

Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (FIG. **2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (Table 6). Often a GR_{50} could not be determined following enzyme treatment; de facto, no detectable herbicide activity remained.

The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (FIG. **3** and Table 7). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3—In Vitro Assay of AAD-1 (v1) Activity Via Colorimetric Phenol Detection

3.1—AAD-1 (v1) Assay.

AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C., the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid+3.73 g KCl+44 ml 1 N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2% 4-aminoantipyrine, then adding 10 μl 0.8% potassium ferricyanide.

3.2—Extraction.

Activity of Recombinant AAD-1 (v1) Expressed in *Escherichia coli.*

E. coli cell pellets were resuspended in 0.1 M Tris, pH 7.4+1 mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at −70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding AAD-1 (v1) was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without AAD-1 (v1) using 1 mM 2,4-D and is shown in FIG. **5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 μl of the *E. coli* extract per assay with a 15 min assay period. FIG. **6**A shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop=mecoprop>diclofop>haloxyfop>2,4-D. Thus AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in FIG. **6**B. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

Example 4—Substrate Specificity of AAD-1 (v1)

4.1—Additional Substrates of AAD-1 (v1).

The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 μg per 160 μl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. Table 3 shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 μg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (Table 8). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7-fold better than 2,4-D as a substrate (Table 3). The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

Table 8:

Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli*.

TABLE 8

Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

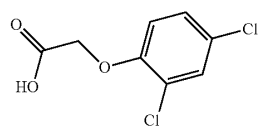
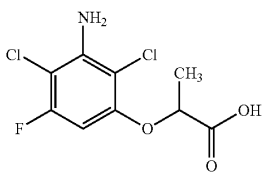
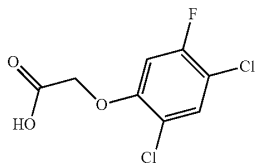
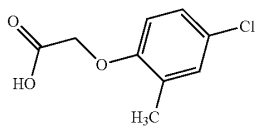
Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125

TABLE 8-continued

Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

4.2—Kinetic Characterization.

The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C.). The dose response curves were fitted using Graft (Erithacus Software, UK), and the graphs and derived constants are shown in FIG. **7** and Table 9 respectively. The K_m values for the four substrates were fairly similar (75-125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in Table 3 and Table 4 as these were performed at high (saturating) substrate concentrations (1 mM).

Table 9:

Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in FIG. **7** using Grafit fitting to the Michaelis-Menten equation.

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mN^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf et al. (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

4.3—Additional Substrates for AAD-1 (v1).

Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in Table 8. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate (Table 3). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

Example 5—Optimization of Sequence for Expression in Plants

5.1—Background.

To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s)

encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

Table 10 illustrates how high the G+C content is in maize. For the data in Table 10, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelerys, San Diego, Calif.). Intron sequences were ignored in the calculations.

TABLE 10

Compilation of G + C contents of protein coding regions of maize genes

Protein Class.sup.a	Range % G + C	Mean % G + C.sup.b
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)

.sup.a Number of genes in class given in parentheses.
.sup.b Standard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 11

Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID

NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

Codon composition comparison of native AAD-1 (v1) coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
22	GCC	10	45.5	8	35	22	CTC	5	22.7	7	32
	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43		CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
23	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	**100**	8	**100**
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53	17	CCC	9	52.9	1	6
19	GAT	4	21.1	9	47		CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0	15	AGT	0	0.0	0	0
1	TAG	1	100.0	0	0		TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69	23	ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29		ACG	5	21.7	0	0
21	GGC	15	71.4	7	33		ACT	1	4.3	8	35
	GGG	3	14.3	2	10	TRP (W)	TGG	5	**100**	5	**100**
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (I)	ATA	0	0.0	3	25	28	GTC	11	39.3	8	29
12	ATC	12	100.0	5	42		GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

moter: Nt OSM 5'UTR: AAD-1 (v3): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: AAD-1 (v3): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

5.3—Completion of Binary Vectors.

5.3.1—Rebuilt AAD-1 (v3).

The plant optimized gene AAD-1 (v3) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-1 (v3) DNA sequence. The AAD-1 (v3) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB720, containing: [AtUbi10 pro-

5.3.2—Native AAD1 (v1) and Modified AAD-1 (v2).

The AAD-1 (v1) gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of AAD-1 (v1). This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation AAD-1 (v2) (SEQ ID NO:4).

A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 AAD-1 (v2) construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

The modified AAD-1 (v2) gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI-NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: AAD-1 (v2): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

5.3.3—Design of a Soybean-Codon-Biased DNA Sequence Encoding a Soybean EPSPS Having Mutations that Confer Glyphosate Tolerance.

This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID NO:21.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 13. Columns D and H of Table 13 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean gene. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 13. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of } C1 = 1/(\%\ C1 + \%\ C2 + \%\ C3 + \text{etc.}) \times \%\ C1 \times 100$$

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 13 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 13. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Ind.).

TABLE 13

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	LYS (K)	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	MET (M)	ATG	100.0	100
	CGT	16.9	14.5	PHE (F)	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	PRO (P)	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	SER (S)	AGC	16.0	15.1

TABLE 13-continued

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	THR (T)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	TRP (W)	TGG	100.0	100
	GGT	30.4	30.4	TYR (Y)	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	VAL (V)	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston Tex.).

5.3.4—Cloning of Additional Binary Constructs.

The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to *Gateway Technology: A universal technology to clone DNA sequence for functional analysis and expression in multiple systems,* © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, Calif. 92008 (printed—2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis et al., 1982). Table 14 lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

The sacB gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay et al., 1983). The sacB gene was recovered on a Hind III fragment from plasmid pRE112 (Edwards, et al., 1998) and cloned into the unique Hind III site in pDAB3289.

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	—	—	—	Erythromycin	—	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—	Spectinomycin	—	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	—	HptII	AtUbi3	Agro binary

TABLE 14-continued

Binary constructs used in transformation of various plant species.

3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Agro binary
3403		Cn, R	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—	Ampi-cillin	—		Same as GOI	Whiskers/ Gun
3404		Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—	Ampi-cillin	—	pat	OsAct1	Whiskers
3415	1283	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—	Ampi-cillin	—	AHAS v3	OsAct1	Whiskers
3602	1421	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—	Spectino-mycin	—	AHAS v3	OsAct1	Agro Super-binary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	—	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	—	—	Erythro-mycin	—	pat	CsVMV	Agro binary

* A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region
(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region
ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

Example 6—Transformation into *Arabidopsis* and Selection

6.1—*Arabidopsis thaliana* Growth Conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, Mo.) solution. The suspended seed was stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2 sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB+erythromycin). The cultures were incubated overnight at 37° C. with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm) An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4 kV, Pulse length: 5 msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C. in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, Mo.) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28° C. for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP+erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10 μl of water, at 100° C. for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Minis Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5× concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C. for 3 minutes, 2) 94° C. for 45 seconds, 3) 55° C. for 30

seconds, 4) 72° C. for 1 minute, for 29 cycles then 1 cycle of 72° C. for 10 minutes. The reaction was maintained at 4° C. after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining A colony was selected whose PCR product was identical to the plasmid control.

6.3—*Arabidopsis* Transformation.

Arabidopsis was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700×g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

6.4—Selection of Transformed Plants.

Freshly harvested T_1 seed (transformed with native [AAD-1 (v2)] or plant optimized [AAD-1 (v3)] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2 s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3) (plant optimized gene) or AAD-1 (v2) (native microbial gene) to provide phenoxy auxin herbicide resistance.

Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, Del.) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200 g ae/ha).

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded in planta.

Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, AAD-1 (v3)-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluroxypyr.

6.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3) (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15). A small lot of AAD-1 (v2) (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

TABLE 15

Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	AAD-1 (v2)	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	AAD-1 (v3)	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	AAD-1(v3)	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	AAD-2 (v1)	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	AAD-2(v2)	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280 g ai/ha glufosinate applied 5-6 + 10 DAP
[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP
[3]PAT protein expression determined by PAT ELISA strips
[4]nd, not determined
[5]codon bias, n-native microbial gene, p = plant optimized

T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 16 compares the response of native AAD-1 (v2) and plant optimized AAD-1 (v3) genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 16 simply to demonstrate the significant difference between the plants transformed with AAD-1 (v2) or AAD-1 (v3) versus the wildtype or PAT/Cry1F transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence AAD-1 (v3) versus the native sequence AAD-1 (v2) (see Table 16). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6× field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

Table 16.

AAD-1 v3 (plant optimized), or AAD-1 v2 (native), or AAD-2 (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 16

AAD-1 v3 (plant optimized), or AAD-1 v2 (native), or AAD-2 (native)- transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev
Native AAD-1 (v2) gene					
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2
Native AAD-2 gene					
Untreated control-buffer	4	1	1	25.0	21.7
50 g ae/ha 2,4-D	1	2	11	68.2	30.2
200 g ae/ha 2,4-D	0	3	11	82.7	28.8
800 g ae/ha 2,4-D	0	0	14	99.8	0.8
Rebuilt AAD-1 (v3) gene					
Untreated control-buffer	9	0	0	0.0	0.0
50 g ae/ha 2,4-D	10	1	5	24.3	35.9
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
Wildtype					
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
PAT/Cry1F (transformed control)					
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.7	4.2
200 g ae/ha 2,4-D	0	0	15	97.2	1.7
800 g ae/ha 2,4-D	0	0	15	100.0	0.0

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some AAD1 (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first in planta data that AAD-1 (v3) will provide AOPP resistance. No resistance was observed with fluroxypyr (a pyridyloxyacetate auxin)

in transformed *Arabidopsis*, consistent with in vitro work using heterologously expressed enzyme.

Table 17.

T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 17

T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100
PAT/Cry1F						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype						
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

Table 18.

T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

TABLE 18

T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100
Wildtype						
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

6.6—AAD-1 (v3) as a Selectable Marker.

The ability to use AAD-1 (v3) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed transformed with PAT and AAD1 (v3) (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50 g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active PAT gene.

Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, PAT. The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (Table 15). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with AAD-1 (v3) or PAT genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

6.7—Heritability.

A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray

application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis (P>0.05).

Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show AAD1 (v3) is stably integrated and inherited in a Mendelian fashion to at least three generations.

6.8—Additional Herbicide Resistance Attributable to AAD-1 in *Arabidopsis*.

The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified in vitro plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id=PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C.) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1% (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, N.J.). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25° C. under continual fluorescent lighting (75 $\mu E\ m^{-2}\ s^{-1}$). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

Assessment of Growth Reduction.

The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction.

Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

In vitro plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

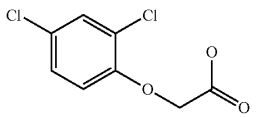
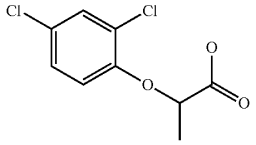
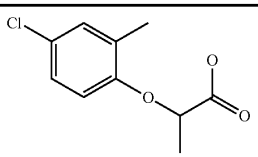
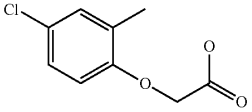
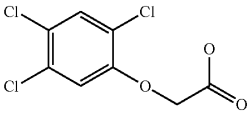
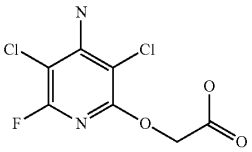
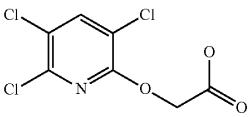
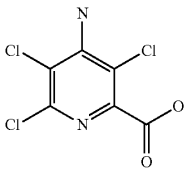
Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	<0.01	0.04	50	>4
dichlorprop		2	5	0.01	1.5	2.5	150

TABLE 19-continued

In vitro plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA
	Pyridine auxins						
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

6.9—Foliar Applications Herbicide Resistance in AAD-1 *Arabidopsis*.

The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for AAD-1 (v3) (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyacetates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

These results (see Table 20) confirm that AAD-1 (v3) in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates the in vitro enzyme and whole plate

substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis*.

TABLE 20

Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Herbicide Treatment	Ave % Injury 14DAT: AAD1.01.315.076.T_4 homozygous AAD1 plants	Ave % Injury 14DAT: PatCry1f-Control
Phenoxypropionic auxins		
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f-Control**
Phenoxyacetic auxins		
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100
Pyridyloxyacetic auxins		
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
Inactive DCP metabolite		
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

6.10—Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, AAD-1 (v3) T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae/ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. FIGS. **8**A and **8**B showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (FIG. **9**A), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

6.11—Molecular Analysis of AAD-1 (v3) *Arabidopsis*.

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-1 (v3) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-1 (v3). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200× 100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

Results showed all 2,4-D resistant plants assayed contained PAT (and thus by inference, AAD-1 (v3)). Copy

number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

6.12—*Arabidopsis* Transformed with Molecular Stack of AAD-1 (v3) and Glyphosate Resistance Gene.

T1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (AAD-1 (v3)+ EPSPS) coding for a putative glyphosate resistance trait. T_1 transformants were selected using AAD-1 (v3) as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-1 (v3)+ PAT T_5 homozygous lines (pDAB721-transformed), and PAT+Cry1F homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by EPSPS molecularly stacked with AAD-1 (v3) (see FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

Example 7—WHISKERS-Mediated Transformation into Maize, and Use of AAD-1 (v3) as a Selectable Marker

7.1—Cloning of AAD-1 (v3).

The AAD-1 (v3) fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbi1/po-aad1/ZmPer5::OsAct1/PAT/ZmLip.

7.2—Callus/Suspension Initiation.

To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu et al., 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter et al., 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

7.3—Cryopreservation and Thawing of Suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C. for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C. in a controlled rate freezer, then allowed to freeze at a rate of −0.5° C./minute until reaching a final temperature of −40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100×25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh

H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

7.4—Dose Response of Non-Transformed Tissue to Haloxyfop Acid.

Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60×20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in FIG. **11**.

Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see FIG. **12**) shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 μg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60×20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C. in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

After 1 week, filter papers were transferred to 60×20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L bialaphos, 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C.) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100×15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C. in dark boxes.

Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60×20 mm plates. If sustained growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}\ s^{-1}$) for one week then higher light (40 $\mu Em^{-2}\ s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150×25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 μg of pDAB3403 and 85 μg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

7.7—Results.

Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with AAD-1 (v3), unequivocally demonstrate stable integration of AAD-1 (v3) following Whiskers-mediated transformation coupled with "fop" selection.

7.8—Quantitative Demonstration of In Vitro Tolerance from AAD-1 (v3) Expressing Callus Events from Bialaphos Selection

Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and AAD-1 (v3) PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in Table 21 below. At least 15 T_0 plants were regenerated from each of these events and sent for spray testing and seed production.

TABLE 21

Event	PAT Copy #	PCR for AAD-1 (v3) PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in FIG. **13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

The ZmUbi1/AAD-1 (v3)/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the AAD-1 (v3) and AHAS Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 μM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression—a subset of the data is shown below. For the events listed in Table 22, the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

TABLE 22

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
1283[1]-001	2	–
1283[1]-002	206	+++
1283[1]-003	1	–
1283[1]-004	90	+++
1283[1]-005	1	–
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	–
1283[1]-011	4	–
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	–
1283[1]-015	194	+++
1283[1]-016	4	–
1283[1]-017	196	+++
1283[1]-018	3	–
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	–
1283[1]-027	1006	++
Neg Control	3	–
Neg Control	3	–
Standard (0.5 μg/mL)		++
Standard (5 μg/mL)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

7.10.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

7.10.2—Invader Assay Analysis.

The DNA samples are diluted to 20 ng/μl then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and

20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma). The plates are then incubated at 63° C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

7.10.3—Polymerase Chain Reaction.

A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRROO1A). Primers for the AAD-1 (v3) PTU were (Forward—ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse—CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. Primers for Coding Region PCR AAD-1 (v3) were (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse—CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

7.10.4—Southern Blot Analysis.

Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling r×n (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

Example 8—In Vivo Tolerance and Field Tolerance Data Generated from PAT-Selected (pDAB3404) AAD-1 (v3) Events

8.1—Tolerance of T_0 Corn Plants to AOPP Herbicides.

If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1× lethal dose (~⅛× field dose) up to 8× field doses (64× lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each AAD-1 (v3)-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8× field dose (64× lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 in vitro.

FIG. **14** shows the responses of several AAD-1 (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

Table 23 shows data for the responses of selected AAD-1 (v3)-transformed T_0 corn events to three AOPP herbicides applied postemergence.

TABLE 23

Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super[#] + 1% COC (v/v)
**Clincher[#] + 1% COC (v/v)
***Assure II + 1% COC (v/v)
[#]Trademark of Dow AgroSciences, LLC

8.2—Field Tolerance of pDAB3404 T_1 Corn Plants to Quizalofop 2,4-D, and Glufosinate Herbicides.

Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II× 5XH571 is of the same parentage as the AAD-1 (v3) event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the sub-plot was AAD-1 (v3) event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

Glufosinate at 560 g ai/ha was applied to AAD-1 (v3) plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

8.2.1—Results.

AAD-1 (v3) event response to the highest rates tested of quizalofop and 2,4-D are shown in Table 24. These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All AAD-1 (v3) events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the AAD-1 (v3) events except with the events noted above.

TABLE 24

	Treatment (rate) =											
	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results			Resistance Segregation Ratios following Liberty Spray		
	Evaluation =											
Event or	% Injury 3 WAA (0-100 scale)		% Leaning 2 DAA (0-100 scale)		Braceroot Deformation 3-4 WAA (0-10 scale)		AAD-1 copy number leaf (Southern	AAD-1 Leaf Western	AAD-1 Leaf	T_1 Population Average		T_2 Population Average
Hybrid	IN	HI	IN	HI	IN	HI	analysis)	Blot T_0	ELISA T_0	IN	HI	HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/−	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51%	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/−	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493	100	100	20	23	8	9						
HI-II X 5XH571	100	100	13	34	7	9						
CROPLAN 585SR	80	96	11	33	9	9						

*Fits single locus dominant trait segregation as determined by chi square analysis ($P > 0.05$)

2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all AAD-1 (v3) events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all AAD-1 (v3) events except one lineage of 3404.001.

Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

These results indicate that most AAD-1 (v3) transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. See also FIG. **16**.

8.2.2—Expected Mendelian Segregation Ratios on Three T_2 Events.

Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see Table 24 above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all AAD-1 (v3) lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see Table 24). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to AAD-1 (v3) Invader assay description for corn). Hemizygous plants were removed and homozygous AAD-1 (v3) plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, AAD-1 (v3), and glufosinate resistance.

8.3—Stacking of AAD-1 (v3) and PAT with Glyphosate Resistance Genes in Corn.

Homozygous T_2 AAD-1 (v3)/PAT corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing AAD-1 (v3), PAT, and glyphosate resistance genes as described in the previous example.

F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where

glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in Table 25.

TABLE 25

		% Injury at 14 DAT	
	Field Rate	Hi II X 5XH751 Ave	RR/PAT/AAD1 Ave
Untreated control	—	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate + 1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X + 4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X + 2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

This study demonstrated that the AAD-1 (v3) gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

8.3.1—Resistance of AAD-1 (v3) Corn Using a Tank Mix of 2,4-D DMA and Quizalofop.

T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

8.3.2—Tolerance of (pDAB3403) T_0 Corn Plants to Quizalofop Herbicide.

A target of approximately eight T_0 plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1× field rate, 4× lethal dose) to 3-leaf, greenhouse-adapted T_0 corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more T_0 clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4× lethal dose of quizalofop at the whole plant level.

8.3.3 Expression of AAD-1 (v3) with Respect to Quizalofop Tolerance.

Three different T2 lineages from 3404 transformations that were pre-screened with

Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see FIG. **15**.). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1× field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

Example 9—*Agrobacterium*-Mediated Transformation of Maize with AAD-1 (v3)

9.1—Plant Material.

Seeds of a "High II" (i.e., Parent A and B) F_1 cross (Armstrong et al., 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

9.2—Tissue Source.

For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

9.3—Preparation of a Superbinary Vector.

Construction of an *Agrobacterium* construct, pDAB2272, containing the AAD-1 (v3) gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/AAD-1 (v3)/ZmPer5 v2 and insert-

ing it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/AAD-1 (v3)/ZmPer5 v2 and the OsAct1 v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~1×10^9 cfu/ml) and 100 μM actetosyringone added to the solution.

9.5—Infection and Cocultivation.

The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$, 1, 100 μM actetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

9.6—Dose Response Using Immature Embryos.

Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously. Once treated, embryos were allowed to co-cultivate for 5 days at 25° C. and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50-100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in FIG. **17**.

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

9.8.—Regeneration and Seed Production.

For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

9.9—Event Recovery and Analysis Particulars; Whole Plant Screening of T_0 Corn Lineages Containing AAD-1 (v3) and AHAS (pDAS1421).

Seventy-two *Agrobacterium*-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid in vitro. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of AAD-1 (v3) into the genome as described previously. Ten single copy events, as indicated in Table 26, were chosen to be regenerated.

TABLE 26

	In vitro Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
Corn event	(nM)	AAD-1	Callus	Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0

Events with more than 1 copy were not taken to the greenhouse

A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the T_0 generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (AHAS) on the function of the AAD-1 (v3).

Example 10—Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000 g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Example 11—Recombinant AAD1 Purification and Antibody Production

Plasmid pDAB3203 containing the AAD-1 (v1) gene was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C. with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C. with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000×g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

All AAD-1 (v1) purifications were conducted at 4° C. as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

11.1—Extracting AAD-1 (v3) from Plant Leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1% Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000×g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 μg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

11.3—Enzyme Linked Immuno-Sorbent Assay (ELISA).

The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C. for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates

were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

11.4—Western Blotting Analysis.

Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

Example 12—Tobacco Transformation

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C. and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, AAD-1 (v3)+PA7), grown overnight in a flask on a shaker set at 250 rpm at 28° C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Fla.) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C. in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C. greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C., 16 hour day, 8 hour night, minimum natural+supplemental light=500 $\mu E/m^2 s^1$).

Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-1 (v3) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

The DNA samples were diluted to 9 ng/μl, then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl2 (Third Wave Technologies). An aliquot of 7.5 μl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 μl of controls, standards, and 20 ng/μl diluted unknown samples. Each well was overlaid with 15 μl of mineral oil (Sigma). The plates were then incubated at 63° C. for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (Table 27).

TABLE 27

Tobacco T0 events transformed with pDAB721 (AAD-1(v3) + PAT).

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (μg AAD-1/ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:	Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

All events were also assayed for the presence of the AAD-1 (v3) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRROO1A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 64° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (Table 27). Protein expression varied across events.

T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5× field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6× field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (Table 27).

Verification of High 2,4-D Tolerance.

Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two AAD-1 (v3) tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (Table 28). No injury was observed at rates below 1× field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by AAD-1 (v3), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

TABLE 28

Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

Herbicide	KY160-Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

A 100 plant progeny test was also conducted on each of the two AAD-1 (v3) lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants

were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with AAD-1 (v3)-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

Example 13—Soybean Transformation

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette et al., 1995), amino acid modification (Falco et al., 1995), and insect resistance (Parrott et al., 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe et al., 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee et al., 1988) or zygotic embryos (Chee et al., 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchee et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-1 (v3) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-1 (v3).

13.1—Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This de novo meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee et al., 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang et al., 1999; Zeng et al., 2004).

13.1.1—*Agrobacterium* Preparation.

The plasmid, pDAB721, contains the AAD-1 (v3) gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the PAT gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

Agrobacterium cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

13.1.2—Plant Transformation.

Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C., with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg et al. (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100×25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The

dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee et al., 1988).

Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES (2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from 10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

13.1.3—Progeny Evaluation.

T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-1 (v3) and PAT genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of AAD-1 (v3) will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

13.2—Transformation Method 2: "No-Shake" *Agrobacterium* Mediated Trans-Formation of Non-Regenerable Soybean Suspension Cells.

The DAS Soybean cell suspensions were cultured on a 3 d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were then transferred to orbital shakers at 140 rpm.

Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100×25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 μL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

13.2.1—Selection of Soybean Suspension Cells and Isolation of Transformed Colonies.

After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100×15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (Table 29) and Western Blotting (FIG. **18**) analysis on two separate soybean calli transformed with AAD-1 (v3) gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

TABLE 29

PCR and ELISA data for transgenic soybean events.

Event	AAD-1 Coding region PCR	PAT Coding region PCR	T SP (μg/mL)	AAD-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	–	–	2074.63	−1.22	−0.0001%

13.3—Transformation Method 3: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Pat. No. 6,809,232 (Held et al.).

Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 μg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

Variations of media referred to in Table 30 were tested, e.g., B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	B3	B5G
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8 g	8 g	
Gelrite			2 g
pH	5.8	5.8	5.8

Example 14—AAD-1 (v3) Enablement in Cotton

14.1—Cotton Transformation Protocol.

Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 31) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 31) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 31) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 31). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

Agrobacterium Preparation.

Inoculate 35 ml of Y media (Table 31) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C., while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 31) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (Table 31), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 31) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

Larger, well-developed embryos are isolated and transferred to DK media (Table 31) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 31

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	

TABLE 31-continued

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

14.2—Experiment Specifics.

For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

14.3—Callus Analysis Results.

The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (Table 32). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (Table 32). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (Table 32). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

TABLE 32

Analysis of cotton callus

Line	Copy #	PTU	Western
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*
13	1	pos	*
14	1	pos	**
15	1	pos	****

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg

TABLE 32-continued

Analysis of cotton callus			
Line	Copy #	PTU	Western
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*
standard	AAD1	5 ug/ml	*****
standard	AAD1	0.5 ug/ml	**

14.4—Plant Regeneration.

Two AAD-1 (v3) cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the AAD-1 (v3) gene provides resistance to 2,4-D in cotton, both the AAD-1 (v3) cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the AAD-1 (v3) plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the AAD-1 (v3) plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

This study demonstrates that the AAD-1 (v3) gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

Example 15—*Agrobacterium* Transformation of Other Crops

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchee et al., 1988. For *Agrobacterium*-mediated transformation of *sorghum*, see, e.g., Zhao et al., 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay et al., 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng et al., 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei et al., 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-1 (v3), for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa, Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum, sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa, perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), *Sorghum* (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans (*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum, Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with AAD-1 (v3) genes, for example, are included in the subject invention.

Example 16—Stacking AAD-1 (v3) with AHAS Herbicide Resistance Gene

Stacking AAD-1 (v3) with an AHAS herbicide resistance gene is described in Example 7.9.

Example 17—Further Evidence of Surprising Results: AAD-1 vs. AAD-2

17.1—AAD-2 (v1) Initial Cloning.

Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 ea. primer 1 n1 (50 ng/μl), gDNA 1 μl (200 ng/μl), H_2O 21 μl, Taq polymerase 1 μl (2.5 units/μl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C. 3.0 minutes heat denature cycle; 95° C. 1.0 minute, 50° C. 1.0 minute, 72° C. 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C. 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~4 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 3 μl Luria Broth+1000 μg/ml Ampicillin (LB Amp), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

17.2—Completion of AAD-2 (v1) Binary Vector.

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

17.3—Comparison of Substrate Specificities of AAD-2 (v1) and AAD-1 (v1)

The activity of an extract from *E. coli* expressing AAD-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 μl (42 μg) of *E. coli* extract per assay with a 15 min assay period. FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. Table 33 shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

TABLE 33

Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

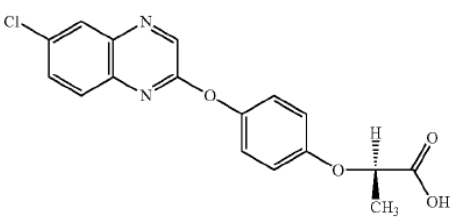
				A510	
STRUCTURE	Reg ID	Compound	Enantiomer	AAD1	AAD2
	18706	quizalofop	R	0.27	0.01

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

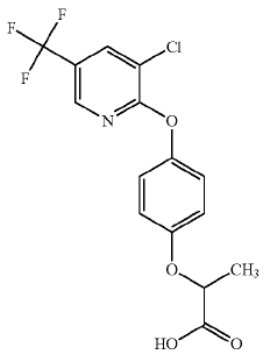
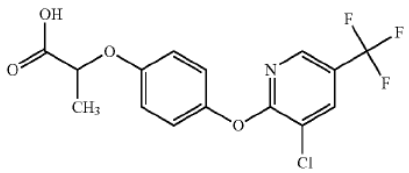
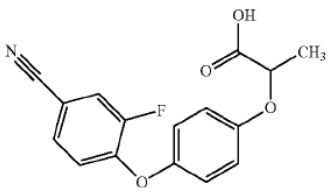
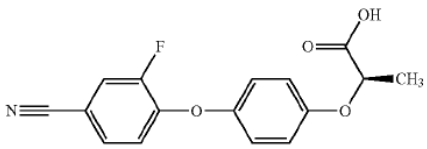
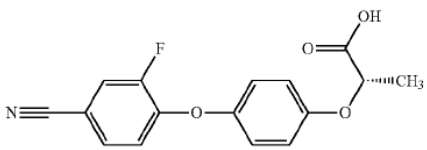
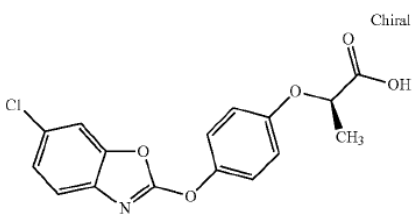
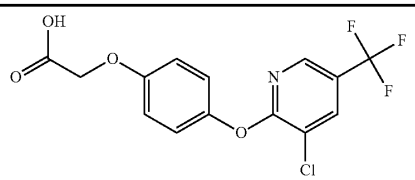
				A510	
STRUCTURE	Reg ID	Compound	Enantiomer	AAD1	AAD2
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
	Chiral 11044492	fenoxaprop	R	0.14	0

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	43865	haloxyfop-acetate	—	0	0.22

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **19**. The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (Table 34). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D in vitro than AAD-1 (v1). This is in surprising contrast to the in planta findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

TABLE 34

Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ± SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes:

Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglulurate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrinc/ferricyanide.

17.4—Evaluation of Transformed *Arabidopsis*.

Freshly harvested T_1 seed transformed with a native [AAD-1 (v2)], plant optimized [AAD-1 (v3)], or native AAD-2 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 hours light:10 dark, minimum 500 μE/m^2 s^1 natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1) to provide phenoxy auxin herbicide resistance.

Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, Del.) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0).

17.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15 in Example 6.5 above). A small lot of native AAD-1 (v2) transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native AAD-2(v1) gene provided a very low transformation frequency when selected for glufosinate tolerance (PAT selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% (see Table 15). Transforma-

tion frequency for native AAD-2 (v1) was only 6% of that of native AAD-1 (v2). The native AAD2 (v1) gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into *Arabidopsis* using methods previously described (see Example 5). The plant optimized AAD2 (v2) (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (see Table 15).

T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 16 (in Example 6.5 above) compares the response of AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) and AAD-1(v2) genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 16 simply to demonstrate the significant difference between the plants transformed with AAD-1 (v2), AAD-1 (v3), AAD-2 (v1) or AAD-2 (v2) versus the wildtype or PAT/Cry1F transformed controls.

Surprisingly, AAD-2 (v1) transformants were far less resistant to 2,4-D than either AAD-1 (v2) or AAD-1 (v3) genes (Table 16) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v1) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) AAD-1 (v2)-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average=34%), and >73% (11 of 15) AAD-1 (v3) T_1 plants were uninjured (population injury average=14%). See FIG. **20**. Tolerance improved slightly for plant-optimized AAD2 (v2) versus the native gene; however, comparison of both AAD-1 and -2 plant optimized genes indicates a significant advantage for AAD-1 (v3) in planta (see Table 16).

These results are unexpected given that in vitro comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. See FIG. **21**. No large difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) in planta for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected.

Example 18—Preplant Burndown Applications

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of Conyza *canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With AAD-1 (v3) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed.

Example 19—In-Crop Use of Phenoxy Auxins Herbicides in AAD-1 (v3) Only Transformed Soybeans, Cotton, and Other Dicot Crops

AAD-1 (v3) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D, dichlorprop, MCPA, et al.) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops

which are naturally tolerant to these herbicides. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3)-transformed dicot crop (i.e., soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of AAD-1 (v3)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20—In-Crop Use of Phenoxy Auxins and AOPP Herbicides in AAD-1 (v3) Only Transformed Corn, Rice, and Other Monocot Species

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-1 (v3) would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. AAD-1 (v3)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, et al. could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of AAD-1 (v3)-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3) transformed monocot crop (e.g., corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the AAD-1 (v3) gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by AAD-1 (v3) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels com-

piled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21—AAD-1 (v3) Stacked with Glyphosate Tolerance Trait in any Crop

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-1 (v3) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops withAAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like Conyza *canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-1 (v3)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 22—AAD-1 (v3) Stacked with Glufosinate Tolerance Trait in any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

By stacking AAD-1 (v3) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by AAD-1 (v3) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 23—AAD-1 (v3) Stacked with AHAS Trait in any Crop

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking AAD-1 (v3) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by AAD-1 (v3) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by AAD-1 (v3), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24—AAD-1 (v3) in Rice

24.1—Media Description.

Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100×20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C., and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) con-

sisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (½MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 μl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 μl spermidine (0.1 M) and 50 μl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 μl cold 100% ethanol and 8-9 μl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

24.4—Tolerance Testing.

Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 μl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in Table 35 below.

TABLE 35

Sample Name	140 g ae/ha quizalofop
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

24.5—Tissue Harvesting, DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

24.6—Southern Blot Analysis.

Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mims TAKRROO1A). Primers for Southern fragment PCR AAD-1 were (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse—GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction

was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in Table 36.

TABLE 36

Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049 bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

24.7—Western Data

Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in Table 37.

TABLE 37

			ELISA		
Lane	Sample Name	TSP (µg/mL)	[AAD1] (ng/mL)	Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	–
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	–
7	AAD1 Standard (0.5 µg/mL)				+++
8	AAD1 Standard (5.01 µg/mL)				+++++

Example 25—Turf Grass Transformation Procedures

Genetic transformation, with AAD-1 (v3) substituted for the "bar" gene as described below, of creeping bentgrass mediated by *Agrobacterium tumefaciens* could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. See "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo et. al., 2004).

Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60-65%) contained only a single copy of the foreign gene with no apparent rearrangements.

25.1—Seed Preparation for Embryogenic Callus Induction.

Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v)

Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-o-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120° C. for 20 min)

25.2—Embryogenic Callus Induction.

The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

25.3—*Agrobacterium* Infection and Co-Cultivation.

One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 µM acetosyringone. A 10-ul aliquot of *Agrobacterium* (LBA4404) suspension (0D=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25° C.

25.4—Resting Stage and *Agrobacterium* Control.

For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

25.5—Selection and Potential Transgenic Colony Identification.

Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

25.6—Regeneration of Transgenic Plants.

For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2-3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

25.7—Root Induction and Transfer to Greenhouse.

Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3-5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

25.8—Vernalization and Out-Crossing of Transgenic Plants.

Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25° C. under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25° C., and T1 plants were grown in the greenhouse for further analysis.

25.9—Other Target Grasses.

Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

Example 26—AAD-1 (v3) in Canola

26.1—Canola Transformation.

The AAD-1 (v3) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation. The construct contained AAD-1 (v3) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C., and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z7075 or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of AAD-1 (v3), whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the AAD-1 (v3) gene.

26.2—"Molecular Analysis": Canola Materials and Methods

26.2.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/ul.

26.2.2—Polymerase Chain Reaction.

A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Minis TAKRROO1A). Primers for Coding Region PCR AAD-1 (v3) were (Forward—ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse—CGGGCAGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-1 (v3) events tested positive. Three negative control samples tested negative.

26.3—ELISA.

Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

TABLE 38

Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (μg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	−
Control 2	61	2549.60	35.00	13.73	−
Control 3	59	2374.41	22.79	9.60	−

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Zipper, C., M. Bunk, A. Zehnder, H. Kohler. 1998. Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by *Sphingobium herbicidovorans* MH. J. Bact. 13:3368-3374

SEQUENCE LISTING

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<210> SEQ ID NO 1
<211> LENGTH: 50
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer used to amplify the rdpA/AAD-1 (v1) gene

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<210> SEQ ID NO 2
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer used to amplify the rdpA/AAD-1

(v1) gene

<400> SEQUENCE: 2

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<222> LOCATION: (1)..(18)
<223> OTHER INFORMATION: Primer linker
<220> FEATURE:
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<222> LOCATION: (907)..(915)
<223> OTHER INFORMATION: Primer linker

<400> SEQUENCE: 3

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atcgcggtcc agccgctgac cggcgtcctg ggcgccgaga tcaccggcgt cgacctgcgc 120

gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300

atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360

accttcctgg acgcaccgcc ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420

ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480

caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660

ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720

ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780

tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840

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cgctagtaac tcgag 915

<210> SEQ ID NO 4
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<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (891)..(897)
<223> OTHER INFORMATION: primer linker

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tgaccggcgt cctgggcgcc gagatcaccg gcgtcgacct gcgcgagccg ctcgacgaca 120

gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccggcc 180

aggcgatcac caacgaacag cacatcgcct tcagccggcg cttcggcccc gtcgatcccg 240

tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300

acgaaagcgg gcgtgtgatc ggtgatgact ggcacaccga cagcaccttc ctggacgcac 360

cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420

ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480

ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540

ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

tgcacccccc ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660

tctattgcca gcgcatcgag ggcatgaccg atgccgaaag caaaccgctg ctgcagttcc 720

tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> SEQ ID NO 5
<211> LENGTH: 919
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 (v3) primary sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(8)
<223> OTHER INFORMATION: additional alanine codon (GCT)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (894)..(919)
<223> OTHER INFORMATION: primer linker

<400> SEQUENCE: 5

ccatggctca tgctgccctc agccctctct cccaacgctt tgagagaata gctgtccagc 60

cactcactgg tgtccttggt gctgagatca ctggagtgga cttgagggaa ccacttgatg 120

acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300

ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420

ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480

aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt 720

tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780

aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca 840

agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta 900

gcttaatcac ctagagctc 919

<210> SEQ ID NO 6

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<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rdpA(ncoI) 5' primer

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 3'saci primer

<400> SEQUENCE: 7

gagctcacta gcgcgccggg cgcacgccac cga 33

<210> SEQ ID NO 8
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BstEII/ Del NotI 5' primer

<400> SEQUENCE: 8

tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> SEQ ID NO 9
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Sphingobium herbicidovorans

<400> SEQUENCE: 9

Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1 5 10 15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
20 25 30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
35 40 45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
50 55 60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65 70 75 80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
85 90 95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
100 105 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
115 120 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
130 135 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145 150 155 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
165 170 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
180 185 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro

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        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 10
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v2 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: Different from v1

<400> SEQUENCE: 10

Met Ala Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1               5                   10                  15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65                  70                  75                  80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220
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Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 11
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: AAD-1 v3 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Different from V1

<400> SEQUENCE: 11

Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1               5                   10                  15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
            20                  25                  30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
        35                  40                  45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
    50                  55                  60

Asn Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro
65                  70                  75                  80

Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
        115                 120                 125

Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
                245                 250                 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
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           260                 265                 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
        275                 280                 285

Val Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 12
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 12

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ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac    120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg    180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg    240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag    300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc    360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc    420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc    480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc    540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc    600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc    660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag    720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc    780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg    840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag                 888


<210> SEQ ID NO 13
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 13

Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                   10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
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    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
                245                 250                 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
            260                 265                 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
        275                 280                 285

Val
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: brjap 5'(speI)

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actagtaaca aagaaggaga tataccatga cgat 34

<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: br jap 3' (xhoI)

<400> SEQUENCE: 15

ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> SEQ ID NO 16
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 forward primer

<400> SEQUENCE: 16

gtaaaacgac ggccagt 17

<210> SEQ ID NO 17
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 reverse primer

<400> SEQUENCE: 17

caggaaacag ctatgac 17

<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NcoI of Brady

<400> SEQUENCE: 18

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 19
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SacI of Brady

<400> SEQUENCE: 19

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 20
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
85 90 95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
100 105 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
115 120 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
130 135 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145 150 155 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
165 170 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
180 185 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
195 200 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
210 215 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225 230 235 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
245 250 255

```
Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525


<210> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine
      183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 21

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                   10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
```

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               85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510
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Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
515 520 525

<210> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence encoding the EPSPSprotien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop codons in all six open reading frames, and to introduce a SacI restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 22

atggctcaag tctcccgtgt tcacaatctt gctcagtcaa cccaaatctt tggacattca 60

agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttcgcccacg cctttgggga 120

gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt 180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc 240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg 300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc 360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga 420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt 480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat 540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga 720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc 780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa 840

atagtggaca agttgatttc tgttccatat gtggaaatga ccctcaaact catggagagg 900

tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag 960

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cttcaaggtg atgtaaagtt tgcagaggtc ttggagaaaa tgggtgccaa ggtcacctgg 1140

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cgtggaattg atgtgaacat gaacaagatg ccagatgtgg ccatgaccct cgctgttgta 1260

gccctgtttg caaatggacc aactgcaatc cgtgatgttg cttcatggag ggtgaaggag 1320

acagagagga tgattgccat ttgcacagaa ctccgcaaac ttggtgcaac agttgaagag 1380

ggaccagatt actgtgtgat aaccccacct gagaagctca atgtgacagc cattgacacc 1440

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tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> SEQ ID NO 23
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 5-3

<400> SEQUENCE: 23

agataccctt ggttggttgc 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 3-3

<400> SEQUENCE: 24

cagatggatc gtttggaagg 20

<210> SEQ ID NO 25
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU forward primer

<400> SEQUENCE: 25

ataatgccag cctgttaaac gcc 23

<210> SEQ ID NO 26
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU reverse primer

<400> SEQUENCE: 26

ctcaagcata tgaatgacct cga 23

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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(RdpAcodF)

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atggctcatg ctgccctcag cc 22

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<223> OTHER INFORMATION: Reverse primer for Coding Region PCR AAD-1
(RdpAcodR)

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cgggcaggcc taactccacc aa 22

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ccatggctac catagcaatc agacagctcc agacccactt tgtgggtcaa gtttctggat 60

tggacctcag aaagccactc actcctggag aagccagaga agttgaatca gctatggaca 120

agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg 180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt 240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg 300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca 360

gcttcagacc aatcccagcc aagttcagcc tccttttctgc tcgtgttgtg aacccaactg 420

gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

ttgccagcat gtccgtccct gaagggaggc tccttttgag ggatttgaat gaacatgcta 720

ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

gtgcaacagt tgctgggacc gagcccacag tgcaacagca agcagcagag tgagtagtta 900

gcttaatcac ctagagctcg gtcaccagat ct 932

<210> SEQ ID NO 30
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Translated AAD-2 v2 protein

<400> SEQUENCE: 30

Met Ala Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln
1 5 10 15

Val Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg
20 25 30

Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
35 40 45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
50 55 60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65 70 75 80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
85 90 95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
100 105 110

Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
115 120 125

Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
130 135 140

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Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
145                 150                 155                 160

Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
                165                 170                 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
            180                 185                 190

Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
        195                 200                 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
    210                 215                 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
225                 230                 235                 240

Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
                245                 250                 255

Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
            260                 265                 270

Ser Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro
        275                 280                 285

Thr Val Gln Gln Gln Ala Ala Glu
    290                 295


<210> SEQ ID NO 31
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 forward primer

<400> SEQUENCE: 31

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<210> SEQ ID NO 32
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 reverse primer

<400> SEQUENCE: 32

gggcaggcct aactccacca a                                               21
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The invention claimed is:

1. A plant cell comprising an expression cassette, wherein said expression cassette comprises a polynucleotide encoding a protein comprising the amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO: 10 and SEQ ID NO: 11, wherein said polynucleotide is operably linked to a heterologous promoter that is functional in a plant cell and expression of said polynucleotide renders said transgenic plant cell tolerant to a phenoxy auxin herbicide as compared to an untransformed plant cell of the same plant.

2. The plant cell of claim **1**, wherein said polynucleotide is selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

3. A plant comprising the plant cell of claim **1**.

4. A seed from the plant of claim **3**, wherein plant cells of said seed comprise said expression cassette.

5. A method of protecting a crop plant from damage by an aryloxyalkanoate herbicide, said method comprising growing the plant of claim **3**, and applying said herbicide to said plant.

6. A method of making the plant cell of claim **1** wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells.

7. The plant cell of claim **1** wherein said promoter is selected from the group consisting of cassava vein mosaic virus promoter (CsVMV), AtUbilO promoter, AtUbi3 promoter, OsActl promoter and ZmUbil promoter.

8. The plant of claim **3** wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus.*

9. The plant of claim **3** wherein said plant further comprises a gene for an agronomic trait selected from the group

consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

10. The plant of claim **3**, wherein said plant is a monocot.

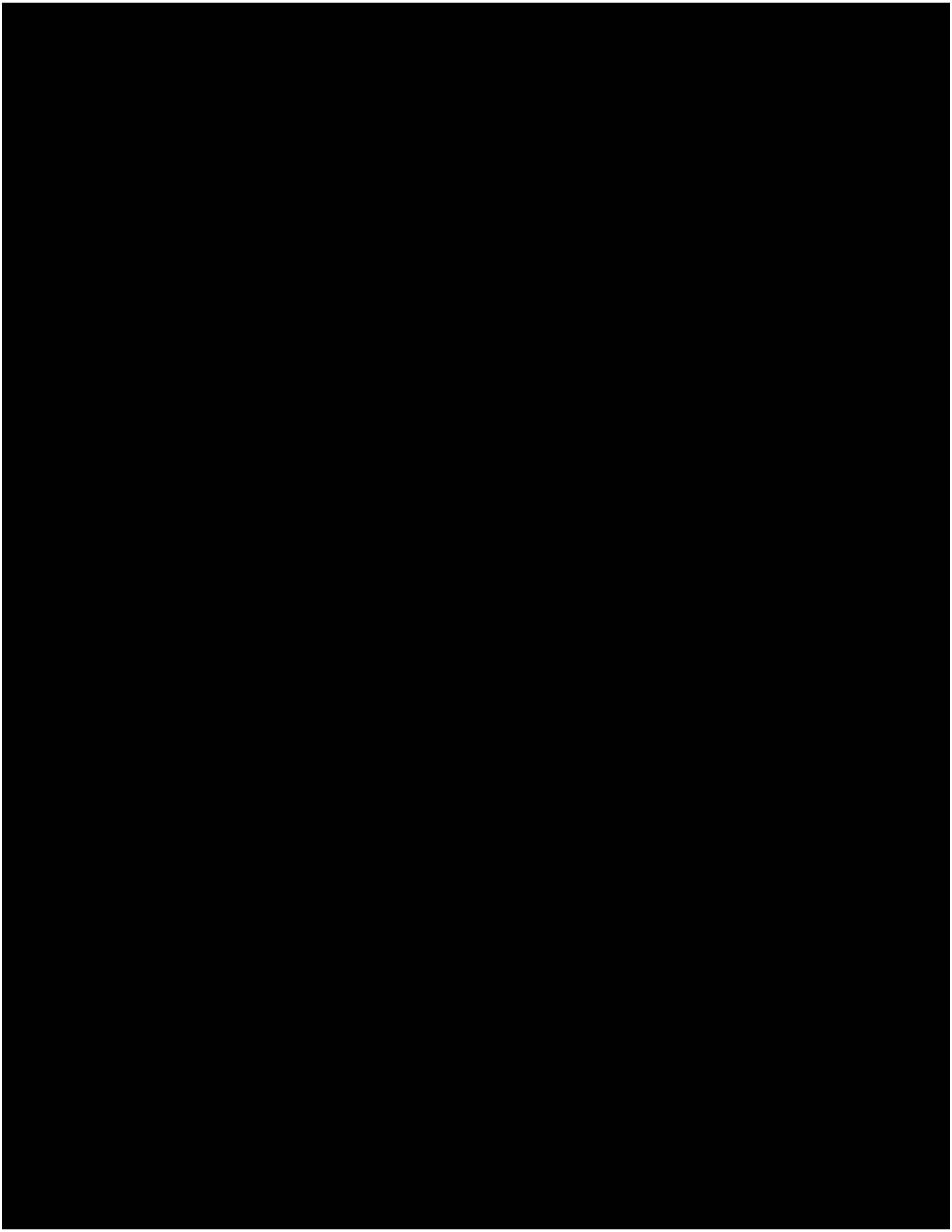
11. The plant of claim **10**, wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, *sorghum*, and pasture grasses.

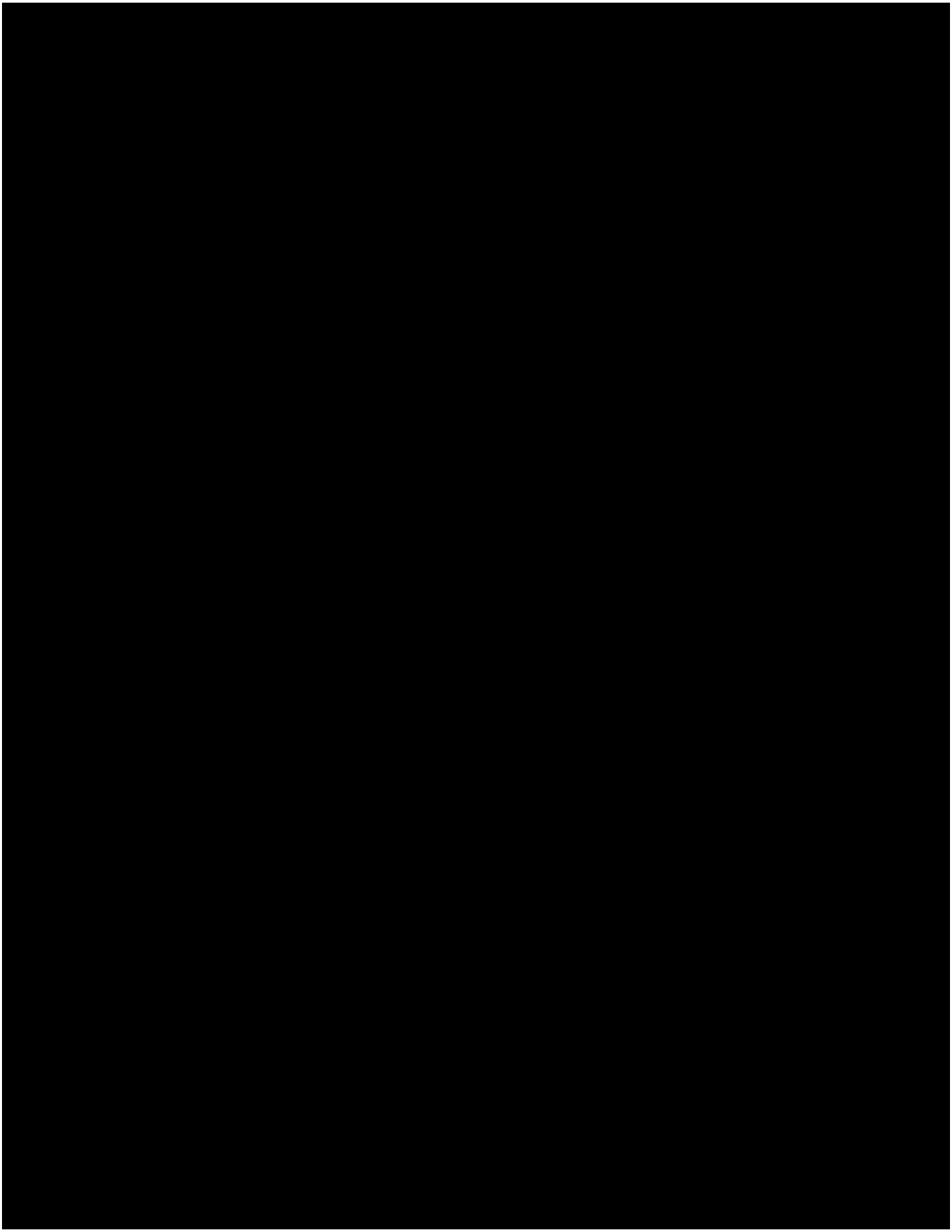
12. A plant grown from the seed of claim **4**.

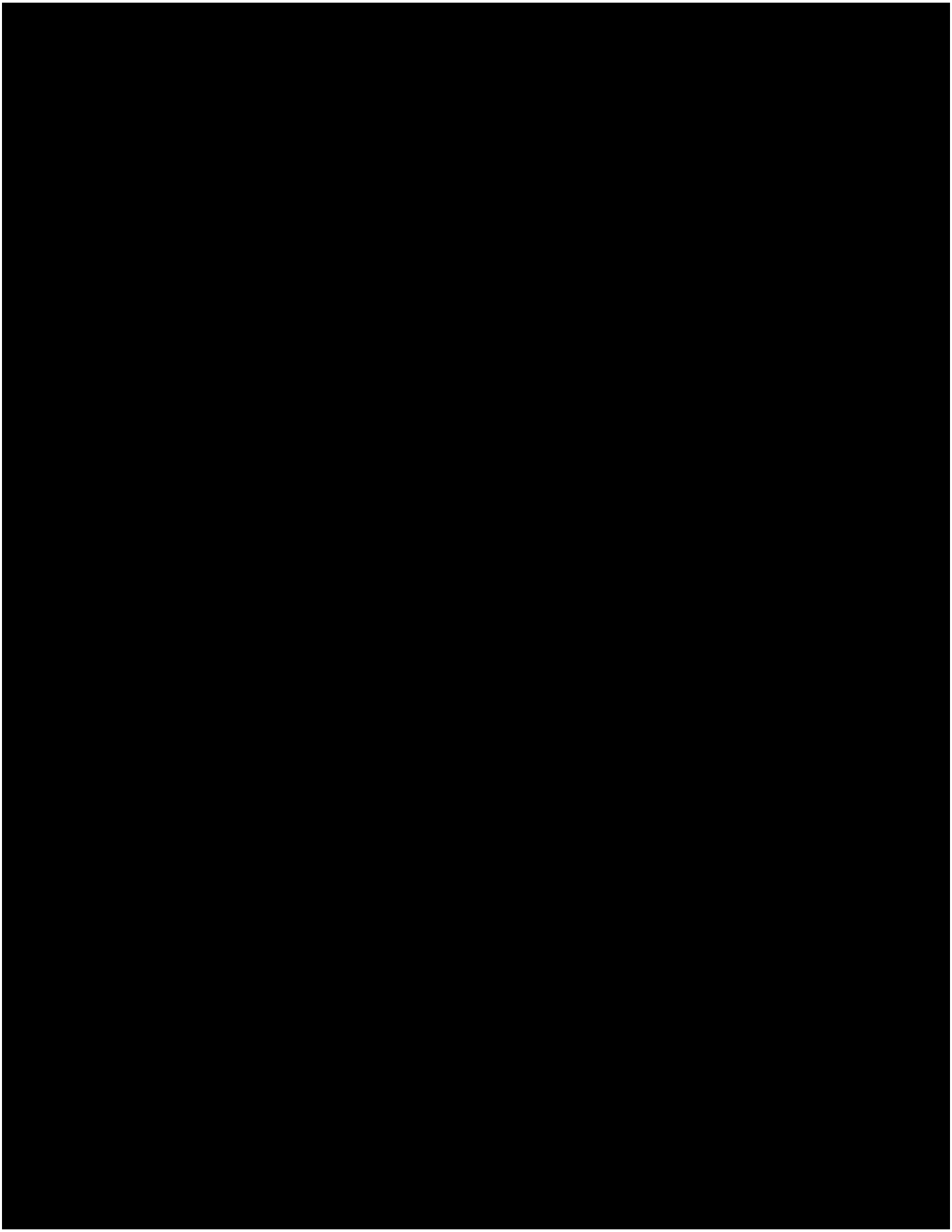
13. A regenerable part, progeny, or asexual propagate of the plant of claim **3**, wherein said regenerable part, progeny, or asexual propagate comprises an expression cassette comprising a polynucleotide encoding a protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO: 10 and SEQ ID NO: 11, wherein said polynucleotide is operably linked to a heterologous promoter that is functional in a plant cell.

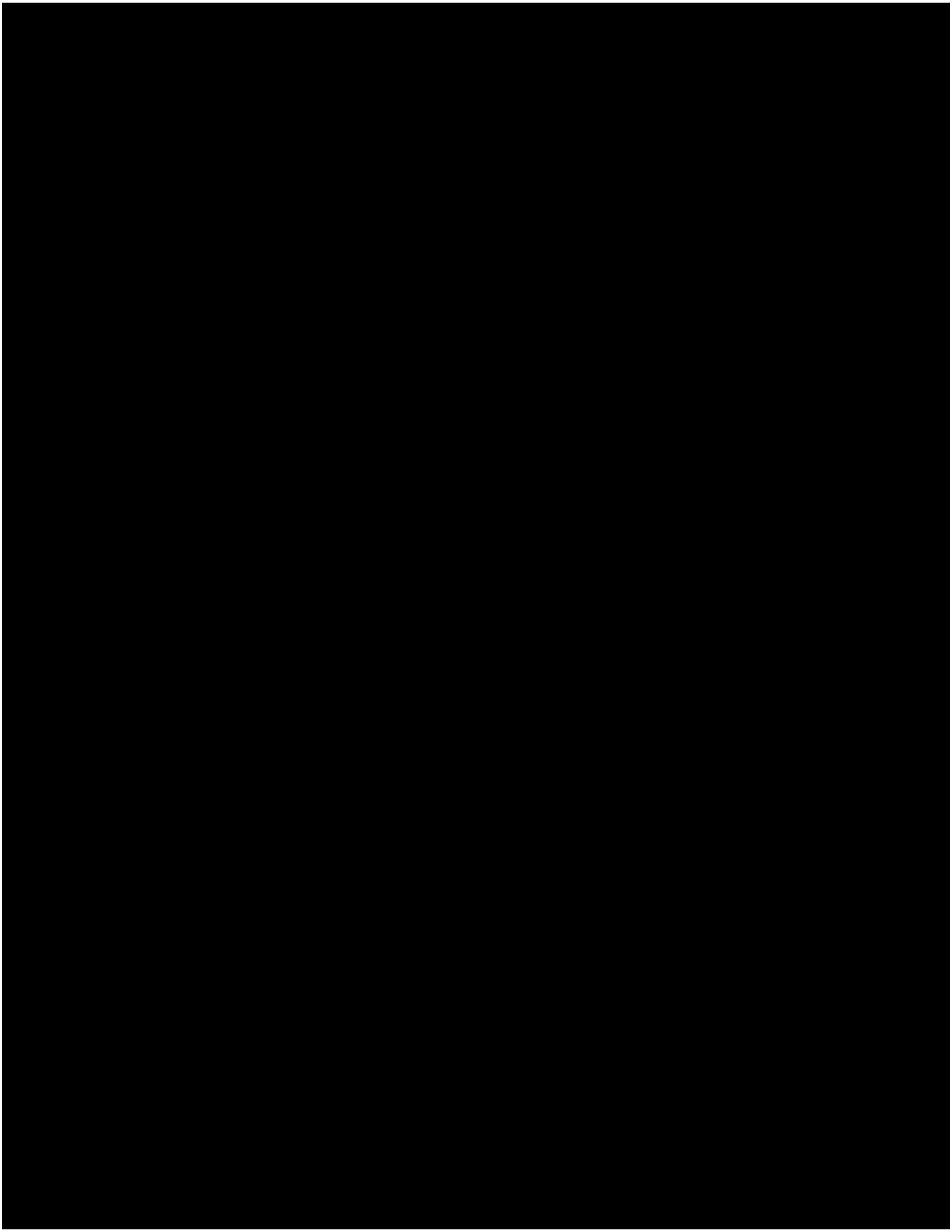
* * * * *

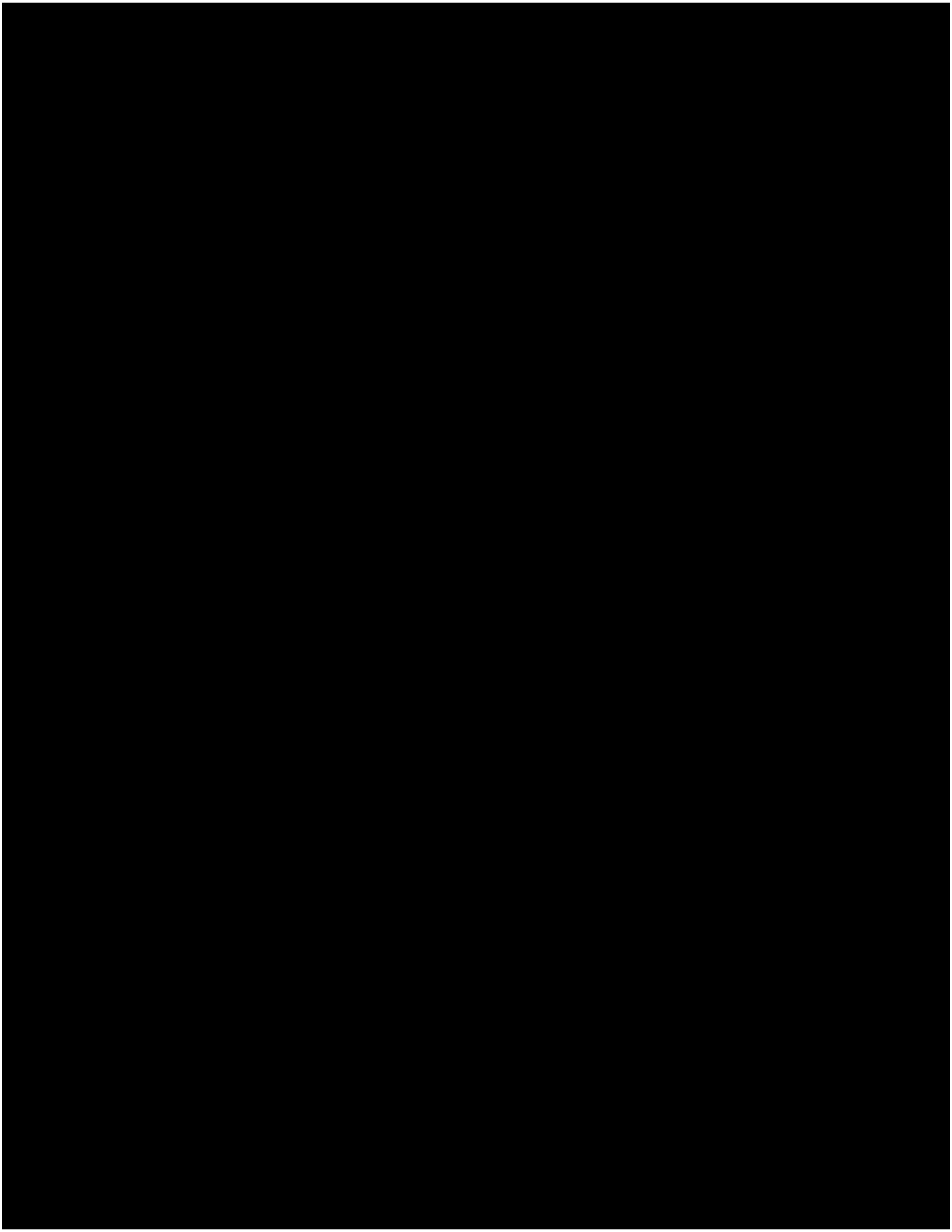
EXHIBIT 15

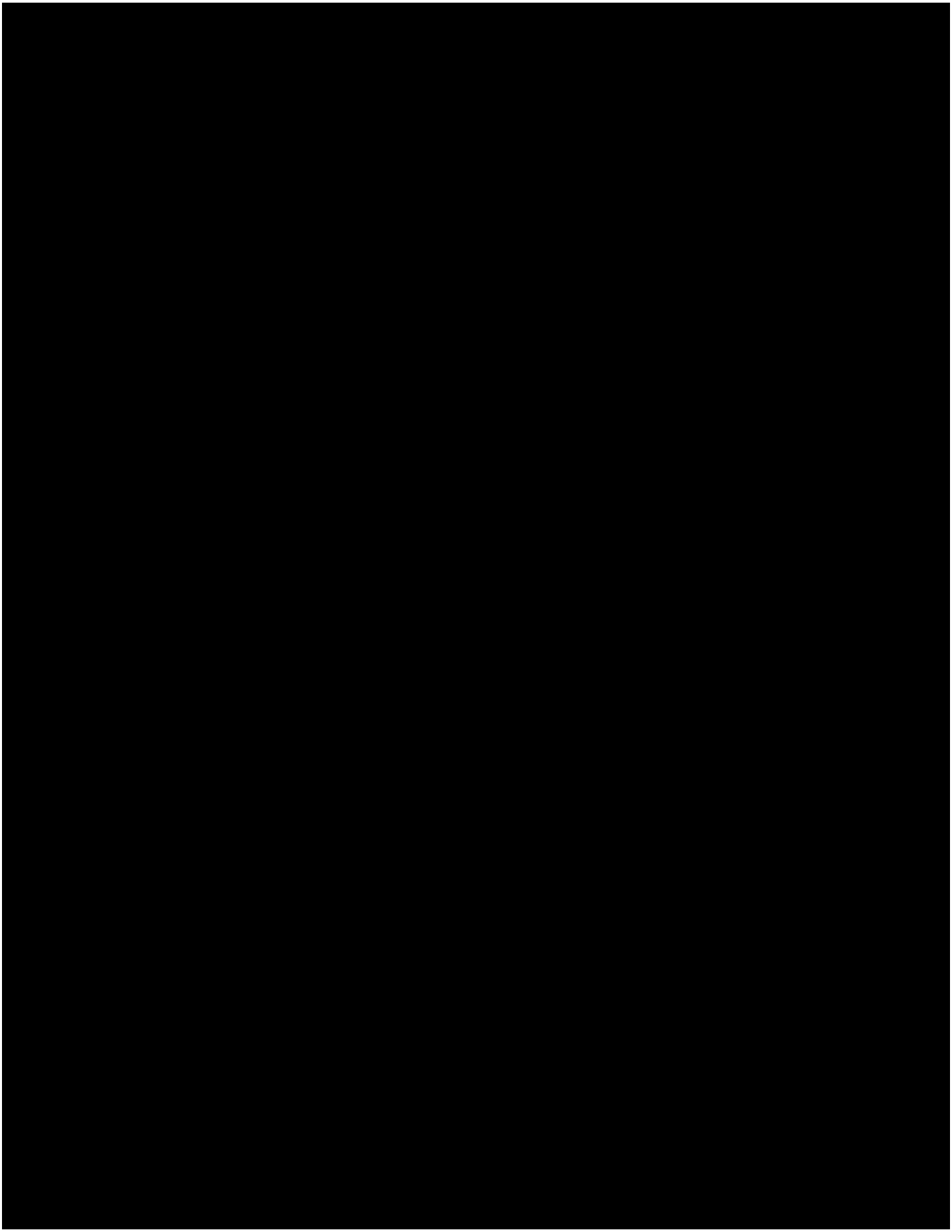


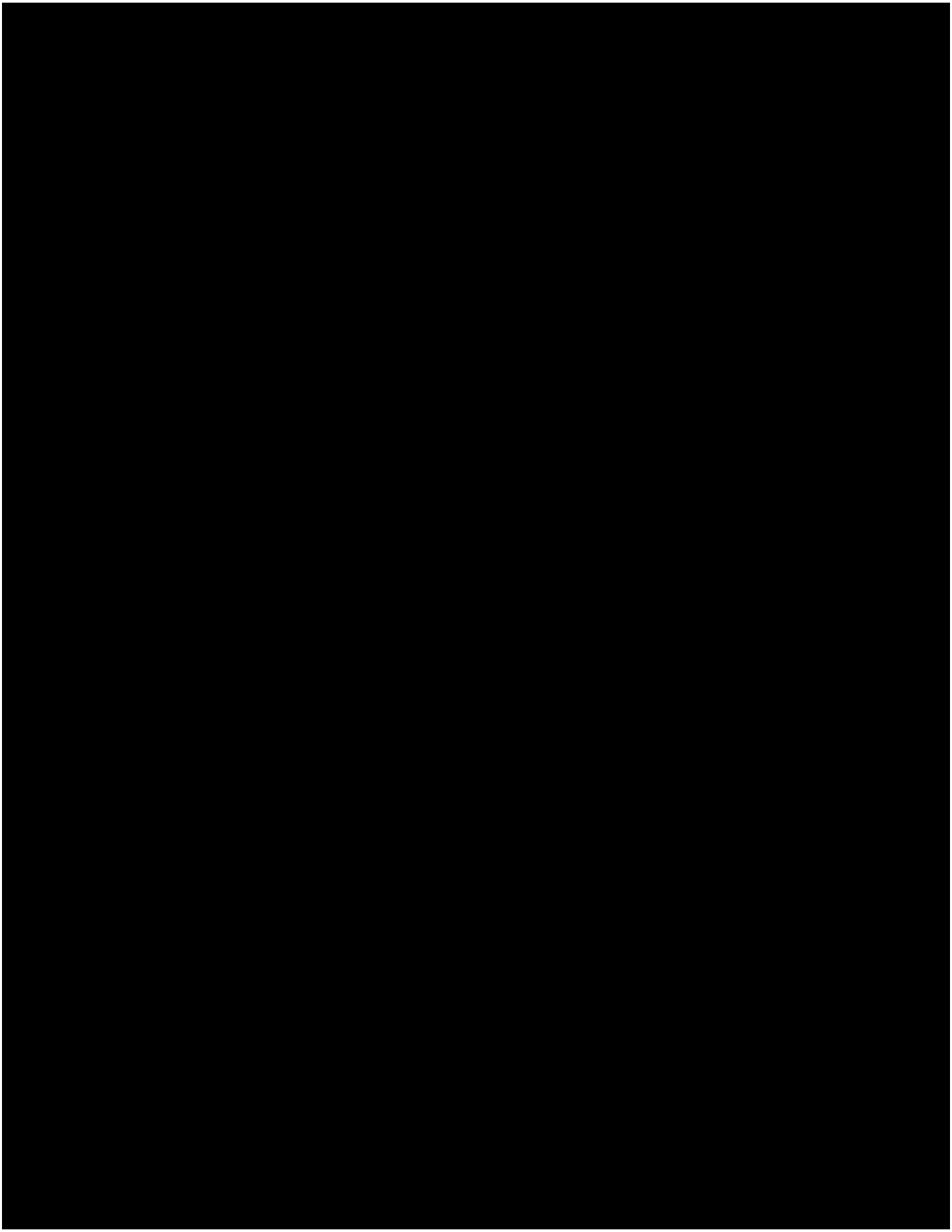


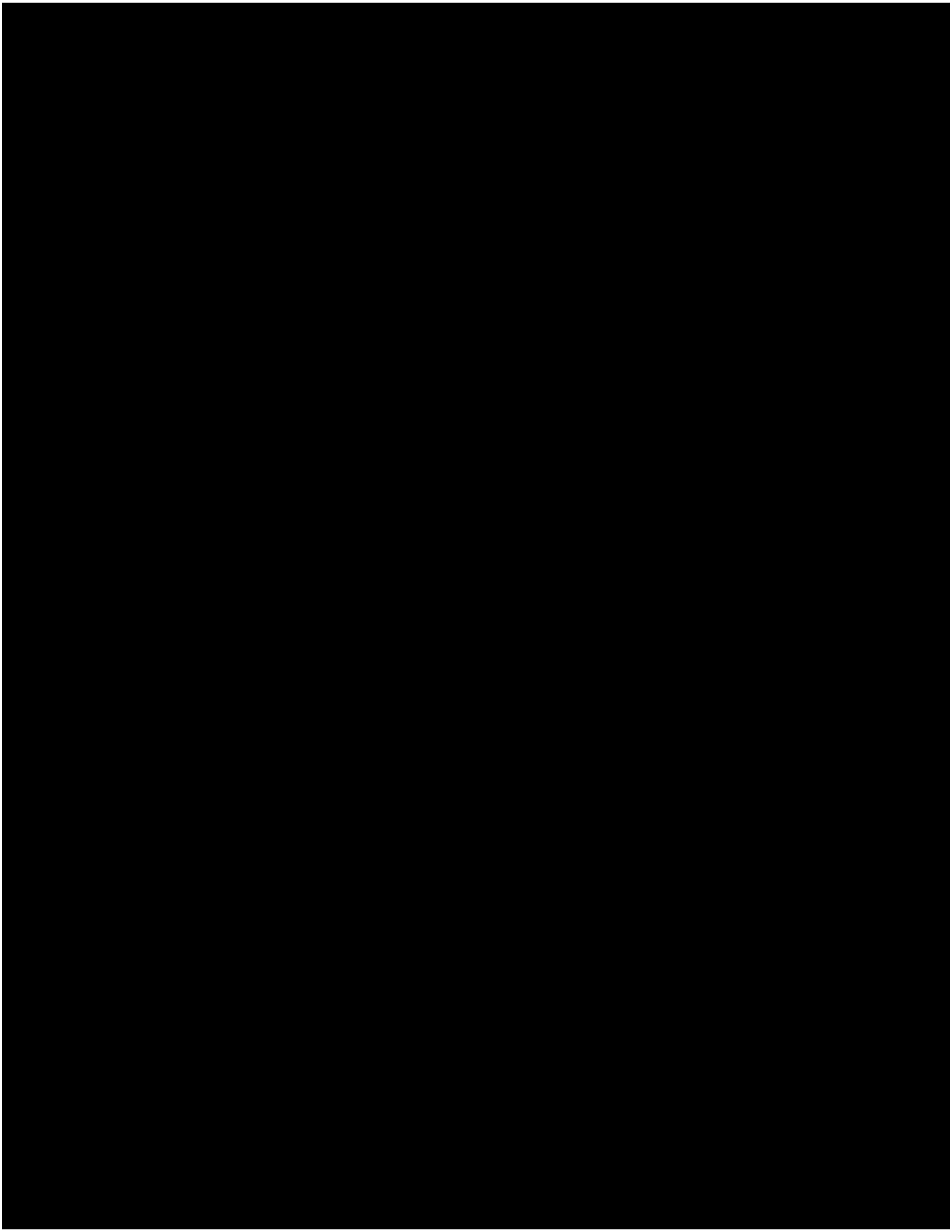


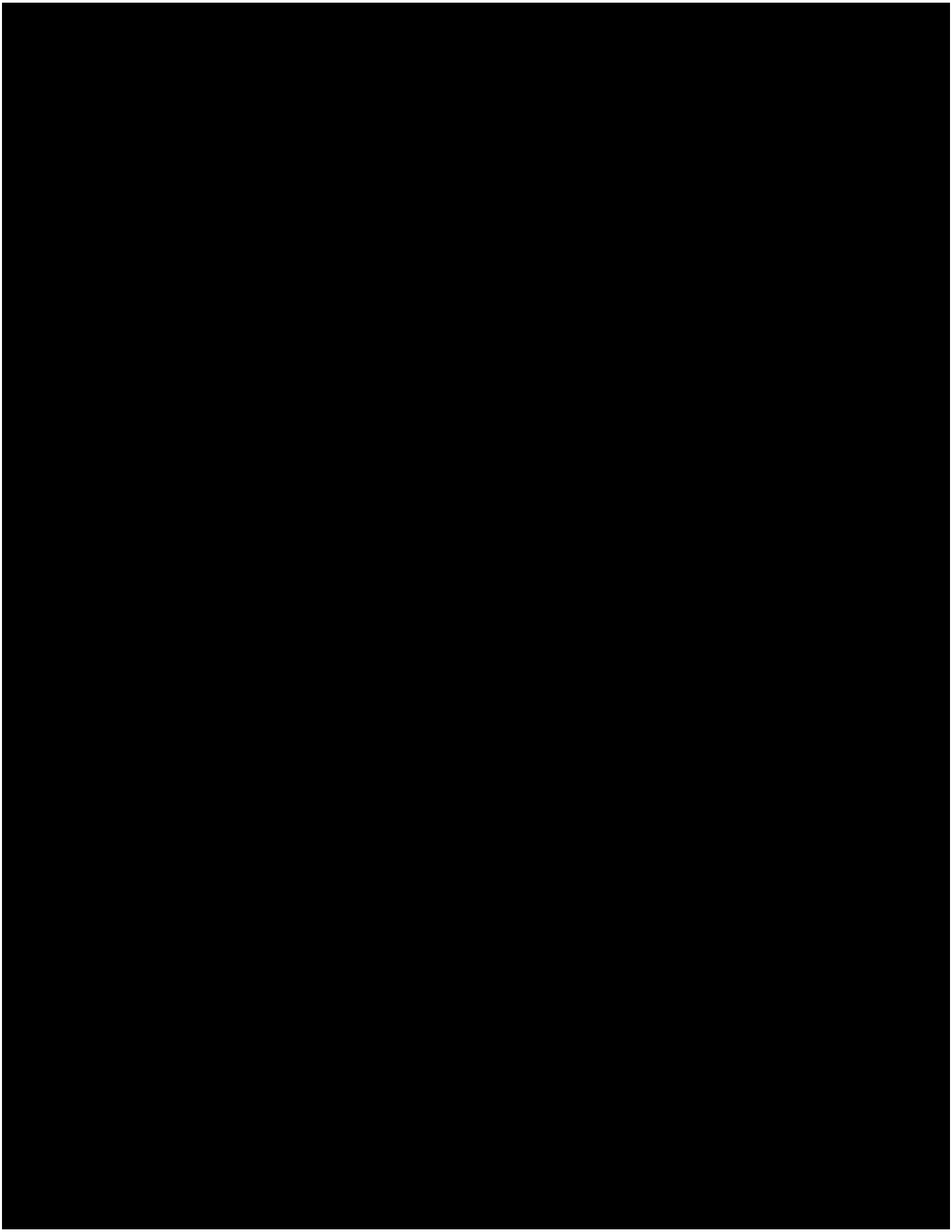


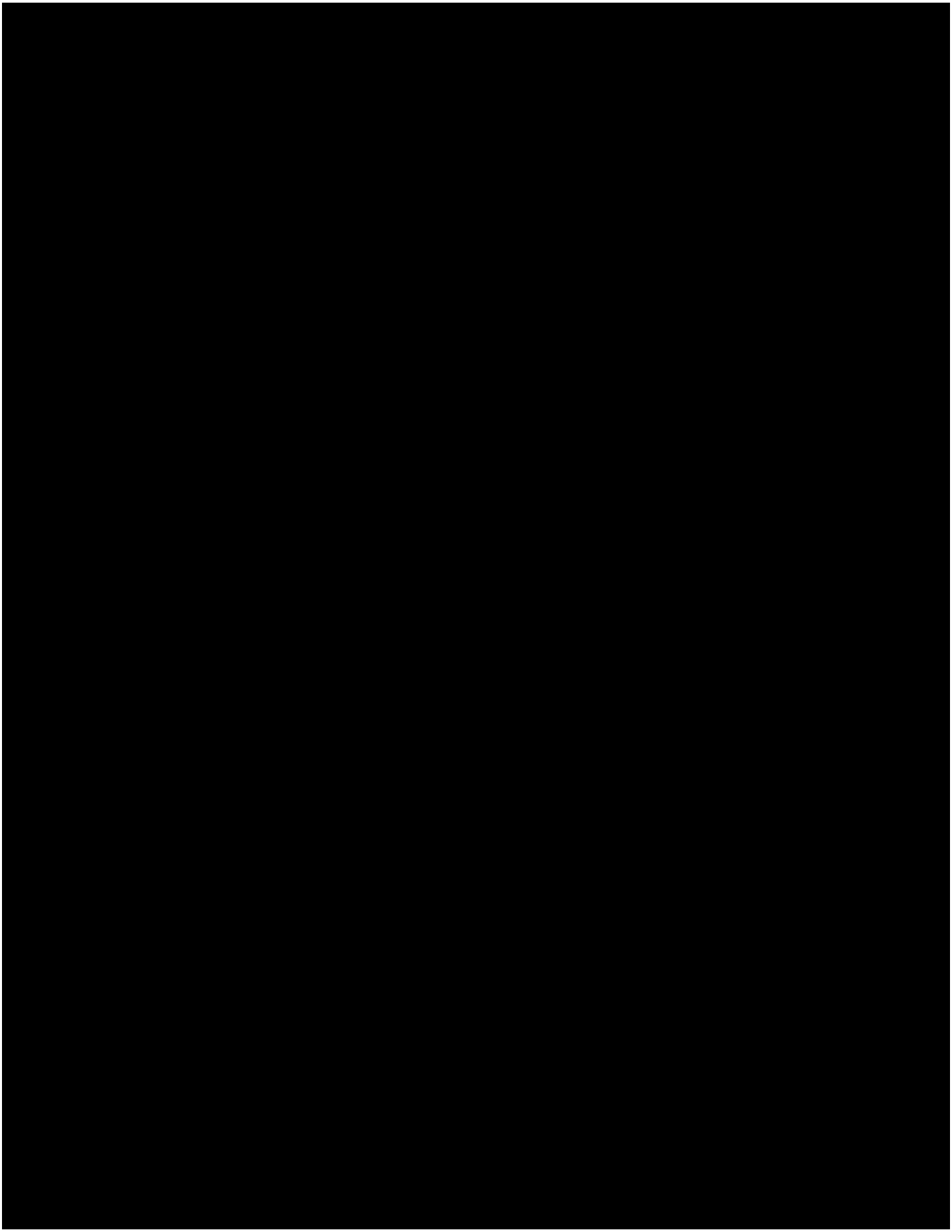


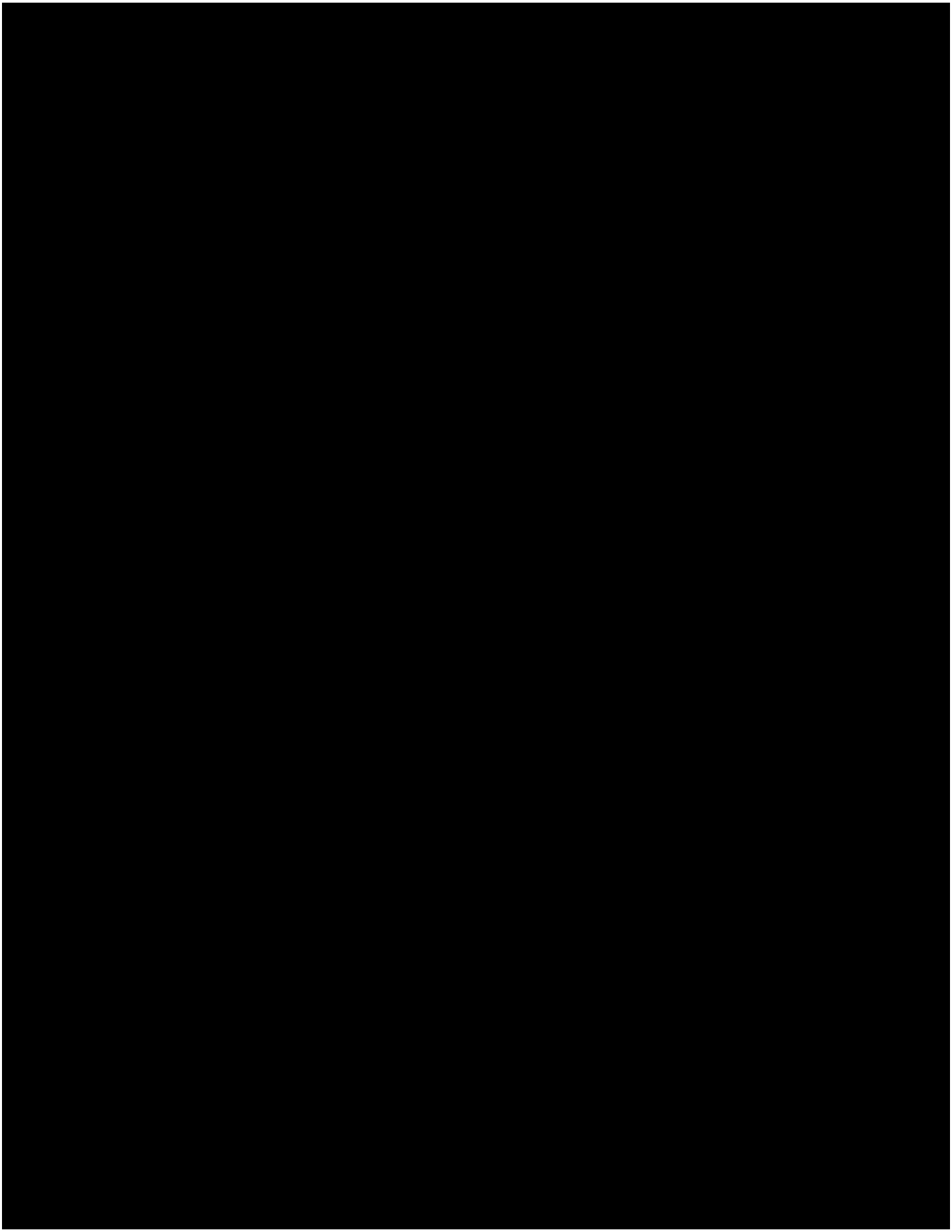












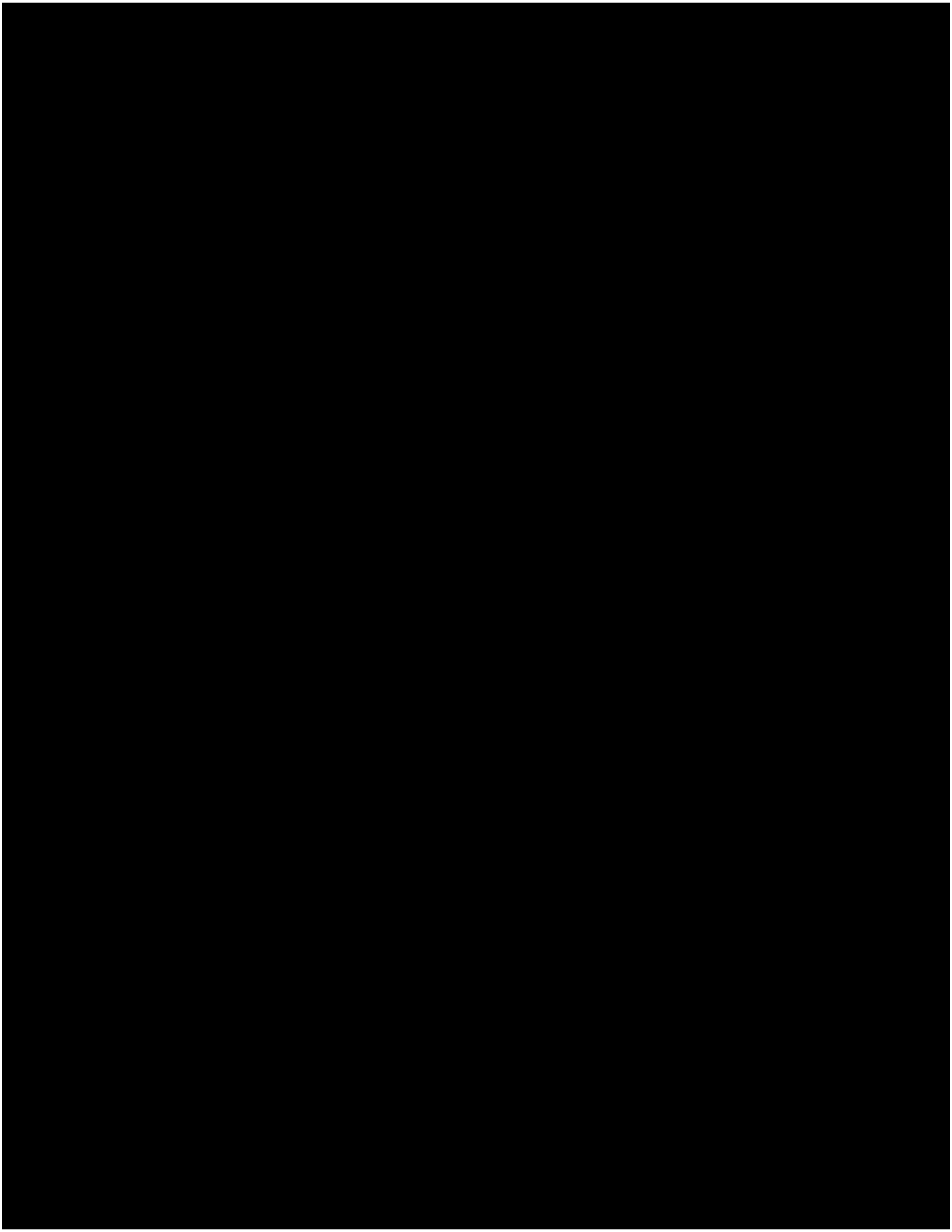


EXHIBIT 16

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 07/12/2019
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER	
KRUSE, DAVID H	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
07/12/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Office Action Summary	**Application No.** 15/288,406	**Applicant(s)** WRIGHT et al.	
	Examiner DAVID H KRUSE	**Art Unit** 1663	**AIA (FITF) Status** No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 12 April 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-16 and 18-48 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-16 and 18-48 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

STATUS OF THE APPLICATION

1. The present application is being examined under the pre-AIA first to invent provisions.

2. This Office action is in response to the Amendment and Remarks filed 12 April 2019.

3. The objection to the Specification is withdrawn in view of Applicant's amendment.

4. The Examiner notes that USP Application 14/989,787 has issued as U.S. Patent 10,323,287. After reviewing the issued claims the Examiner has determined that there is no double patenting issue.

5. The Examiner notes that USP Application 14/820,893 has issued as U.S. Patent 10,174,337.

6. The rejection of claims 32, 35, 38 and 46 under 35 U.S.C. 103(a) is withdrawn in view of Applicant's arguments.

Double Patenting

7. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed.

Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

8. Claims 15, 15 and 18-48 remain rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-43 of U.S. Patent No. 7,838,733.

Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '733 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

9. Claims 15, 16 and 18-48 remain rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 9,127,289. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '289 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

10. Claims 15, 16 and 18-48 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 10,174,337 (which issued from US Patent Application 14/820,893). Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in

the '337 patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

This rejection has been modified because the Application has issued as a U.S. Patent. Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 10, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

Claim Rejections - 35 USC § 112

11. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

12. Claims 15, 16 and 18-48 remain rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is repeated for the reason of record as set forth in the last Office action mailed 13 November 2018.

Applicant's arguments filed 12 April 2019 have been fully considered but they are not persuasive.

Applicant claims a transgenic plant cell, plant and method of using wherein the plant cell comprises a recombinant polynucleotides encoding an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 and an AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$.

Applicant describes the amino acid sequence of the AAD-1 (syn. RdpA) enzyme in instant SEQ ID NO: 9 and two variants thereof in instant SEQ ID Nos. 10 and 11 which are 98.8% and 99.7% identical respectively to said SEQ ID NO: 9.

Applicant does not describe the claimed genus of AAD-1 proteins or the AAD-1 motif (in the specification). The specification describes a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases on page 17, paragraph 0085, $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed.

Applicant argues that the original specification highlights the key amino acid residues of aryloxyalkanoate dioxygenases by providing the structure of a motif comprising the active site of the enzyme. Applicant argues that one of ordinary skill in the art would know that the motif provided on page 17 in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs; 9, 10 and 11 to allow one to generate a "AAD-1 motif" of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, that is specific to the claimed genus of AAD-1 proteins. Applicant argues that the claims have been narrowly tailored to be directed to AAD-1 proteins that

enzymatically degrade a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide by requiring the claimed peptides to comprise the functional element of the AAD-1 motif (page 11, 1st paragraph of the Remarks).

Applicant argues that in support of the objective teachings relating to the structure/function relationship of the AAD-1 genes, Applicant submits herewith additional data and a Declaration under 37 CFR 1.132. Applicant argues that an expert's Declaration is relevant to the issue of written description when it is direct to specific statements in the originally filed application and used to explain how, in his opinion, a person of ordinary skill in the art would understand those statements (see *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009)) (page 11, 2nd paragraph of the Remarks).

Applicant argues that as described in paragraph 14 of the accompanying (Wright) Declaration, an experiment was conducted to confirm the objective teachings of the present application that the defined residues in the AAD-1 motif $HXDX_{24}TX_{13}1HX_{10}R$ are important to the desired dual MOA herbicide degradation activity. Applicant argues that nine variants of AAD-1 (SEQ ID NO: 9) were prepared and tested for enzymatic activity against 2,4-D and (R)-quizalofop. Applicant argues that alignments of all nine variants and the original AAD-1 (SEQ ID NO: 9) are shown in Appendix III of the accompanying Declaration. Applicant argues that the data presented with the accompanying Declaration (see the bar graph provided in Appendix III and paragraphs 15 and 16 of the accompanying Declaration) confirm the importance of the amino acids at positions 111, 113, 138, 270 and 281 in AAD-1 proteins for maintaining the claimed enzymatic activity. Applicant argues that the skilled practitioner readily appreciates that other

regions of the AAD-1 protein would be more tolerant to modification without loss of the claimed activity (page 11, 4th paragraph of the Remarks).

Applicant argues that applicant respectfully submits that they have provided a substantial amount of structure of the members of the claimed genus.

Applicant argues that each peptide of the claimed genus is required to have at least 85% sequence identity with an amino acid of SEQ ID NO: 9. Applicant argues that applicant has provided guidance as to where modifications can be made in the AAD-1 sequence requiring all peptides of the claimed genus to comprise the AAD-1 motif. Applicant argues that those skilled in the art are well aware that proteins can be modified while retaining the activity of the original protein, provided that the active site remains intact. Applicant argues that the claimed genus is further limited by the requirement that the claimed analogs of SEQ ID NO: 9 comprise the AAD-1 motif and retain enzymatic activity for degrading phenoxy auxin herbicides **as well as** (R)-aryloxyphenoxypropionate herbicides (emphasis added by the Examiner). Applicant argues that as indicated in the accompanying Declaration and data presented in Appendix III, applicant has established a structure function relationship for the desired dual MOA herbicide degradation activity.

Applicant argues that the teachings regarding the AAD-1 motif, in conjunction with the disclosed exemplified species, provide sufficient guidance to those skilled in the art, that one of skill in the art would recognize that applicant was in possession of the necessary common attributes possessed by the each member of the genus (page 12 of the Remarks).

Applicant's arguments have been fully considered but are not found to be fully persuasive. The Examiner has reviewed *Martek Biosciences Corp. v. Nutrinova, Inc.*, 579 F.3d 1363 (Fed. Cir. 2009), and acknowledges that that court states that a patent claim is not necessarily invalid for lack of written description just because it is broader than the specific example disclosed (at 1371). But, the written description must allow one of skill in the art to envision the claimed invention based on the text of the application. See *University of Rochester v. G.D. Searle & Co.,* 68 USPQ2d 1424, 1433 (DC WNY 2003) which teaches knowing the "starting point" is not enough; that is little more than a research plan. The court held that the disclosure of screening assays and general classes of compounds was not adequate to describe compounds having the desired activity: without disclosure of which peptides, polynucleotides, or small organic molecules have the desired characteristic, the claims failed to meet the description requirement of § 112.

While the recited AAD-1 motif is similar to other enzymes having a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases the generic motif does not itself define a specific function and Applicant does not describe what structure in the AAD-1 motif provides the specific activity that degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide. While a description of a representative number of species is adequate to represent an entire genus, wherein there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus (see MPEP 2163). Compared to instant SEQ ID NO: 9, the claims encompass (20^{165})-1 variations of just

the AAD-1 motif. Therefore, it is the Examiner's opinion that Applicant has failed to adequately describe the variation within the claimed genus.

Claim Rejections - 35 USC § 103

13. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

14. Claims 15, 16 18-30, 32-34, 36, 37, 39-45, 47 and 48 remain rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jepson *et al* (U.S. Patent Application US 2003/0041357 filed on 23 February 2001) in view of Kohler (Genbank Accession No: Q8KSC8 first published on 1 October 2002). This rejection is repeated for the reason of record as set forth in the last Office action mailed 13 November 2018. Applicant's arguments filed 12 April 2019 have been fully considered but they are not persuasive.

Jepson *et al* teach a method of controlling at least one weed in an area by applying a phenoxy auxin herbicide said area comprising a transgenic plant comprising a recombinant polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity (see paragraph 0027 on pages 3-4, especially lines 18-24 on page 4). Jepson *et al* teach said method using a plant transformed with an additional transgene that provides glyphosate, glufosinate, imidazolinone or sulfonylurea herbicide tolerance (page 4, left column, lines 18-27). Jepson *et al* teach applying the phenoxy auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Jepson *et al* teach said method can

be practiced using turf grass or forage grass (page 3, paragraph 0024, last sentence; claim 15). Jepson *et al* teach said method can be practiced using a plant transformed with a transgene that produces tolerance to a pre-emergence herbicide (claim 12).

Jepson *et al* do not teach a transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9.

Kohler teaches a polynucleotide that encodes a protein comprising the amino acid sequence of instant SEQ ID NO: 9. Kohler teaches that said protein has R-2,4-dichlorophenoxypropionate dioxygenase activity (i.e. degrades the phenoxy auxin herbicide (R)-dichlorprop).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicant's invention to modify the teachings of Jepson *et al* to substitute the tfdA herbicide tolerance gene for a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase taught by Kohler. It would have been obvious to combine a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase with another gene for tolerance to additional herbicide tolerances as taught by Jepson *et al*. Jepson *et al* teach that one would apply one (claim 16) or two (claim 17) herbicides in the method of controlling weeds. Jepson *et al* teach that at the time of Applicant's invention it was desirable to produce plants tolerant to a phenoxy auxin herbicide. Given the high level of skill in the instant art at the time of Applicant's invention, one would have had a reasonable expectation of success.

Applicant argues that the Examiner fails to provide any rationale for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO: 9, or a derivative thereof having at least 85% sequence identity to a peptide of SEQ ID NO: 9, to substitute for the *tfdA* gene disclosed in Jepson. Applicant argues that there are many other known sequences that share sequence identity with the *tfdA* gene and serve as possible candidates for providing aryloxyalkanoate dioxygenase activity. Applicant argues that at the time of applicant's invention it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants.

Applicant argues that at the time of the invention there was simply no motivation to select a bacterial gene having specific sequence of SEQ ID NO: 9, (or a peptide having at least 85% sequence identity with SEQ ID NO: 9) for combination with a heterologous promoter that is functional in a plant cell for use in producing transgenic plants that are tolerant to damage by an aryloxyalkanoate herbicide.

Applicant argues that Example 17 of the instant specification provides data comparing the subject AAD-1 enzyme to another enzyme referred to as AAD-2, wherein AAD-2 has a higher percentage sequence identity with *tfdA* relative to AAD-1. Applicant argues that, AAD-2 is a protein that falls outside the scope of the current claims, as AAD-2 does not have at least 85% sequence identity with SEQ ID NO: 9, yet is one with a sequence identity closer to *tfdA*. Significantly, AAD2 also does not comprise the AAD-1 motif that is present in AAD-1 (see paragraph 12 and Appendix II of the accompanying Declaration).

Applicant argues that AAD-2 was one of several genes identified from BLAST search results using *tfdA* as the reference sequence (page 13 of the Remarks).

Applicant argues that the subject AAD-1 enzyme was a choice of one of over 100 other possible genes.

Applicant argues that 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). Applicant argues that 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). Applicant argues that 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. Applicant argues that using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. Applicant argues that these range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level.

Applicant argues that traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with <50% homology. Applicant argues that we (the inventors) go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

Applicant argues that the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with *tfdA*. Applicant argues that there were great numbers of other possible choices that could have been selected.

Applicant argues that the subject invention offers surprising advantages that neither Jepson nor Kohler taught. Applicant argues that as illustrated by the subject specification, particularly by Table 1 and Table 2, mecoprop and dichlorprop have phenoxy auxin chemistries, while the aryloxyphenoxypropionates or "AOPPs" are in a different class of chemistries. Applicant argues that, thus, it is not seen how Kohler would support the reasonable expectation of success to produce a plant that is resistant to aryloxyphenoxypropionates and thus protecting a crop plant from damage by an aryloxyalkanoate herbicide (page 14 of the Remarks).

Applicant argues that the subject invention relates in part to the identification of an enzyme AAD-1 that is not only able to degrade 2,4-D, but also aryloxyphenoxypropionate (AOPP) herbicides, thus distinguishing the enzyme of the subject invention from previously known *tfdA* proteins. Applicant argues that no alpha-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Applicant argues that there was no reason to select the nucleic acid of SEQ ID NO: 9, or derivative thereof, to be operably linked to a plant specific promoter, as there was no reasonable expectation that such a construct would produce the desired effect when transfected into plant cells (page 15, 3rd paragraph of the Remarks).

Applicant's arguments have been fully considered but are not found to be persuasive. The examiner has withdrawn the instant rejection for instant claims 31, 35,

38 and 46 because Kohler, as asserted by Applicant, does not teach that the dioxygenase taught could be capable of degrading an AOPP herbicide. Kohler does specifically teach that the dioxygenase degrades the phenoxy auxin herbicides (R)-dichlorprop and (R)-mecoprop herbicides. The fact that Kohler did not recognize that the dioxygenase could also degrade AOPP herbicides does not mean that the use of the dioxygenase to degrade phenoxy auxin herbicides would not be obvious. Based on the teachings of Jepson *et al*, it would have been obvious to transform a plant cell with a recombinant polynucleotide that encodes a phenoxy auxin herbicide degrading enzyme, to produce a plant from said plant cell and to use the plant in a method to control at least one weed in a field by applying a phenoxy auxin herbicide. The fact that Applicant has recognized another advantage which would flow naturally from following the suggestion of the prior art cannot be the basis for patentability when the differences would otherwise be obvious. See *Ex parte Obiaya*, 227 USPQ 58, 60 (Bd. Pat. App. & Inter. 1985).

The limitations in instant claim 24, for example, do not actually structurally or functionally modify the claimed plant.

Applicant's argument that the subject AAD-1 enzyme was a choice of one of over 100 other possible genes is not persuasive because it would have been routine in the art at the time of Applicant's invention to screen multiple candidate coding sequences (obvious to try), and given the teachings of Kohler where the function had been confirmed there would have been an even greater motivation to try. Nothing in the prior art would suggest that there **would not have been** a reasonable expectation of success

in substituting the *tfdA* gene taught by Jepson *et al* with the gene taught by Kohler. The rejection is maintained for the reasons of record.

Claim Rejections - 35 USC § 102

15. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

16. Claim(s) 15, 16, 18-25, 28-31, 45, 47 and 48 remain rejected under pre-AIA 35 U.S.C. 102(a) and (e) as being anticipated by Ellis *et al* (US 2016/0108422 published 21 April 2016).

Ellis *et al* disclose polynucleotides that exhibit aryloxyalkanoate dioxygenase activity having at least 85% sequence identity with instant SEQ ID NO: 9 at SEQ ID Nos. 22, 25, 28, 31 and 34, for example, (claim 1). Ellis *et al* disclose a transgenic plant cell comprising said polynucleotides at claims 9-12. Ellis *et al* disclose a maize transgenic plant comprising said polynucleotides in Figure 1. Ellis *et al* discloses a method of controlling weeds using said transgenic plant using at least herbicide sleected from AOPP (aryloxyphenoxypropionate) herbicides, phenoxy acid herbicides and pyridinyloxy acid herbicides at claim 20. Hence, Ellis *et al* had previously disclosed the claim limitations.

Applicant argues that the present specification is substantively identical to that of PCT/US2005/014737. The early disclosure of PCT/US2005/014737 provides the sequence of SEQ ID NOs: 9, 10 and 11 and the formulation of the α-ketoglutarate-dependent dioxygenase motif. Applicant argues that as noted above those of ordinary skill would understand how to apply the α-ketoglutarate-dependent dioxygenase motif to the AAD-1 proteins sequences of SEQ ID NOs: 9, 10 and 11 to generate the AAD-1 motif listed in the current claims. Applicant argues that the current claimed invention is entitled to the priority date of May 2, 2005, and Ellis *et al* is not a proper prior art document.

This argument is not found to be persuasive. Each claim is given only one priority date. The Examiner has provided evidence that the instant claims have the priority of the filing date of the instant Application, that being 7 October 2016. Consequently, Ellis *et al* represent prior art under 35 U.S.C. 102(a) and (e).

17. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

18. Claim(s) 15, 16 and 18-48 remain rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Wright *et al* (EP 1 740 039 B1).

Wright *et al* disclose a transgenic plant cell comprising instant SEQ ID NO: 9 at claims 1-15 and 46-48, in addition to a second herbicide resistance gene at claims 10-15. Wright *et al* disclose a method of controlling weeds using a plant comprising said

transgenic plant cell at claims 16-45. Hence, Wright *et al* had previously disclosed the claimed invention.

Applicant argues that EP 1740039 is a regionalized application of PCT/US2005/014737. Accordingly, EP 1740039 represents the same application as the present application. Applicant argues that Wright et al (EP 1740039) is not a proper prior art reference to the present application.

This argument is not found to be persuasive. Each claim is given only one priority date. The Examiner has provided evidence that the instant claims have the priority of the filing date of the instant Application, that being 7 October 2016. Given the fact that Wright *et al* had previously disclosed a species of the instantly claimed invention and thus anticipate the instantly claimed genus.

Conclusion

19. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

20. No claims are allowed.

21. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

EXHIBIT 17

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 03/10/2020

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER	
KRUSE, DAVID H	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
03/10/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Office Action Summary	**Application No.** 15/288,406	**Applicant(s)** WRIGHT et al.	
	Examiner DAVID H KRUSE	**Art Unit** 1663	**AIA (FITF) Status** No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 10 January 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 15-16 and 18-49 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 15-16 and 18-49 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

STATUS OF THE APPLICATION

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 10 January 2020 has been entered.

2. The rejection under 35 U.S.C. 102(a) and (e) over Elliss *et al* and 102(b) over Wright *et al* are withdrawn in view of Applicant's arguments.

Double Patenting

3. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper time-wise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

4. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-43 of U.S. Patent No. 7,838,733. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the ‘733 Patent represents a

subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

5. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 9,127,289. Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '289 Patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

6. Claims 15, 16 and 18-48 remain and claim 49 is rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-13 of U.S. Patent No. 10,174,337 (which issued from US Patent Application 14/820,893). Although the claims at issue are not identical, they are not patentably distinct from each other because the genus claimed in the '337 patent represents a subgenus of the instantly claimed invention and thus both anticipates and renders obvious the instantly claimed invention.

This rejection has been modified because the Application has issued as a U.S. Patent. Applicant states that Applicant will file the necessary terminal disclaimer to overcome the rejection upon a finding of allowable subject matter (page 13, 2nd paragraph of the Remarks). Because the instant rejection is not provisional it is maintained for the reasons of record.

Claim Rejections - 35 USC § 112

7. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claims 15, 16 and 18-48 remain and claim 49 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant claims a transgenic plant cell, plant and method of using wherein the plant cell comprises a recombinant polynucleotides encoding an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity having at least 85% sequence identity

with instant SEQ ID NO: 9 and an AAD-1 motif of HX112D(X)114-137T(X)139-269H(X)271-280R. New claim 49 claims wherein said motif has 95% sequence identity with corresponding amino acid positions 11-281 of SEQ ID NO: 9.

Applicant describes the amino acid sequence of the AAD-1 (syn. RdpA) enzyme in instant SEQ ID NO: 9 and two variants thereof in instant SEQ ID Nos. 10 and 11 which are 98.8% and 99.7% identical respectively to said SEQ ID NO: 9.

Applicant does not describe the claimed genus of AAD-1 proteins or the AAD-1 motif (in the specification). The specification describes a motif comprising three conserved histidine residues found in α-ketoglutarate-dependent dioxygenases on page 17, paragraph 0085, $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed.

Applicant argues that one skilled in the art would recognize the much more limited group having the desired functional dual-MOA activity would be defined by the more refined structure of [the] AAD-1 motif. Applicant argues that the comparison of the AAD-1 enzymes and AAD-2 reveal while the two proteins retain all five active site residues, there is a difference in the 131 amino acid ($(X)_{139-269}$) interval in the AAD-1 motif $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ vs the spacing in AAD-2 which is a 2,4-D-degrading Fe/α-KG dioxygenase that cannot also catalyze (R)-AOPP herbicides. Applicant argues that those skilled in the art would have compared and recognized the importance of the sequence differences between AAD-1 variants [(R)-specific dioxygenase] having dual herbicide MOA Fe/α-KG dioxygenase activity and (S)-specific

dioxygenases not having dual herbicide MOA Fe/α-KG dioxygenase activity..." (page 16, 2nd paragraph of the Remarks).A

Applicant argues that support for a claimed genus is not limited to information contained within the specification but also includes knowledge of protein structure, such as consensus sequences, that are well-known to those of skill in the art (page 16, 3rd paragraph of the Remarks). Applicant argues that the AAD-1 proteins of the present invention bind to the same phenoxy auxin herbicide substrates as the tfdA protein and presumably retain similar structural domains as the homologous tfdA proteins (page 17, 1st paragraph of the Remarks).

Applicant argues that the skilled practitioner would know not to make modifications to amino acids that are conserved, and thus applicant's required functional limitations effectively add these structural elements to the claimed genus of proteins. Applicant argues that this information, in combination with applicant's description of the AAD-1 motif (defining the key structural elements of the enzyme active site), provides a detailed description of the structural elements of the claimed genus members (page 18, 1st paragraph of the Remarks). Applicant argues that one skilled in the art appreciates that regions of conservation between homologous proteins suggests a structure/function role for such sequences (page 18, 3rd paragraph of the Remarks).

Applicant argues that the motif disclosed in paragraph [0085] of the specification highlights important amino acids comprising the active site of a-ketoglutarate-dependent dioxygenases. Applicant argues that one of ordinary skill in the art would know that the motif provided in paragraph [0085] provides a roadmap to identify the corresponding positions in the AAD-1 proteins of SEQ ID NOs: 9, 10 and 11 thus identifying the

specific "AAD-1 motif" of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$. Applicant argues that this motif provides structural elements of the active site of the unique AAD-1 proteins of the present invention that enzymatically degrade phenoxy auxin herbicides and (R)-aryloxyphenoxypropionate herbicides (page 20, 2nd paragraph of the Remarks).

Applicant argues that the difference of the 131 amino acid spacing $(X)_{139-269}$ in the AAD-1 motif) becomes evident with alignment of R vs S-type αKG-dioxygenases that have activity on 2,4-D; as the R-AOPP activity has only been seen where the 131 amino acid spacing exists (page 21, 2nd paragraph of the Remarks). Applicant argues that the fact pattern of Example 11B (claim 2) of the 2008 written description guidelines is closely aligned with the claimed subject matter of the present invention (page 22, 3rd paragraph of the Remarks).

Applicant's arguments have been considered but are not found to be persuasive. Applicant never specifically identifies the 131 amino acid spacing $(X)_{139-269}$ in the AAD-1 motif, or what structural feature therein defines the R-AOPP activity. While the prior art recognized a structure function relationship for the α-ketoglutarate-dependent dioxygenases motif $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$, the prior art does not describe a structure function relationship for specific enzymatic activity without actual sequence information. The α-ketoglutarate-dependent dioxygenases define a large family of enzymes with diverse enzymatic activities. Further, applicant does not claim conservative substitutions as exemplified in the 2008 WD guidelines. The decision in *AbbVie Deutschland GmbH & Co. v. Janssen Biotech, Inc.* 111 USPQ2d 1780 (Fed. Cir. 2014) seems to be germane to the instant case. In *Abbvie*, an analogy is drawn between a claimed genus and a plot of land (see pages 1789-1791). Using this analogy,

the Court offered that "[I]f the disclosed species only abide in a corner of the genus, one has not described the genus sufficiently to show that the inventor invented, or had possession of, the genus. He only described a portion of it." Similar to the analogy drawn in *Abbvie*, in the case of *Ariad Pharmaceuticals, Inc. v. Eli Lilly & Co.,* 94 USPQ2d 1161 (Fed. Cir. 2010), the court (at page 1171) offered: [M]erely drawing a fence around the outer limits of purposed genus is not an adequate substitute for describing a variety of materials constituting the genus and showing that one has invented a genus and not just a species. Applicant has only described a very limited "corner" of the claimed genus.

Claim Rejections - 35 USC § 103

9. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 15, 16 18-30, 32-34, 36, 37, 39-45, 47 and 48 remain and claim 49 is rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jepson *et al* (U.S. Patent Application US 2003/0041357 filed on 23 February 2001) in view of Kohler (Genbank Accession No: Q8KSC8 first published on 1 October 2002).

Jepson *et al* teach a method of controlling at least one weed in an area by applying a phenoxy auxin herbicide said area comprising a transgenic plant comprising a recombinant polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity (see paragraph 0027 on pages 3-4, especially lines 18-24 on page 4). Jepson *et*

al teaches one of skill in the art can use mutagenized or modified variants thereof (page 2, paragraph 0012). Jepson *et al* teach said method using a plant transformed with an additional transgene that provides glyphosate, glufosinate, imidazolinone or sulfonylurea herbicide tolerance (page 4, left column, lines 18-27). Jepson *et al* teach applying the phenoxy auxin herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Jepson *et al* teach said method can be practiced using turf grass or forage grass (page 3, paragraph 0024, last sentence; claim 15). Jepson *et al* teach said method can be practiced using a plant transformed with a transgene that produces tolerance to a pre-emergence herbicide (claim 12).

Jepson *et al* do not teach a transgenic plant comprising a recombinant polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9.

Kohler teaches a polynucleotide that encodes a protein comprising the amino acid sequence of instant SEQ ID NO: 9. Kohler teaches that said protein has R-2,4-dichlorophenoxypropionate dioxygenase activity (i.e. degrades the phenoxy auxin herbicide (R)-dichlorprop).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicant's invention to modify the teachings of Jepson *et al* to substitute the *tfdA* herbicide tolerance gene for a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase taught by Kohler. It would have been obvious to combine a gene encoding the R-2,4-dichlorophenoxypropionate dioxygenase with another gene for tolerance to additional herbicide tolerances as taught by Jepson *et al*. Jepson *et al* teach that one would apply one (claim 16) or two (claim 17) herbicides in the method of controlling

weeds. Jepson *et al* teach that at the time of Applicant's invention it was desirable to produce plants tolerant to a phenoxy auxin herbicide. Given the high level of skill in the instant art at the time of Applicant's invention, one would have had a reasonable expectation of success.

Applicant argues that the Examiner fails to provide any rational for why one of ordinary skill would specifically select a gene encoding a peptide of SEQ ID NO; 9 to substitute for the *tfdA* gene disclosed in Jepson (page 24, 4th paragraph of the Remarks). Applicant's argument is not found persuasive because substitution of equivalents having the same or similar function would have been obvious to one of ordinary skill in the art at the time of Applicant's invention. The Examiner expressed that the reason would be for herbicide tolerance of similar function.

Applicant argues that it cannot be reasonably said that one would have turned to the specific gene disclosed in Kohler with the expectation that it would be expressed in plants, particularly to convey herbicide tolerance to the plants (page 25, 1st paragraph of the Remarks). Applicant's argument is not found persuasive because there is no requirement for an absolute expectation of success, just a reasonable expectation. Jepson *et al* had taught that a bacterial R-2,4-dichlorophenoxypropionate dioxygenase enzymes can be used to produce a transgenic plant resistant to the herbicide 2,4-D.

Applicant argues that not all known α-ketoglutarate-dependent dioxygenases exhibit the ability to degrade phenoxy auxin herbicides when inserted into plant cells (page 25, 3rd paragraph of the Remarks). Applicant argues that there was no teaching provided by the prior art to indicate that the rdpA bacterial gene would provide tolerance to phenoxy auxin herbicides upon introduction into plant cells (page 25, 5th paragraph of

the Remarks). Applicant's arguments are not found persuasive because Applicant is arguing that the claims are not anticipated by the prior art, the instant rejection is directed to an issue of obviousness. Many of the genes used to produce herbicide tolerant transgenic plants at the time of Applicant's invention encode bacterial enzymes, including the *tfdA* taught by Jepson *et al*. Further, given the generally high level of skill in the art at the time of Applicant's invention it would have been obvious to try genes encoding other bacterial genes that degrade herbicides of agricultural importance such as 2,4-D.

Applicant argues that Applicant's experience expressing the AAD-2 enzyme in a plant not providing herbicide tolerance is evidence of unpredictability (page 26 of the Remarks). This argument is not persuasive because it does not provide evidence that it would not have been obvious to try the enzyme taught by Kohler, just that there would not have been an **absolute** expectation of success, just that one of ordinary skill in the art would have had to **try** by making the transgenic plant.

Applicant argues that the subject AAD-1 enzyme was only 1 of over 100 BLAST search results having some degree of identity with tfdA (page 27 of the Remark). This argument is not found persuasive because transforming plant with transgenes encoding 100 potential enzymes would have been routine in the art at the time of Applicant's invention, and further Kohler had taught a function that would be useful for producing a transgenic plant as suggested by Jepson *et al*.

Conclusion

11. No claims are allowed.

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

EXHIBIT 18

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/288,406	10/07/2016	Terry WRIGHT	14764-258662	5402

107540 7590 08/31/2020
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

EXAMINER	
KRUSE, DAVID H	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
08/31/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Applicant-Initiated Interview Summary	**Application No.** 15/288,406	**Applicant(s)** WRIGHT et al.	
	Examiner DAVID H KRUSE	**Art Unit** 1663	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) DAVID H. KRUSE.

(2) John Breen (attomey).

(3) Terry Wright (inventor).

(4) Bill Poirier (company rep).

Date of Interview: 26 August 2020.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☑ Yes ☐ No.
If Yes, brief description: Graph of enzymatic activity comparison of variants of the AAD1 enzyme (see attached).

Issues Discussed ☐101 ☑112 ☐102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 15.

Identification of prior art discussed: Hotopp 2002.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant presented the attached exhibit demonstrating that the ADD1 enzyme could be modified down to 82% identical to instant SEQ ID NO: 9 and retain dioxygenase activity in the presence of both 2,4-D and R-quizalofop. The Inventor expressed that the illustrated variants have multiple modifications throughout the polypeptide sequence (but not the specific amino acids in the recited AAD-1 motif) and that the variants maintained the recited activity in instant claim 15 (for example). The Inventor asserted that the critical structural feature of a-ketoglutarate-dependent dioxygenases were well understood at the time of the invention (Hotopp 2002) and thus the Specification had adequately described the claimed genus in instant claim 15..

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☑ Attachment

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

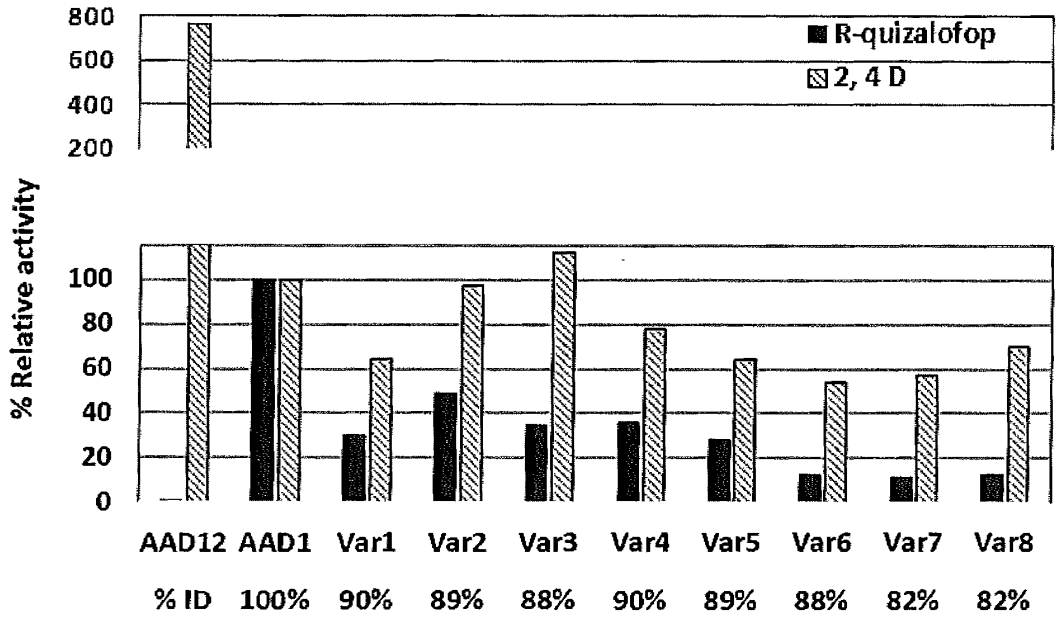
08-25-20;14:59 ;From: To:15712730799 ;4342202866 # 2/ 2

For Discussion Purposes Only

For purposes of discussion during an Examiner interview on August 26, 2020 at 10 AM

[redacted]

Dual MOA retained for variants with AAD-1 motif



PAGE 2/2 * RCVD AT 8/25/2020 3:44:20 PM [Eastern Daylight Time] * SVR:IW-PTOFAX-001/19 * DNIS:2730799 * CSID:4342202866 * ANI:4342202866 * DURATION (mm-ss):00-46