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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the <u>1</u> separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
Novel Herbicide Resistance Genes					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
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ENCLOSED APPLICATION PARTS (check all that apply)					
☒ Specification Number of Pages <u>104</u>		☐ CD(s), Number _____			
☒ Drawing(s) Number of Sheets <u>13</u>		☒ Other (specify) Tables (16 pages) Sequences (5 pages)			
☐ Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
☐ Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE	
☐ A check or money order is enclosed to cover the filing fees.				AMOUNT (\$)	
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
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☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

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Date April 30, 2004REGISTRATION NO. 39,355

(if appropriate)

Docket Number: DAS-115P**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

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Inari Exhibit 1004
Inari v. Corteva

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Additional Page

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Docket Number

DAS-115P

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[Page 2 of 2]

Number 2 of 2

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : DAS-115P
Applicant(s) : Terry Wright, Justin Lira, Donald Merlo
For : Novel Herbicide Resistance Genes

Mail Stop PROVISIONAL PATENT APPLICATION
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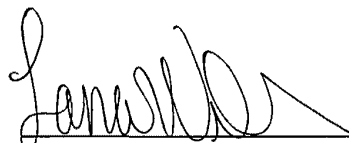
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NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

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

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

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.*, wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

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

[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata

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

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agrilience, 2003).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is an auxin derivative that

acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

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

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

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

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are many homologues that have a significantly lower homology to *tfdA* (25-50%), yet have the characteristic residues associated with α -ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (28% amino acid identity) is *rdpA* from *Sphingobium herbicidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes, or transformation of this gene into plants, has not yet been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids.

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

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridinyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and dichlofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject

invention also includes plants that produce one or more enzymes of the subject invention “stacked” together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidizolanone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] A preferred embodiment of the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (e.g., glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, ACCases, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise (without the *AAD-1* gene) be sensitive to that herbicide (such as 2,4-D).

[0017] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

[0018] There was no prior motivation to produce plants comprising an *AAD-1* gene (preferably an *AAD-1* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an

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AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxypionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an *AAD-1* gene. The subject invention also provides methods of using an *AAD-1* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-1*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of a herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

[0021] **Figure 2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

[0022] **Figure 3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

[0023] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0024] **Figure 5** shows 2,4-dichlorophenol production by recombinant AAD-1.

[0025] **Figure 6** shows phenol production by recombinant AAD-1 from various herbicide substrates.

[0026] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

[0027] **Figure 8** shows dose response of callused maize suspensions to R-haloxyfop.

[0028] **Figure 9** is a bar graph of the responses of *AAD-1* (v3)-transformed and non-transformed corn callus to R-haloxyfop.

[0029] **Figure 10** shows the responses of several *AAD-1* (v3)-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

[0030] **Figure 11** shows fitted curves for 2,4-D degradation rates of AAD-2 vs. AAD-1 (v1).

[0031] **Figure 12** shows dose responses of *AAD-1* v3 (plant optimized), *AAD-1* v2 (native), or *AAD-2* (native)-transformed T₁ *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T₁ family. The relative protective capability from 2,4-D injury of individual genes is plant optimized *AAD-1* (v3) > native *AAD-1* (v2) > native *AAD-2*.

[0032] **Figure 13** shows western blot analysis of individual native *AAD-2*-transformed T₁ *Arabidopsis* plants. This shows that plants expressing the AAD-2 protein are suffering severe injury from 200 g ae/ha 2,4-D treatments, which normally causes little injury to native *AAD-1* (v2) or plant optimized *AAD-1* (v3)-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in *AAD-2*-transformed and *Pat/Cry1F*-transformed samples.

Brief Description of the Sequences

[0033] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA/AAD-1* (v1) gene.

[0034] **SEQ ID NO:2** is the sequence of a reverse primer used to amplify the *rdpA/AAD-1* (v1) gene.

[0035] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1* (v1) from *Sphingobium herbicidovorans*.

[0036] **SEQ ID NO:4** is the nucleic acid sequence of the native *AAD-1* gene with internal NotI restriction site removed. This gene is designated *AAD-1* (v2). DNA sequencing confirmed that the correct PCR product was generated. But an inadvertent change was made at amino acid #212 from arginine to cysteine.

[0037] **SEQ ID NO:5** is a “plant-optimized” DNA sequence (“*AAD-1 (v3)*”). This “gene” encodes essentially the identical protein (disclosed herein as SEQ ID NO. 10) as the native gene (disclosed herein as SEQ ID NO. 9), except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

[0038] **SEQ ID NO: 6** (“rdpA(ncoI)”) and **SEQ ID NO:7** (“3’ sacI”) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[0039] **SEQ ID NO:8** is another PCR primer (“BstEII/ Del NotI”) that was used with the “3’ SacI” primer.

[0040] **SEQ ID NO:9** is the native amino acid sequence encoded by the *AAD-1 (v1)* gene from *Sphingobium herbicidovorans*.

[0041] **SEQ ID NO:10** is the amino acid sequence encoded by the *AAD-1 (v2)* DNA sequence of SEQ ID NO:4.

[0042] **SEQ ID NO:11** is the amino acid sequence encoded by the *AAD-1 (v3)* plant-optimized DNA sequence of SEQ ID NO:5.

[0043] **SEQ ID NO:12** is the DNA sequence of the native *AAD-2* gene.

[0044] **SEQ ID NO:13** is the amino acid sequence of the *AAD-2* protein.

[0045] **SEQ ID NO:14** is a forward primer used to amplify *AAD-2* DNA for cloning.

[0046] **SEQ ID NO:15** is a reverse primer used to amplify *AAD-2* DNA for cloning.

[0047] **SEQ ID NO:16** is the M13 forward primer.

[0048] **SEQ ID NO:17** is the M13 reverse primer.

[0049] **SEQ ID NO:18** is a forward primer used to amplify *AAD-2* DNA for cloning.

[0050] **SEQ ID NO:19** is a reverse primer used to amplify *AAD-2* DNA for cloning.

Detailed Description of the Invention

[0051] Development of a 2,4-D resistance gene and subsequent resistant crops could provide an excellent opportunity to control broadleaf glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be

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

[0052] An additional benefit of the *AAD-1* gene is its unprecedented ability to concomitantly degrade a whole host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). *See Table 2.* This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing *AAD-1* where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

[0053] A single gene (*AAD-1*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-1* can provide protection *in planta* to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing *AAD-1* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae (acid equivalent)/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds.

Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combination of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

[0054] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has and will continue to select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-1* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides; or, broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and AOPP herbicide(s) (e.g., quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0055] It is important to have flexibility in controlling a broad spectra of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-1* stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The

optimal combination(s) and timing of these application(s) will be situation, species, and environmentally-dependent and best made by a person skilled in the art of weed control.

[0056] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0057] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glufosinate tolerance (*e.g.*, *Pat*, *bar*), ALS-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, and other chemistries = *AHAS*, *Csr1*, *SurA*, *et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD enzyme, resistance to inhibitors of phytoene desaturase, resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to PROTOX-inhibiting herbicides (*e.g.*, AcuronTM), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0058] Additionally, *AAD-1* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits to provide a complete agronomic package of improved crop quality with the ability to cost effectively and with flexibility control any number of agronomic pests.

[0059] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α -ketoglutarate-dependent dioxygenases. This family of proteins is

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characterized by three conserved histidine residues and in a “HX(D/E)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R” motif which comprises the active site. The histidine coordinate Fe⁺² ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes.

[0060] More specifically, the subject invention relates to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0061] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

[0062] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol (“DCP”; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100% conversion) *in vitro*. An additional advantage that *AAD-1* plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0063] The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an *AAD-1* gene.

[0064] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art. This highly novel discovery is the basis of significant HTC trait opportunities.

[0065] The exemplified genes and proteins, and future homologues discovered because of the benefit of the subject disclosure, will provide great benefit to the agricultural community. Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0066] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. See U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α -ketoglutarate dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α -ketoglutarate dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0067] *RdpA*, from *Sphingobium herbicidovorans* (Westendorf *et al.*, 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R vs. S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that *rdpA* or *AAD-1* genes could be successfully expressed in

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plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0068] As described in more detail in the Examples below, *rdpA* was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α -ketoglutarate dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α -ketoglutaratedependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. *RdpA*' has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs.

[0069] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

[0070] The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

[0071] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0072] *AAD-1*, and future homologues discovered because of the benefit of the subject disclosure, can provide great benefit to the agricultural community. The subject enzymes allow transgenic

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

[0073] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis* and corn have been transformed with *AAD-1/PAT* constructs and have demonstrated resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to “plant optimized” genes that encode proteins of the subject invention. As shown below in Example 5, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[0074] The genes of the subject invention can be used to delivery a transgenic trait that imparts 2,4-D (or other aryloxyalkanoate auxinic herbicides) tolerance to plants. The results disclosed herein demonstrate the ability of AAD-1 to degrade representative AOPP herbicides, and specifically the herbicidally active R-enantiomer. Practicing aspects of the subject invention provides an opportunity to impart grass selectivity to the graminicidal AOPP herbicides. This concept is also unique in that resistance to multiple herbicide modes of action can be imparted by the same gene or by genes encoding enzymes with a similar mechanism.

[0075] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by “acid trapping,” a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0076] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cultures, greenhouses, and in the field. In addition to HTC traits, methods for controlling weeds using

herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, is within the scope of the invention. Likewise, combination of said HTC trait is useful when combined with other HTC traits (including but not limited to glyphosate tolerance) for the purpose of controlling weed (and like) species with newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate).

[0077] Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

[0078] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0079] The transgenic expression of the subject *AAD-1* genes are exemplified in *Arabidopsis* and corn (maize). However, the subject invention can be applied to any other desired types of plants, such as tobacco, cotton, canola, and soybeans. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

[0080] The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes. In fact, there is high inherent value for the subject genes simply as a selectable marker for biotechnology projects.

[0081] Perhaps more importantly is the presently demonstrated activity on AOPP herbicides. This provides a tool for imparting grass selectivity to AOPP herbicides where there previously was none.

[0082] Still further, the subject invention provides a single gene that can provide resistance to broadleaf weed herbicides (auxins) and grass weed herbicides (AOPPs). This gene may be utilized

in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology. Examples include oxyalkanoate herbicides with mode of action as inhibitors of protoporphyrinogen IX oxidase, or herbicides with other modes of action.

[0083] One gene of the subject invention, now coined *AAD-1* (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli*. When this gene is overexpressed (by induction of 1mM IPTG and culture lysate combined with the following reaction mix: 112.5ug/ml 2,4-D, 1mM Ascorbic acid, 1mM α -ketoglutarate, 50uM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$), the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

[0084] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By “functional activity” (or “active”) it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce “an effective amount” of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002, for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations, or, because of the subject invention, the level and/or concentration can be higher than that which was previously used. Preferably, plant cells and plants of the subject invention are protected against growth inhibition or

injury caused by herbicide treatment. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

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

[0086] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0087] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0088] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated,

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*, *Rodoferrax*, and *Comamonas* for example, can be used as source isolates.

[0089] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0090] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0091] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

[0092] Polynucleotides and probes. The subject invention further provides nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0093] The polynucleotides of the subject invention can be used to form complete “genes” to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-1* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[0094] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The “coding strand” is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the “anti-sense” strand of DNA. The “sense” or “coding” strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0095] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention

and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[0096] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal proteins of the subject invention.

[0097] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

[0098] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) DNA Probes, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as

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would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example).

The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0099] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[00100] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[00101] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ³²P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25°C below the melting temperature (T_m) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983:

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

[00102] Washes can typically be carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).
- (2) Once at $T_m - 20^\circ \text{C}$ for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[00103] For oligonucleotide probes, hybridization can be carried out overnight at $10-20^\circ \text{C}$ below the melting temperature (T_m) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T_m for oligonucleotide probes can be determined by the following formula:

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

(Suggs *et al.*, 1981).

[00104] Washes can typically be out as follows:

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

[00105] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

- | | |
|-----------|--------------------------------------|
| Low: | 1 or 2x SSPE, room temperature |
| Low: | 1 or 2x SSPE, 42°C |
| Moderate: | 0.2x or 1x SSPE, 65°C |
| High: | 0.1x SSPE, 65°C . |

[00106] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

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[00107] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al, 1985. PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[00108] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[00109] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.

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

[00110] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene “shuffling,” which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[00111] “Shuffling” can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, “focused shuffling” can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[00112] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these “gene shuffling” techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

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14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[00113] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal31* or site-

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directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[00114] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By “truncated protein” it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* 1985. As used herein, the term “protein” can include functionally active truncations.

[00115] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[00116] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity

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ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[00117] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, , modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[00118] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 5** provides a listing of examples of amino acids belonging to each class.

Table 5	
Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[00119] In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the functional/biological activity of the protein.

[00120] As used herein, reference to “isolated” polynucleotides and/or “purified” proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to “isolated” and/or “purified” signifies the involvement of the “hand of man” as described herein. For example, a bacterial “gene” of the subject invention put into a plant for expression is an “isolated polynucleotide.” Likewise, a protein derived from a bacterial protein and produced by a plant is an “isolated protein.”

[00121] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled “Optimization of sequence for expression in plants.”

[00122] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species.

[00123] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, and the like.

[00124] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant,

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transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00125] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00126] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00127] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in United States Patent No. 5,135,867.

[00128] Vectors comprising an *AAD-1* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or

other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema,(1985); Fraley *et al.*, (1986); An *et al.* (1985).

[00129] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters , 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00130] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00131] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00132] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00133] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Pat. No. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500 all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other

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transformation technology includes whiskers technology. See U.S. Pat. Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. Nos. 5,569,597 to Mycogen Plant Science and Ciba-Giegy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00134] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00135] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker

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employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00136] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00137] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00138] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonyleureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00139] In addition to a selectable marker, it may be desirable to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00140] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin,

CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00141] Promoter regulatory elements may also be active during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00142] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. Nos. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00143] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00144] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - PCR and cloning of gene into pET

[00145] A single gene (*rdpA*) was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession # AF516752) as a homologue with only 28% amino acid identity to *tfdA* from *Ralstonia eutropha*. Percent identity was determined by first translating both the *rdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00146] The strain of *Sphingobium herbicidovorans* containing the *rdpA* gene was obtained from ATCC (American Type Culture Collection strain# 700291). The lyophilized strain was revived according to ATCC protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days.

[00147] A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28°C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat.# 69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

[00148] Twenty microliter reactions were set up as follows: MasterMix 8 µl, ea. primer 1 µl (50 pmoles/µl), gDNA 2.5 µl, H₂O 7.5 µl. PCR was then carried out under the following conditions: 94°C 45 sec, 52°C 1.5 minute, 72°C 1.5 minute, for 30 cycles, followed by a final cycle of 72°C 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1kb PCR product was cloned into pCR 2.1 (Invitrogen cat.# K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00149] Ten of the resulting white colonies were picked into 4 ml Luria Broth + 50 µg/ml Kanamycin (LB K), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. # A1460) and following included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. # 608120) using M13

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Forward (5' GTA AAA CGA CGG CCA GT 3') and Reverse (5' CAG GAA ACA GCT ATG AC 3') primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein was given a new general designation for internal consistency *AAD-1* (*v1*) (AryloxyAlkanoate Dioxygenase).

[00150] Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) *AAD-1* (*v1*) was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 µg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between *AAD-1* (*v1*):pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37°C for 4 hours with agitation.

[00151] Each culture was then spotted onto LB K plates, which were incubated at 37°C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2 – Expression and testing

2.1 – HPLC analysis.

[00152] Plasmid pDAB 3203 was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat.# PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat.# C6010-03) following manufacturer's protocol. After transformation, 50 µl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

[00153] The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37°C/200 rpm for 1 hr. Gene expression was then induced with 1mM IPTG, and incubated for 4 hrs at 30°C/200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was

pipetted off and stored at 4°C. To check for recombinant expression, a 20 µl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat.# EC60255).

[00154] After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat.# 17-0435-01). This was then used for subsequent herbicide enzyme reactions.

[00155] For each reaction, the following were combined: 2,4-D (125 µg/ml), [Ascorbate (1 mM), Ferrous ion (50 µM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 µl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 µl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat.# 11-131-6), eluting with 400 µl of Acetonitrile + 0.05% TFA.

[00156] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10µ C18(2) 250 x 4.6mm (Phenomenex cat.# 00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H₂O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[00157] To evaluate AAD-1 (v1) for degradation of any other herbicide for utilization as a substrate , the same conditions as above were used except that the 2,4-D in the assay was substituted with an appropriate amount of the other herbicide. Concentrations of these herbicides in the assay were dependent on its relative activity in the plate bioassay. Most were tested at 500 µg/ml.

2.2 – Plate test bioassays for herbicide degradation.

[00158] Plant bioassays were used to determine if *in vitro* enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (*i.e.*, monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis thaliana* var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

[00159] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 µL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @

250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

[00160] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO₃, 2.5 mM KH₂PO₄, 50 mM FeSO₄, 10 mM NaEDTA (pH 8.0), 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 70 μM H₃BO₃, 14 μM MnCl₂, 0.5 μM CuSO₄, 1 μM ZnSO₄, 0.2 μM NaMoO₄·2H₂O, 10 μM NaCl, 10 nM CoCl₂·H₂O, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR₅₀ rate (50% growth reduction) approximately in the center of the range.

[00161] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day⁻¹, 100 μE/m²s⁻¹, 23°C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR₅₀ determined.

[00162] This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. **Tables 6 and 7** represent herbicidal substrates tested.

2.3 – HPLC results.

[00163] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme from being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

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[00164] Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (**Figure 2**). Regardless of the specific conditions of the assay, (*i.e.*, longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

[00165] After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (*i.e.*, oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the 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).

[00166] ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (**Figure 3**) and diclofop. These results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

2.4 – Plate test bioassays for herbicide degradation.

[00167] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR₅₀ for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio

of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR₅₀ could not be determined following enzyme treatment; *de facto*, no detectable herbicide activity remained.

[00168] The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (**Figure 3 & Table 7**). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3 – Testing with colorimetric assay

3.1 – AAD-1 (v1) Assay.

[00169] AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) to enable deployment in a 96-well microplate format. The subject invention includes the use of *AAD-1* in detection assays such as these. Assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μ M NH₄FeSO₄, 200 μ M sodium ascorbate, 1 mM α -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α -ketoglutarate at time zero. After 15 minutes of incubation at 25°C, the reaction was terminated by addition of 10 μ l 100 mM sodium EDTA. Color was developed by addition of 15 μ l pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH), 1.5 μ l 2% 4-aminoantipyrine and 1.5 μ l 8% potassium ferricyanide. After 10 to 20 minute, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μ l of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2 % 4-aminoantipyrine, then adding 10 μ l 0.8 % potassium ferricyanide.

3.2 – Phenol Detection.

[00170] The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the phenol product, 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides (**Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μ M in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μ M NH_4FeSO_4 , 200 μ M sodium ascorbate. The absorbance is shown in **Table 8**.

[00171] The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so are readily detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. Thiophenols were also not detected. Some phenols equivalent to those found in certain protoporphyrinogen IX oxidase inhibitors produced faint pink colors of significantly less intensity than the 2,4-dichlorophenol. Some phenols produced entirely different colors with differing λ_{max} values (2-Cl,4-F, 5-nitrophenol, yellow, 420 nm; 6-hydroxy-2,4-D, blue, 580 nm; 3-amino-2,4-dichlorophenol, orange, 480 nm).

[00172] Further experiments showed that color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to $\sim$ 500 μ M. A calibration curve performed under standard assay conditions (160 μ l final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μ M phenol.

[00173]

3.3 – Extraction from *Escherichia coli*.

[00174] *E. coli* cell pellets were resuspended in 0.1M Tris, pH 7.4 + 1mg/ml lysozyme (5 ml/ cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and MgCl_2 to 1mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at -70°C . Assays were either performed with these unpurified desalted extracts or with purified enzymes.

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

[00175] A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding *AAD-1 (v1)* was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without *AAD-1 (v1)* using 1 mM 2,4-D and is shown in **Figure 5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

[00176] The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 µl of the *E. coli* extract per assay with a 15 min assay period. **Figure 6A** shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop = mecoprop > diclofop > haloxyfop > 2,4-D. Thus AAD-1 (v1) has activity on AOPPs as well as phenoxy auxins.

[00177] The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in **Figure 6B** as well. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

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

4.1--Expanded substrates

[00178] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 µg per 160 µl assay and each substrate was tested at 1 mM with an assay time of 15 minutes.

[00179] **Table 3** shows the A₅₁₀ detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced

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small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 µg) of AAD-1 (v1).

[00180] From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates (X188476 and X398166, sesone). **Table 4** shows similar data for a variety of AOPP graminicide herbicides, and also the safener cloquintocet (albeit to a lesser extent). All the commercial AOPP herbicides tested were cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins.

[00181] AAD-1 (v1) activity on quizalofop was the greatest (76% of the dichlorprop rate) and cyhalofop the least (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 µg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Very little, if any, activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, little activity was noted against the quinolinoxycetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

4.2—Catalytic characterization

[00182] The K_m and k_{cat} values of purified AAD-1 (v1) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 µM Na ascorbate; 200 µM Fe²⁺; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 – 125 µM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in Tables 4 and 5, as these were performed at high (saturating) substrate concentrations (1 mM).

[00183] The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf *et al.* (2003). The

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published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

4.3 – Additional substrates for AAD1.

[00184] Three additional substrates were tested at 1 mM using 27 µg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 10**. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >700 fold better than 2,4-D as a substrate (**Table 8**). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

Example 5 – Optimization of Sequence for Expression in Plants

5.1 – Background.

[00185] To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00186] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small

nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00187] **Table 11** illustrates how high the G+C content is in maize. For the data in **Table 11**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelrys, San Diego, California). Intron sequences were ignored in the calculations.

[00188] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This “codon bias” is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of “minor” codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00189] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 12**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein,

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but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

[00190] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00191] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

5.2 – Rebuild analysis.

[00192] Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native *AAD-1* (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a “plant-optimized” DNA sequence (SEQ ID NO:5) was developed that encodes essentially the identical protein (disclosed herein as SEQ ID NO:11) as the native gene (disclosed herein as SEQ ID NO:9), except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and **Table 13** shows the differences in codon compositions the native and plant-optimized sequences.

5.3 – Completion of binary vectors.

[00193] 5.3.1 – Rebuilt *AAD-1* (v3). The plant optimized gene *AAD-1* (v3) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward and M13 Reverse primers using the Beckman Coulter “Dye Terminator Cycle Sequencing with Quick Start Kit” reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-1* (v3) DNA sequence. The *AAD-1* (v3) gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB720, containing: [AtUbi10 promoter: Nt OSM 5’UTR: *AAD-1* (v3): Nt OSM3’UTR: ORF1 polyA 3’UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5’UTR: *AAD-1* (v3): Nt OSM 3’UTR: ORF1 polyA 3’UTR: CsVMV promoter: *PAT*: ORF25/26 3’UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, Hind III, Pac I, and Xmn I) for verification of

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the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[00194] 5.3.2 – Native *AAD-1* (v2). The *AAD-1* (v1) gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[00195] The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

[00196] Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of *AAD-1* (v1). This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/ Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" were used.

[00197] A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation *AAD-1* (v2) (SEQ ID NO:4).

[00198] A sequencing reaction using the M13 Reverse primer and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 *AAD-1* (v2) construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

[00199] The modified *AAD-1* (v2) gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct

was then cloned into the binary pDAB3038 as a NotI - NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1* (v2): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

Example 6 – Transformation into *Arabidopsis* and Selection

6.1 – *Arabidopsis thaliana* Growth Conditions

[00200] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00201] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00202] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu\text{mol}/\text{m}^2\text{sec}$ under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

[00203] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) containing a streaked DH5 α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37°C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00204] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and

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Glazebrook (2002). 50 µl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00205] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00206] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10µl of water, at 100°C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94°C for 3 minutes 2) 94°C for 45 seconds 3) 55°C for 30 seconds 4) 72°C for 1 minute, for 29 cycles then 1 cycle of 72°C for 10 minutes. The reaction was maintained at 4°C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

6.3 – *Arabidopsis* transformation.

[00207] *Arabidopsis* was transformed using the floral-dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28°C with constant agitation at

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220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28°C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044µM benzylamino purine (10 µl/liter of 1 mg/ml stock in DMSO) and 300 µl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22°C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

6.4 – Selection of transformed plants.

[00208] Freshly harvested T₁ seed [transformed with native [*AAD-1* (v2)] or plant optimized [*AAD-1* (v3)] gene] was allowed to dry for 7 days at room temperature. T₁ seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T₁ seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4°C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00209] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00210] Five to six days after planting (DAP) and again 10 DAP, T₁ plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted

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 $\mu\text{E}/\text{m}^2\text{s}^{-1}$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1* ($\nu 3$) (plant optimized gene) or *AAD-1* ($\nu 2$) (native microbial gene) to provide phenoxy auxin herbicide resistance.

[00211] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR₉₉ for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[00212] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

[00213] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta*.

[00214] Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1* ($\nu 3$)-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and

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death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above. Injury ratings were taken 7 and 14 days after treatment. Likewise, T₁ individuals were treated with the pyridinyloxyacetate auxin herbicide, fluroxypyr.

6.5 – Results of selection of transformed plants.

[00215] The first *Arabidopsis* transformations were conducted using *AAD-1* (v3) (plant optimized gene). T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T₁ seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see **Table 14** below in Example 15). A small lot of *AAD-1* (v2)(native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T₁ individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

[00216] T₁ plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 15** compares the response of native *AAD-1* (v2) and plant optimized *AAD-1* (v3) genes to impart 2,4-D resistance to *Arabidopsis* T₁ transformants. Both genes imparted resistance to individual T₁ *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 15** simply to demonstrate the significant difference between the plants transformed with *AAD-1* (v2) or *AAD-1* (v3) versus the wildtype or *PAT/CryIF* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1* (v3) versus the native sequence *AAD-1* (v2) (see **Table 15**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T₁ individuals expressing *AAD-1* (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these

elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00217] **Table 16** shows a similar dose response of T₁ *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 – 1,600 g ae/ha (**Table 17**). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some *AAD1* (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first *in planta* data that *AAD-1* (v3) will provide AOPP resistance. No resistance was observed with fluroxypyr (a pyridinyloxyacetate auxin) in transformed *Arabidopsis*, consistent with *in vitro* work with heterologously expressed enzyme.

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

[00218] The ability to use *AAD-1* (v3) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T₁ seed transformed with *PAT* and *AAD1* (v3) (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[00219] Fifty three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for *PAT* protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, *PAT*. The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (**Table 14**). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs glufosinate (0.12%); however, the range of selection rates across all experiments

would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with *AAD-1* (*v3*) or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

6.7 – Heritability.

[00220] A variety of T₁ events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂ plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T₁ families (T₂ plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00221] Seed were collected from 12 to 20 T₂ individuals (T₃ seed). Twenty-five T₃ siblings from each of eight randomly-selected T₂ families were progeny tested as previously described. Approximately one-third of the T₂ families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1* (*v3*) is stably integrated and inherited in a Mendelian fashion to at least three generations.

Example 7 – Transformation into Maize

7.1 – Cloning of *AAD-1* (*v3*).

[00222] The *AAD-1* (*v3*) fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5 α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 were digested with NotI. The 3442bp band from pDAB3403 and the 11017bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5 α , and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404.

7.2 – Callus/suspension initiation.

[00223] To obtain immature embryos for callus culture initiation, F₁ crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersion in 70% ethanol for 2-3 minutes, followed by immersion in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00224] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Complete Medium (Phyto Technology Laboratories C167), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L AgNO₃, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Complete Medium (Phyto C167), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00225] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS Basal Salt Mixture (Murashige and Skoog, 1962. Phyto* M524), Modified MS Vitamins, 2.0 mg/L (2,4-D), 2.0 mg/L α -naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium using a 50 ml pipette. Once the suspensions were fully established, they were cryopreserved for future use.

7.3 – Cryopreservation and thawing of suspensions.

[00226] One day post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled the vials were placed in a controlled rate freezer set to freeze at a rate of -0.5°C/minute until reaching a final temperature of -40°C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with Liquid nitrogen vapors.

[00227] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until “boiling” subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 1 or 4) in 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 solid medium (N6 Complete Medium (Chu *et al.*, 1975. Phyto C167), 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite (omitted for liquid medium), pH 5.8. The tissue paper containing the cells was transferred to fresh medium after one week. After approximately 2 weeks, the tissue was re-initiated into 125 ml flasks. Three milliliters PCV was subcultured every 3.5 days until a total of 12 ml PCV was obtained. Then subculturing was conducted as described previously.

7.4 – WHISKERS-mediated transformation

[00228] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28°C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Complete Medium (Phyto C167), 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of

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GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00229] After incubation in GN6 S/M, the contents of each flask was pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00230] Depending on the parameter being tested, either 3 or 6 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 solid medium. Plates were cultured for 1 week at 28°C in a dark box covered with a single layer of plastic.

7.5 – Selection of transformed tissue.

[00231] After 1 week, the filter papers were transferred to 60x20 mm plates of GN6 solid medium containing 2,4-D and/or dicamba plus 1 mg/L bialaphos [in the form of Herbiace, GN6 1H (N6 Complete Medium (Phyto C167), 2.0 mg/L 2,4-D, 30 g/L Sucrose, 100 mg/L myo-inositol, 1.0 mg/L (active ingredient) Herbiace, 2.5 g/L Gelrite, pH 5.8) or GN6D 1H (N6 Complete Medium (Phyto C167), 8.0 mg/L dicamba, 0.8 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L (active ingredient) Herbiace, 2.5 g/L Gelrite, pH 5.8)] or 100 nM haloxyfop-R methyl ester (in the form of Gallant* Super, same as GN6 1H and GN6D 1H except active ingredient is 100nM Gallant).

[00232] Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the plate or else the entire plate into 3.0 of melted GN6 Agarose medium (N6 Complete Medium (Phyto C167), 2.0 mg/L 2,4-D, 30 g/L Sucrose, 100 mg/L myo-inositol, 7 g/L SeaPlaque Agarose, pH 5.8, Autoclaved for only 10 minutes at 121° C) containing the same concentration of selection agent as in the previous transfer. The

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tissue was broken up and evenly poured onto the surface of a 100 x 15 mm plate of GN6 medium containing the same level of selection agent as was added to the agarose. This was repeated for all remaining tissue. Once embedded, plates were individually sealed, and incubated in the dark at 28° C.

[00233] Transgenic isolates are typically recovered 5-7 weeks post-embedding. Any putative isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth was evident, an event was deemed to be resistant and was submitted for molecular analysis.

7.6 – Dose response of non-transformed tissue to haloxyfop

[00234] Three established Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 solid medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 450 mg of callused suspension tissue was transferred per treatment to selection media in 100 x 15 mm plates containing the following concentrations of haloxyfop-R methyl ester from GallantTM* RLPU: 0, 10, 50, 100, 200, and 400 nM. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos, GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded.

7.7 – Results.

[00235] 7.7.1 – Dose response to haloxyfop-R methyl ester. A dose response was implemented in order to determine the level of selection needed for WHISKERS-treated suspension tissue. Results from an initial dose response testing levels from 0 to 400 nM haloxyfop-R are shown in **Figure 8**.

[00236] These data show that growth appears to hit a plateau at levels of 100 nM and higher, and suggests that this level might be a good starting point for direct recovery of transformed isolates from selection. Although a plateau is evident, growth reduction at 100 nM haloxyfop was still only 87% of the control whereas the Herbiace treatment showed 98% growth reduction as compared to the control.

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[00237] 7.7.2 – Transformant selection strategy using pDAB3404. Two selection strategies were used for WHISKERS-mediated transformation (Frame *et al.*, 1994) of embryogenic suspensions. For Strategy I, only Herbiace selection was implemented; however, 2,4-D and dicamba were compared as auxins. [2,4-D is a critical component of maize tissue culture media because it keeps the callus tissue from differentiating prematurely. Since 2,4-D is degraded by AAD-1 (V3) and dicamba is not, dicamba (at 8 mg/L dicamba + 0.8 mg/L 2,4-D) was assessed for its ability to served as a growth regulator in 2,4-D maintained cells.] Strategy II implemented 75% Herbiace selection and 25% haloxyfop selection while comparing 2,4-D and dicamba within each selection agent. A total of 3 bottles were WHISKERS-treated for each strategy for a total of 6 bottles. The tissue from Strategy I was embedded whereby each filtered plate produced 2 embedded plates. However, the tissue from Strategy II was embedded whereby each filtered plate produced only 1 embedded plate. Therefore, a total of 360 embedded plates were produced from each strategy. Herbiace-resistant events were recovered approximately 3 to 6 weeks post-embedding. They appeared as light yellow sectors proliferating against a background of darker yellow-to-brown growth-inhibited tissue. The putatively transformed events were subcultured onto fresh GN6 (1H) medium in 60x20 mm plates every 10-14 days using a dissecting scope. They became established and ready for analysis after approximately 2-6 weeks.

[00238] Ninety-six isolates were recovered from Herbiace selection and 3 isolates were recovered from direct selection on haloxyfop-R. All were submitted for copy number analysis for the *PAT* gene. In addition, full length plant transformation unit (PTU) PCR analysis was performed [for the *AAD-1* (*v3*) gene] concurrently using primers that amplified 1520 bp of the Ubi promoter, all of the *AAD-1* (*v3*) coding region and 25 bp of the rice actin promoter, with a final product of 2880 bp. Thirty-four isolates, all of which were selected upon Herbiace, were identified to contain 3 copies or less of the *PAT* gene plus an intact PTU for the *AAD-1* (*v3*) gene. Most, but not all of these callus events were also submitted for Western/ELISA screening.

[00239] Fourteen Western positive events that contained 3 copies or less of *PAT* plus an intact PTU for *AAD-1* (*v3*) were regenerated and advanced to the greenhouse. Two additional events that contained 3 copies or less of *PAT* plus an intact PTU for *AAD-1* (*v3*) but were never analyzed for expression were also advanced. These latter 2 events were subsequently tested for expression using

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T₀ leaf tissue and were found to express AAD-1 (v3). A final characterized event that showed expression during the callus stage but not in leaf tissue was also advanced to the greenhouse.

[00240] Dose Response of Transformed Tissue to Haloxyfop. Approximately 150-200 mg of transgenic callus tissue was transferred to 60 x 20 mm plates containing the following concentrations of haloxyfop-R methyl ester from Gallant* Super: 0, 30, 100, 300 nM. Three replicates were used per concentration. An additional medium containing 1mg/L bialaphos (from a Herbiace formulation) was also included for comparison. These steps were repeated using non-transgenic maize tissue derived from callused embryogenic suspensions.

[00241] After 2 weeks, tissue from each plate was removed, weighed, and then transferred to fresh media of the same concentration for an additional 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. One example of results after 4 weeks is provided in **Figure 9**. Event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited ~97% versus untreated control at this rate. The expression of AAD-1 (v3) enzyme allowed protection of the corn callus from haloxyfop.

[00242] Regeneration of Transformed Tissue. Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, "28", containing 1 mg/L bialaphos. Cells were allowed to grow in low light ($13 \mu\text{Em}^{-2}\text{s}^{-1}$) for one week then higher light ($40 \mu\text{Em}^{-2}\text{s}^{-1}$) for another week before being transferred to hormone-free regeneration medium, "36", containing the same level of selection. Small (3-5 cm) plantlets were removed and placed into 150 x 25 mm culture tubes containing SHGA (Schenk and Hildebrandt salts: 10 g/L sucrose, 1 g/L myo-inositol, 2 g/L Gelrite, pH 5.8) medium. Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse and allowed to acclimate to greenhouse conditions under a 0.5 L clear plastic cup for 5-10 days. Once plants were growing rapidly, cups were removed and plants that typically possessed 4-10 axillary shoots were thinned to 1-2 primary shoots. In most cases 15 plants were produced for each event. A total of 251 plants representing 17 events were chosen for advancement to the next generation. Reciprocal crosses with a commercial-type hybrid shall be made to generate sufficient F₁ seed for field tolerance testing.

[00243] Tolerance of T₀ 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 T₀ corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure II, Clincher*, or Gallant Super* for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1X lethal dose (~1/8 X field dose) up to 8X field doses (64 X lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[00244] AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each *AAD-1* (*v3*)-transformed line tested, survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived, with no visible injury (14 DAT) even when treated with an 8X field dose (64X lethal dose) of quizalofop. Several individual clones from events '017' and '038', however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

[00245] The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T₀ stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for *AAD-1* *in vitro*.

[00246] **Figure 10** shows the responses of several *AAD-1* (*v3*) –transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

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[00247] **Table 18** shows data for the responses of selected *AAD-1* (v3)-transformed T₀ corn events to three AOPP herbicides applied postemergence.

7.8 – *Agrobacterium*-mediated transformation of maize

7.8.1 Plant Material

[00248] Seed of two S₃ parents of "High II" (i.e., Parent A and B) or the F₁ cross (Armstrong et al., 1991) are planted directly into 5 gallon-pots containing commercial soil mix (Conrad Fafard, Inc., Springfield; Soil Mix #3). The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps. The illumination is approximately 1,500 ft-candles. The day and night temperature is maintained at 27/20 + 2° C. The plants are watered as needed and a soluble fertilizer (20-20-20) along with chelated iron (8 mg/L) is applied at regular intervals.

7.8.2 Tissue Source

[00249] For obtaining immature embryos, controlled pollinations are performed. Plants at anthesis are prepared for pollination by cutting back silks one day prior to pollination, thereby producing a full brush of silks for maximum fertilization and seed set. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. When the embryos reach 1.0-1.2 mm size (9-10 days after pollination), the ear is excised and surface sterilized. Briefly, ears are washed with diluted "Liquinox" soap and subjected to immersions in 70% ethanol for 2-5 min. and 20% commercial bleach (0.1% sodium hypochlorite) for 30-45 minutes followed by 3 rinses in sterile, distilled water. Following sterilization, immature embryos (1.0-1.2 mm) are isolated.

7.8.3 Bacterial Supply

[00250] All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Patent No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of bacteria from a YP streak plate is put into 5ml of LS-inf. Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture is vortexed until a uniform suspension is achieved. The bacterial concentration was taken using a Klett-Summerson

Photoelectric Colorimeter by reading the density of the solution. The solution is adjusted to a concentration of Klett 200 ($\sim 1 \times 10^9$ cfu/ml) with 100 μ M actetosyringone added to the solution.

7.8.4 Infection and Cocultivation

[00251] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod, which is a high osmotic liquid medium. Each tube, containing ~ 100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5-10 minute incubation at room temperature, the embryos are transferred to LS-As Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L AgNO₃, 1, 100 μ M actetosyringone, 3.0g/L Gelrite, pH 5.8) for the co-cultivation step which lasts for 5-7 days at 25° C.

7.8.5 Selection

[00252] After co-cultivation, the embryos are moved through a 3-step selection scheme after which transformed isolates will be obtained. For selection, LSD Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L AgNO₃, 250 mg/L cephotaxime, 100-600 nM Pursuit[®], 2.5 g/L Gelrite, pH 5.7) is used along with one of three Pursuit[®] selection levels as well as cefotaxime to stop further growth of the *Agrobacterium*. Throughout the selection phase, the embryos are cultured in the dark at 27° C. Pursuit[®] is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,18. The embryos are first put on a very "low" level of selection (~ 50 nM Pursuit[®]) for 5-7 days, moved up to a "moderate" selection level of 300 nM Pursuit[®] for 2 weeks and then taken through 2 week cycles of the "high" selection level of 600 nM Pursuit[®] until embryogenic colonies are obtained. These colonies are maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies are bulked up by transferring to fresh selection medium at 2 week-intervals for regeneration and further analysis.

7.8.6 Regeneration and Seed Production

[00253] For regeneration, the cultures are transferred to 28 "induction" medium (MS Basal salts, 0.25 mg/L 2,4-D, 5 mg/L BAP, 30.0 g/L sucrose, 250 mg/L cephotaxime, 100-600 nM Pursuit[®], 2.5 g/L

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Gelrite, pH 5.7) and incubated at 28° C in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). The callus cultures are then non-selectively transferred to the 36 “regeneration” medium (MS Basal salts, 30.0 g/L sucrose, 250 mg/L cephotaxime, 50-600 nM Pursuit®, 2.5 g/L Gelrite, pH 5.7) for differentiation of plantlets. The composition of the ‘regeneration’ medium is similar to the “induction” medium except that it did not contain any plant growth regulators. Both “induction” and “regeneration” media contain Pursuit® selection along with continued use of cefotaxime. When plantlets are established, they are transferred to SHGA tubes to allow for further growth and development of the shoot and roots. After a healthy shoot has been obtained, it is moved to the greenhouse where it is first grown in 10 cm pots containing commercial soil mix (Fafard #3). At the 3-5 leaf stage, they are transplanted to larger pots (5 gallon) and grown to maturity in the greenhouse. Controlled pollinations for seed production and tolerance testing can be conducted as described above in Example 7.2.

Example 8 – Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[00254]

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

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Example 9 – Western Blot and ELISA Analysis for AAD-1 Expressed in *Arabidopsis* and Maize9.1 – Antibody production.

[00255] Five milligrams purified AAD-1 (v1) delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for polyclonal antibody production. Two rabbits each received 5 injections in the period of 10 weeks with each injection contained 0.5 mg of the purified AAD-1 (v1) protein resuspended in 1 ml of Incomplete Freund's Adjuvant. Sera after injections were shipped back for analysis.

9.2 – Antibody purification and biotinylation.

[00256] Rabbit anti-serum was purified using ImmunoPure® (G) IgG Purification Kit (Pierce Biotech). One ml of the serum was diluted to 5 ml with the Binding buffer and the purification was conducted according to the manufacturer's instructions. The purified fractions were pooled and dialyzed against PBS using dialyzing cassette (Pierce Biotech.) with MWCO of 100,000 for overnight at 4° C. One milligram of the purified antibody was biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin labeling kit (Pierce Biotech.) following the manufacturer's instruction. The reaction proceeded on ice for 2 hours and the product was dialyzed against PBS as above.

9.3 – Extraction of AAD-1 v3 from plant materials.

[00257] Transgenic *Arabidopsis* leaves or corn calli were collected from different transgenic and 2 control lines. Approximately 30 to 40 mg of plant material was weighed and put into a cluster tube containing one steel bead. A tungsten bead was put on top of the plant material and extraction buffer (PBS containing 0.1% Triton X-100 and 10 mM DTT) was added (10 µl per mg of plant material). All the samples were put into cluster box with the lids secured. The box was put into shaker to pulverize the plant tissue for 4 minutes at maximum speed. Tubes were then centrifuge for 10 min at 2500 rpm. The supernatant was used directly for Western Blot or ELISA analysis.

9.4 – Western blots.

[00258] All steps were conducted at room temperature except as otherwise stated. Various concentrations of AAD-1 (v1), namely 1.0, 0.1, 0.01 and 0.001 ng, were denatured in sample buffer (Bio-Rad; Hercules, CA) and loaded onto pre-casted Novex® Tris-Glycine polyacrylamide gel (Invitrogen; Carsberg, CA). Ten µl SeeBlue® Plus Pre-stained Standard (Invitrogen; Carsberg, CA) was loaded in separate well to monitor the electrophoresis, which was conducted at 200 V. The

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proteins were then electro-transferred onto nitrocellulose paper. The blots were blocked with 4% skim milk dissolved in phosphate buffered saline (MPBS) for 1 hour and probed with rabbit anti-sera diluted 5000-fold in PBS. After 1 hour incubation, the blots were washed 3 times with PBS containing 0.05% Tween 20 (PBST). Bound antibodies were detected by goat anti-rabbit antibodies conjugated to horseradish peroxidase (GAR/HRP; Pierce; Rockford, IL). The blot was incubated for 1 hour and followed by 3 washes. ECL Western Blotting Detection Reagents (Amersham; Piscataway, NJ) were used to visualize the protein detected. Manufacturer's instructions were followed. To detect the AAD-1 (v3) in transgenic plant extracts, 20 μ l of plant extract (see below) was added to 10 μ l of 3X sample buffer. After incubated at 95° C for 5 minutes, 20 μ l of the mixture was loaded onto gel for electrophoresis.

9.5 – Sandwich ELISA.

[00259]

All steps were conducted at room temperature except otherwise stated. Purified rabbit antibody was immobilized on microtiter wells to capture AAD-1 (v3) in plant extract. The purified antibody was diluted to 1 μ g/ml in PBS and 100 μ l was incubated in each well at 4° C for 16 hr. After 4 washes, each well was blocked with 200 μ l MPBS for 1 hour. Various concentrations of plant extracts were incubated in the wells. A 2-fold serial dilution of the extracts was performed on the plate, with the highest concentrations at the top of the plate. To prepare a standard curve, different concentrations of purified AAD-1 (v3) (100, 50, 25, 12.5, 6.25, 3.125, 1.0625 and 0 ng/ml) were also put into the wells in duplicate. The plate was incubated for 1 hour and washed 4 times with PBST. Biotinylated antibody was used to detect the bound AAD-1 (v3). Diluted to 1 μ g/ml, 100 μ l of the biotinylated antibody was incubated in each well for 1 hour. After another wash, neutravidin conjugated to horseradish peroxidase (Pierce Biotech.) was added to each well and incubated for another hour. TMB (3,3',5,5'-Tetramethylbenzidine) substrate (100 μ l) was added and, after the 15 min incubation, 100 μ l 0.4 N sulfuric acid was added to stop the reaction. The absorbance at 450 nm was measured using a microtiter plate reader. The concentration of AAD-1 (v3) in plant extract was extrapolated from the standard curve on the same plate.

9.6 – Results of Western and ELISA data.

[00260]

Polyclonal antibodies specific to AAD-1 (v1) were generated by immunizing rabbits with recombinant AAD-1 (v1) purified from *E. coli*. After 5 separate boosts, the sera from both rabbits

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were able to detect 1 ng of AAD-1 (v3) in Western Blot and direct ELISA analysis. Serum from second rabbit was further purified by Protein G affinity chromatography and an aliquot of it was biotinylated. A sandwich ELISA was developed, which can quantify AAD-1 (v3) in the linear range of 5 to 100 ng/ml.

[00261] Eight and 20 different transgenic *Arabidopsis* leaves and corn calli, respectively, were analyzed by both Western Blot and the Sandwich ELISA. Results indicated that the expression level of AAD-1 (v3) in *Arabidopsis* ranged from 2 to 50 µg/ml of the plant extract, which is equivalent to 0.08 to 2.0% of total soluble protein extracted from the leaf tissue. Similarly, AAD-1 (v3) was found to be 0.7 to 2.1% of total soluble protein in corn calli tested.

Example 10 – Tobacco Transformation Protocol

[00262] Tobacco transformation with *Agrobacterium tumefaciens* can be carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) can be surface sterilized and planted on the surface of TOB- medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants can be grown for 6-8 weeks in a lighted incubator room at 28-30°C and leaves collected sterily for use in the transformation protocol. Pieces of approximately one square centimeter will be sterily cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721), grown overnight in a flask on a shaker set at 250 rpm at 28° C, should be pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces should be dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces are to be moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30°C in the light. Leaf pieces should be moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small

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plants arising from transformed foci will be removed from this tissue preparation and planted into medium TOB- containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets will be grown in a lighted incubator room. After 3 weeks, stem cuttings will be taken and re-rooted in the same media. Plants should be ready to send out to the greenhouse in 2-3 additional weeks.

[00263] Plants can be moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30 C greenhouse for one week. After one week the bag should be opened; the plants were fertilized and allowed to grow in the open bag until the plants are greenhouse-acclimated, at which time the bag can be removed. Plants are grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu\text{E}/\text{m}^2\text{s}^{-1}$). T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic tobacco. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 50-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn.

Example 11 – Soybean Transformation

[00264] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen,

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1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00265] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-1* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-1*.

[00266] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of axillary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the

Agrobacterium-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchey *et al.*, 1988), only very recently it is optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00267] *Agrobacterium* Preparation. The plasmid, pDAB721, contains the *AAD-1* gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the *PAT* gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00268] *Agrobacterium* cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00269] Plant Transformation. Seeds of 'Thorne,' 'Williams82,' or 'NE3001,' public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C, with a 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, Cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma Cat #A 8678). Alternatively certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl₂) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added drop wise along the side of

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the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl₂ gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00270] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-Cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 µM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00271] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00272] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging 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.

[00273] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00274] Progeny Evaluation. T₀ plants will be allowed to self fertilize in the greenhouse to give rise to T₁ seed. T₁ plants (and to the extent enough T₀ plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T₀ plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T₁ seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T₀ and T₁ plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for Arabidopsis and corn. Determination of the inheritance of AAD-1 will be made using T₁ and T₂ progeny segregation with respect to herbicide tolerance.

Example 12 – Cotton Transformation Protocol

[00275] Seeds are surface-sterilized in 95% alcohol for 1 minute, rinsed, sterilized with 50% commercial bleach for 20 minutes, then rinsed 3 times with sterile distilled water before being germinated on G-medium (Murashige and Skoog (MS) basal salts, B5 vitamins, 3% sucrose) in Magenta GA-7 vessels and maintained under a high light intensity of 40 - 60 $\mu\text{E}/\text{m}^2\text{s}$, with a photoperiod of 16 hours light and 8 hrs dark at 28° C.

[00276] Cotyledon segments (~5 mm) square are isolated from 7 - 10 day old seedlings into liquid M-medium (MS-based medium with 1-5 μM 2,4-D and 1-5 μM Kinetin) in Petri plates. Cut segments are treated with an *Agrobacterium* solution then transferred to semi-solid M-medium and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to M-medium containing 5 mg/l glufosinate-ammonium medium and 500 mg/l carbenicillin (MG5 medium). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00277] After 3 weeks, callus from the cotyledon segments is isolated and transferred to fresh MG5 medium. The callus is transferred for an additional 3 weeks on MG5 medium. Callus is then transferred to C-medium (MS-based medium containing 10-20 μM NAA and 5-10 μM Kinetin) containing glufosinate-ammonium and carbenicillin, and transferred again to fresh selection medium after 3 weeks. After another 3 weeks the callus tissue is transferred to culture medium lacking plant

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growth regulators for embryogenic callus induction. After 4 - 8 weeks on this medium, embryogenic callus is formed; and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00278] Larger, well-developed embryos are isolated and transferred to basal medium for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to basal medium for shoot and root development. After 4 - 8 weeks, any well-developed plants are transferred into soil and grown to maturity.

[00279] T₀ plants will be allowed to self-pollinate in the greenhouse to give rise to T₁ seed. T₁ plants (and to the extent enough T₀ plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic cotton. Rates of 2,4-D used on T₀ plants will typically use one or two selective rates in the range of 50-400 g ae/ha. T₁ seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T₀ and T₁ plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in example 9 for *Arabidopsis* and corn.

Example 13 -- *Agrobacterium* Transformation of Other Crops

[00280] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated transformation of rye, *see e.g.* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see e.g.* Hinchee *et al.* 1988. For *Agrobacterium*-mediated transformation of sorghum, *see e.g.* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see e.g.* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see e.g.* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see e.g.* Hiei *et al.*, 1997.

Example 14 – Vector Construction and Stacking *AAD-1* With Other Herbicide Resistance Genes

[00281] *AHAS* Vector construction: Whiskers transformation. A corn *AHAS* gene having a mutation coding for asparagine instead of serine at residue 621 of the protein (see US Patent Nos. 5,731,180, 5,767,361 and 5,928,937 to Cyanamid) was received from BASF in plasmid pAC1565. Five

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restriction enzyme recognition sites were removed from the *AHAS* gene without altering the amino acid sequence, and both a multiframe stop codon and a *SacI* site were added to the 3' end of the gene. A sixth modification of AAT to AAC at amino acid 621 was also completed. The *AHAS* gene was then used to replace the *PAT* gene in pDAB8504 (RB7 MAR/OsAct1/*PAT*/Zmlip/RB7 MAR) to create a whiskers backbone vector containing *AHAS*.

[00282] A short PCR fragment was designed to add the multiframe stop codons and a *SacI* site at the 3' end of the *AHAS* gene. The sense primer (*AHAS* 5') and the antisense primer (*AHAS* 3') were used to amplify the 3' end of the BASF *AHAS* gene to create the 3' *AHAS* Adapter. The adapter was then ligated into pDAB8504 on an *NcoI*/*SacI* fragment replacing the *PAT* gene to create pDAB2210. A unique enzyme, *RsrII*, was left in the adapter to enable the remainder of the *AHAS* gene to be easily ligated into pDAB2210 (RB7 MAR/OsAct1/*AHAS* Adapter/Zmlip/RB7 MAR) on an *NcoI*/*RsrII* digest after the rest of the modifications were completed.

[00283] Modifications to remove the 5 restriction enzyme recognition sites and alter amino acid 621 were done with the QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Changes M1-3, 5, 6 were done in succession with the starting material being pAC1565. M4 was done on pDAB2210. These changes are summarized in Table 19.

Table 19.		
Modification	Goal	Primers
M1	Remove <i>NcoI</i> site at bp1115 of pAC1565	M1 & M1R
M2	Remove <i>NcoI</i> site at bp1310 of pAC1565	M2 & M2R
M3	Remove <i>HindIII</i> site at bp1993 of pAC1565	M3 & M3R
M4	Modify AA 621 of <i>AHAS</i> gene from AAT to AAC	M4 & M4R
M5	Remove <i>FspI</i> site at bp1677 of pAC1565	M5 & M5R
M6	Remove <i>FspI</i> site at bp1786 of pAC1566	M6 & M6R

[00284] Upon completion of all 6 changes, the gene was reassembled in pDAB2210 by ligating the *NcoI*/*RsrII* fragment containing the modified gene from pAC1565 into pDAB2210 to create pDAB2212 (RB7 MAR/OsAct1/*AHAS*/Zmlip/RB7 MAR).

[00285] Stacking *AAD-1* and *AHAS*. The ZmUbi1/*AAD-1*/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1* and *AHAS* Whiskers transformation vector pDAB3415.

[00286] Stacking *AAD-1* and *EPSPS*. A gene encoding a glyphosate tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is isolated, e.g., from the soil bacterium *Agrobacterium tumefaciens* strain CP4 and modified for plant-preferred codons (see, e.g., U.S. Patent Nos. 5,804,425 and 5,633,435). Native *EPSPS* genes can also be engineered into glyphosate-tolerant forms, as is described, e.g., in WO9506128, WO 9704103, WO9854330, and U.S. Patent No. 6,040,497. These *EPSPS* genes are preferably fused to a chloroplast transit peptide to allow subcellular targeting of the glyphosate-tolerant EPSPS proteins into the chloroplast (see, e.g., US Patent Nos. 4,940,835, 5,188,642 and 6,063,601), then ligated, for example, into plasmid pDAB2212 to replace the *PAT* gene using standard cloning techniques (see, e.g., Sambrook and Russell2000). The ZmUbi1/*AAD-1*/ZmPer5 cassette is removed from pDAB3404 with AscI/SwaI and inserted into the above vector to create the *AAD-1* and *EPSPS* Whiskers transformation vector.

[00287] *AHAS* Vector construction: *Agrobacterium* Transformation. A binary vector was constructed containing the *AHAS* selectable marker for use in the Maize *Agrobacterium* mediated transformation system. Plasmid pDAB2212 was digested with PmeI to isolate the OsAct1/*AHAS*/Z mliip cassette and the fragment ligated into pDAB8546 to create pDAB8548 (RB7 MAR/OsAct1/*AHAS*/Z mliip with Agro backbone).

[00288] The artificial MAR sequence was isolated from pdartMAR with a PmeI digest. The MAR was inserted into pDAB8548 at the MluI (blunted) to create pDAB8549. The resulting base vector, pDAB8549, contained non-identical MAR regions, RB7 MAR 5' and artificial MAR 3'.

[00289] Stacking *AAD-1* and *AHAS*. The ZmUbi1/*AAD-1*/ZmPer5 cassette was isolated from pDAB3404 with a NotI digest and inserted into pDAB8549 to create pDAB2272. This intermediate vector was then transformed into *Agrobacterium* strain LBA4404 containing the pSB1 plasmid (U.S. Patent 5,591,616).

Example 15 – Further Evidence of Surprising Results: *AAD-1* vs. *AAD-2*

[00290] *AAD-2* initial cloning. Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession # AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00291] The strain of *Bradyrhizobium japonicum* containing the *AAD-2* gene was obtained from Northern Regional Research Laboratory (NRRL, strain # B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28°C on a floor shaker at 150rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat.# 69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(brjap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

[00292] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/ul), H2O 21 µl, Taq polymerase 1 ul (2.5 units/ ul). Three Fail Safe Buffers; A, B, and C were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. # FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat.# K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00293] Ten of the resulting white colonies were picked into 3 μ l Luria Broth + 1000 μ g/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. # K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. # R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. # 608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2* for internal consistency.

[00294] Completion of *AAD-2* Binary Vector. The *AAD-2* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/ SacI *AAD-2* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HindIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00295] Functional enzyme assay (colorimetric). Colorimetric assays were conducted as previously described in Example 3. Assays were performed with unpurified desalted extracts. Results are reported in **Table 20**.

Table 20. Comparison of the K_m and “ V_{max} ” values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	K_m (μM) $\pm$ SE	V_{max} (A510 units)	K_m/V_{max} (arbitrary units)
AAD-2	2,4-D	423 (± 1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (± 21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α -ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe^{2+} and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[00296] Current data indicate that AAD-2 is an S-enantiomer-specific enzyme whereas AAD-1 is R-enantiomer-specific. Therefore dichlorprop K_m values were calculated based on the single enantiomer concentration rather than the total racemic mix used.

[00297] The fitted curves from the data are shown in **Figure 11**, which illustrates the relative rates of herbicide degradation for the various substrates by the two enzymes (AAD-2 vs. AAD-1). The concentrations of AADs in each extract are approximately equivalent, which suggests that AAD-2 is a better degrader of 2,4-D than in AAD-1. However, these *in vitro* results are in surprising contrast to the *in planta* findings reported below, where AAD-1 is surprisingly much superior to AAD-2 in regards to 2,4-D resistance.

[00298] Evaluation of transformed Arabidopsis. Freshly harvested T_1 seed transformed with a native [AAD-1 (v2)], plant optimized [AAD-1 (v3)], or native AAD-2 gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00299] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00300] Five to six days after planting (DAP) and again 10 DAP, T₁ plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml / tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5°C, 50±30% RH, 14 hours light:10 dark, minimum 500 µE/m²s⁻¹ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1* (v3), *AAD-1* (v2), or *AAD-2* to provide phenoxy auxin herbicide resistance.

[00301] Random individual T₁ plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

[00302] Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). mixed in 200 mM Tris buffer (pH 9.0).

Results of testing.

[00303] Results of selection of transformed plants. The first *Arabidopsis* transformations were conducted using *AAD-1* (v3). T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T₁ seed were screened and 493 glufosinate resistant plants were identified (*PAT* gene), equating to a transformation / selection

frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (**Table 14**). A small lot of *AAD-1* (v2) transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T₁ individuals were identified out of 84,000 seed screened (0.33% transformation / selection frequency). Surprisingly, *Arabidopsis* transformations using the *AAD-2* gene provided a very low transformation frequency when selected for glufosinate tolerance (*PAT* selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered equating to a transformation / selection frequency of 0.018% (see **Table 14**). Transformation frequency for native *AAD-2* was only 6% of that of *AAD-1* (v2).

Table 14: Selection of *AAD-1* v3 (plant optimized), or *AAD-1* v2 (native), or *AAD-2* (native)-transformed T₁ individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Total seed sown and screened	Number of resistant T ₁	Transformation/ Selection rate	Selection rate range	% of selected plants expressing PAT ³
Glufosinate ¹	<i>AAD-1</i> v3	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D ²	<i>AAD-1</i> v3	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate ¹	<i>AAD-1</i> v2	84,000	278	0.33%	0.33%	nd ⁴
Glufosinate ¹	<i>AAD-2</i>	1,301,500	228	0.018%	0.07 to 0.09%	100%

¹Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP

²2,4-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

⁴nd, not determined

[00304]

T₁ plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 15** compares the response of *AAD-1* (v2) with *AAD-1* (v3) and *AAD-2* genes to impart 2,4-D resistance to *Arabidopsis* T₁ transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T₁ *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 15**

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simply to demonstrate the significant difference between the plants transformed with *AAD-1* (v2), *AAD-1* (v3), or *AAD-2* versus the wildtype or *PAT/CryIF* transformed controls.

[00305] Surprisingly, *AAD-2* transformants were far less resistant to 2,4-D than either *AAD-1* (v2) or *AAD-1* (v3) genes (**Table 15**) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1* (v2)-transformed T₁ plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1* (v3) T₁ plants were uninjured (population injury average = 14%). See **Figure 12**.

[00306] These results are unexpected given that *in vitro* comparison of native *AAD-1* and *AAD-2* indicated 2,4-D was better degraded by *AAD-2*. *AAD-2* is expressed in individual T₁ plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. See **Figure 13**. These data corroborate earlier findings that make the functional expression of *AAD-1* *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected and not obvious.

Example 16 – Preplant Burndown Applications

[00307] This and the following Examples are specific examples of novel herbicide uses made possible by the subject *AAD-1* invention.

[00308] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several

herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-1* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agrilience Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1* gene if stably transformed.

Example 17 – In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[00309] *AAD-1* can enable the use of phenoxy auxin herbicides (e.g., 2,4-D, dichlorprop, MCPA, *et al.*) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present

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in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1*-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (*see* weed control spectra by reference to Agrilience Crop Protection Guide performance ratings).

[00310] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/*AAD-1* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the art of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[00311] Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (*see* example 16); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-1*-transformed dicot crop volunteers.

[00312] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1* gene if

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stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1* Only Transformed Corn, Rice, And Other Monocot Crops

[00313]

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

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agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agrilience Crop Protection Guide performance ratings).

[00314] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-1* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the art of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as “rat-tailing,” crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[00315] Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the *AAD-1* gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

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Example 19 – AAD-1 stacked with glyphosate tolerance trait in any crop

[00316]

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-1* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1* and a GT trait are stacked in any monocot or dicot crop species:

- a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.
- b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica*, 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

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

[00317] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTC's by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-1* Stacked with Glufosinate Tolerance Trait in Any Crop

[00318] Glufosinate tolerance (*PAT* or *bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves

24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2003).

[00319] By stacking *AAD-1* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

- a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
 - i) Inherently tolerant grass weed species (e.g., *Echinochloa spp* or *Sorghum spp*) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
 - ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.
- b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00320] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTC by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 - *AAD-1* Stacked with *AHAS* Trait in Any Crop

[00321] Imidazolinone herbicide tolerance (*AHAS*, *et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agrilience, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) for which many weeds have developed resistance to (Heap, 2004). By stacking *AAD-1* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control

options can be envisioned where *AAD-I* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

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

[00322] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by *AAD-I* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop

Protection Guide from Agrilience (2003). Each alternative herbicide enabled for use in HTC's by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

References

- Adang, M.J., M.J. Staver, T.A. Rocheleau, J. Leighton, R.F. Barker, and D.V. Thompson.** 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene* 36:289-300.
- Agrilience Crop Protection Guide.** 2003. Agrilience, LLC. St Paul, MN. 588 p.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D.J. Lipman.** 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Altschul, S. F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman.** 1997. Gapped BLAST and PSIBLAST: a new generation of protein databasesearch programs. *Nucl. Acids Res.* 25:3389-3402.
- An, G., B.D. Watson, S. Stachel, M.P. Gordon, E. W. Nester.** 1985. New cloning vehicles for transformation of higher plants. *EMBO J.* 4:277-284.
- Armstrong C.L., C.E. Green, R.L. Phillips.** 1991. Development and availability of germplasm with high Type II culture formation response. *Maize Genet Coop News Lett* 65:92-93.
- Beltz, G.A., K.A. Jacobs, T.H. Eickbush, P.T. Cherbas, and F.C. Kafatos.** 1983 *Methods of Enzymology*, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285
- Birch R.G. and T. Franks.** 1991. Development and optimization of microprojectile systems for plant genetic transformation. *Aust. J. Plant Physiol.* 18:453-469.

- CDMS.** Crop Data Management Systems Labels and MSDS. Online. Internet. March 13, 2004. Available at net/manuf/manuf.asp.
- Chee P.P., K.A. Fober, J.L. Slightom.** 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. *Plant Physiol.* 91:1212-1218.
- Cheng M., J.E. Fry, S. Pang, H. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Conner, and Y. Wan.** 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* 115: 971-980.
- Chu C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, F.Y. Bi.** 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sinica* 18:659-668.
- Clemente T.E., B.J. LaVallee, A.R. Howe, D. Conner-Ward, R.J. Rozman, P.E. Hunter, D.L. Broyles, D.S. Kasten, M.A. Hinchee.** 2000. Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium*-mediated transformation. *Crop Sci* 40: 797-803.
- CPR: Crop Protection Reference.** 2003 Chemical and Pharmaceutical Press, New York, NY. 2429 p.
- Didierjean L, L. Gondet, R. Perkins, S.M. Lau, H. Schaller, D.P. O'Keefe, D. Werck-Reichhart.** 2002. Engineering Herbicide Metabolism in Tobacco and Arabidopsis with CYP76B1, a Cytochrome P450 Enzyme from Jerusalem Artichoke. *Plant Physiol* 2002, 130:179-189.
- Ditta, G. S. Stranfield, D. Corbin, and D.R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium leguminosarum*. *PNAS* 77:7347-7351.

- Falco S.C., T. Guida, M. Locke, J. Mauvais, C. Sanders, R.T. Ward, P. Webber.** 1995. Transgenic canola and soybean seeds with increased lysine. *Bio/Technology* 13:577-582.
- Finer J. and M. McMullen.** 1991. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In Vitro Cell. Dev. Biol.* 27P:175-182.
- Fraley, R.T., D.G. Rogers, and R. B. Horsch.** 1986. Genetic transformation in higher plants. *Crit. Rev. Plant Sci.* 4:1-46.
- Frame B.R., P.R. Drayton, S.V. Bagnall, C.J. Lewnau, W.P. Bullock, H.M. Wilson, J.M. Dunwell, J.A. Thompson, and K. Wang.** 1994. Production of fertile maize plants by silicon carbide whisker-mediated transformation. *Plant J.* 6:941-948.
- Fukumori, F., and R.P. Hausinger.** 1993. Purification and characterization of 2,4-dichlorophenoxyacetate/ α -ketoglutarate dioxygenase. *J. Biol. Chem.* 268: 24311-24317.
- Gamborg, O.L., R. A. Miller, and K. Ojima.** 1968. Nutirent requirements of suspensions of cultures of soybean root cells. *Exp. Cell Res.* 50:151-158.
- Heap, I.** The International Survey of Herbicide Resistant Weeds. Online. Internet. March 18, 2004 . Available at weedsience.com.
- Hiei Y., T. Komari, and T. Kubo.** 1997. Tranformation of rice mediated by *Agrobacterium tumefaciens*, *Plant Mol. Biol.* 35:205-218.
- Hinchee M.A.W., D.V. Conner-Ward, C.A. Newell, R.E. McDonnell, S.J. Sato, C.S. Gasser, D.A. Fischhoff, D.B. Re, R.T. Fraley, R.B. Horsch.** 1988. Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Bio/Technology* 6:915-922.

- Höfte, H. and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. 1989. Microbiol. Rev. 53:242-255.
- Hoekema, A.** 1985. In: *The Binary Plant Vector System*, Offset-drukkerij Kanters B.V., Alblasterdam, Chapter 5.
- Hogan, D.A.; S.R. Smith, E.A. Saari, J. McCracken, R.P. Hausinger.** 2000. Site-directed mutagenesis of 2,4-dichlorophenoxyacetic acid/a-ketoglutarate dioxygenase. Identification of residues involved in metallocenter formation and substrate binding. J. Biol. Chem. 275:12400-12409.
- Holsters, M., D. De Waele, A. Depicker, E. Messens, M. Van Montagu, and J. Schell.** 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen. Genet. 163:181-187.
- Horsch, R., J. Fry, N. Hoffman, J. Neidermeyer, S. Rogers, and R. Fraley.** 1988. In Plant Molecular Biology Manual, S. Gelvin *et al.*, eds., Kluwer Academic Publishers, Boston
- Horvath, M., G. Ditzelmüller, M. Lodl, and F. Streichsbier.** 1990. Isolation and characterization of a 2-(2,4-dichlorophenoxy)propionic acid-degrading soil bacterium. Appl. Microbiol Biotechnol. 33:213-216.
- Jefferson, R. A. M. Bevan, and T. Kavanagh.** 1987. The use of *Escherichia coli* β -glucuronidase gene as a gene fusion marker for studies of gene expression in higher plants. Biochem. Soc. Trans. 15: 17-19.
- Karlin, S. and S.F. Altschul.** 1990. Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. PNAS 87:2264-2268.

- Karlin, S. and S.F. Altschul.** 1993. Applications and statistics for multiple high-scoring segments in molecular sequences. PNAS 90:5873-5877
- Keller, G.H., and M.M. Manak.** 1987. DNA Probes. Stockton Press, New York, NY, pp. 169-170.
- Kohler, H.P.E.** 1999. *Sphingobium herbicidovorans* MH: a versatile phenoxyalkanoic acid herbicide degrader. J. Ind Microbiol and Biotech. 23:336-340.
- Lorraine-Colwill, D. F., S. B. Powles, T. R. Hoawkes, P.H. Hollingshead, S.A.J. Arner, and C. Preston.** 2003. Investigations into the mechanism of glyphosate resistance in *Lolium rigidum*. Pestic. Biochem. Physiol. 74:62-73.
- Lyon, B.R., D. J. Llewellyn, J. L. Huppatz, E. S. Dennis, and W. J. Peacock.** 1989. Expression of a bacterial gene in transgenic tobacco confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid. Plant Mol. Bio. 13:533-540.
- Lyon, B.R., Y. L. Cousins, D. J. Llewellyn, and E. S. Dennis.** 1993. Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophenoxyacetic acid. Transgenic Res. 2: 166-169.
- Maniatis, T., E.F. Fritsch, J. Sambrook.** 1982. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Martin, J.R. and W.W. Witt.** 2002. Response of glyphosate tolerant and susceptible biotypes of horseweed (*Conyza canadensis*) to foliar applied herbicides. Proc.North Cent.Weed Sci.Soc. 57:185.
- Martinell B.J., L.S. Julson, C.A. Emler, H. Yong, D.E. McCabe, E.J. Williams.** 2002. Soybean *Agrobacterium* transformation method. U.S. Patent Application No. 6384301

- McCabe D.E., W.F. Swain, B.J. Martinell, P. Christou.** 1988. Stable transformation of soybean (Glycine max) by particle acceleration. *Bio/Technology* 6:923-926.
- Miller S.D., P.W. Stahlman, P. Westra, G.W. Wicks, R.G. Wilson, J.M. Tichota.** 2003. Risks of weed spectrum shifts and herbicide resistance in glyphosate-resistant cropping systems. *Proc.West.Soc.Weed Sci.* 56:61-62.
- Muller R, S. Jorks, S. Kleinsteuber, W. Babel.** 1999. *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichlorprop and mecoprop and the herbicides 2,4-D and MCPA. *Microbiol. Res.* 154:241-246.
- Murashige T. and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Murphy G.P., T.E. Dutt, R.F. Montgomery, T.S. Willard, G.A. Elmore.** 2002. Control of horseweed with glyphosate. *Proc.North Cent.Weed Sci.Soc.* 57:194.
- Ng, C.H., R. Wickneswari, S. Salmigah, Y.T. Teng, and B.S. Ismail.** 2003. Gene polymorphisms in glyphosate-resistant and -susceptible biotypes of *Eleusine indica* from Malaysia. *Weed Res.* 43:108-115.
- Olhoft P.M. and D.A. Somers.** 2001. L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Rep* 20: 706-711
- Olhoft, P.M., L.E. Flagel, C.M. Donovan, and D.A. Somers.** 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta* 216:723-735
- Padgett S.R., K.H. Kolacz, X. Delannay, D.B. Re, B.J. LaVallee, C.N. Tinius, W.K. Rhodes, Y.I. Otero, G.F. Barry, D.A. Eichholtz, V.M. Peschke, D.L. Nida, N.B. Taylor, and**

- G.M. Kishore.** 1995. Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci.* 1995;35:1451-1461.
- Parrott W.A., J. N. All, M.J. Adang, M.A. Bailey, H.R. Boerma, and C.N. Stewart, Jr.** 1994. Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. kurstaki insecticidal gene. *In Vitro Cell. Dev. Biol.* 30P:144-149.
- Popelka, J. C. and F. Altpeter** 2003. *Agrobacterium tumefaciens*-mediated genetic transformation of rye (*Secale cereale* L) *Mol. Breed* 11:203-211
- Saari, R.E., D.A. Hogan, and R.P. Hausinger.** 1999. Stereospecific degradation of the phenoxypionate herbicide dichlorprop. *J. Mol. Catal. B: Enz.* 6:421-428.
- Saiki, R. K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim.** 1985. Enzymatic Amplification of β -Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. *Science* 230:1350-1354.
- Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press.
- Sambrook, J. and D.W. Russell.** 2000. *Molecular Cloning*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sfiligoj, E.** 2004. Spreading resistance. *Crop Life*. March issue.
- Simarmata, M., J.E. Kaufmann, and D. Penner.** 2003. Potential basis of glyphosate resistance in California rigid ryegrass (*Lolium rigidum*). *Weed Sci.* 51:678-682.
- Singh R.J., T.M. Klein, C.J. Mauvais, S. Knowlton, T. Hymowitz, C.M. Kostow.** Cytological characterization of transgenic soybean. *Theor. Appl. Genet.* 1998; 96:319-324.

- Smejkal, C.W., T. Vallaey, S.K. Burton, and H.M. Lappin-Scott.** 2001. Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria is not dependent upon phylogenetically related *tfdA* gene types. *Biol. Fertil. Sols* 33:507-513.
- Streber, W. and L. Willmitzer.** 1989. Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. *Bio/Technology* 7:811-816.
- Streber, W.R., K.N. Timmis, and M. H. Zenk.** 2000. Microorganisms and plasmids for 2,4-dichlorophenoxyacetic acid (2,4-D) monooxygenase formation and process for the production of these plasmids and strains. US Pat. 6,153,401.
- Streber, W.R., K.N. Timmis, and M.H. Zenk.** 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenylacetic monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. *J. Bacteriol.* 169:2950-2955.
- Suggs, S.V., T. Miyake, E.H. Kawashima, M.J. Johnson, K. Itakura, and R.B. Wallace.** 1981. *ICN-UCLA Symp. Dev. Biol. Using Purified Genes*, D.D. Brown [ed.], Academic Press, New York, 23:683-693.
- Tingay, S., D. McElroy, R. Kalla, S. Fieg, W. Mingbo, S. Thornton, and R. Bretell.** 1997. *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J.* 11:1369-1376.
- Weigel, D. and J. Glazebrook.** 2002. *Arabidopsis: A Laboratory Manual*. Cold Spring Harbor Lab Press, Cold Spring Harbor, N. Y.. 358 pp.
- Weising, K., J. Schell, and G. Kahl.** 1988. Foreign genes in plants: transfer, structure, expression and applications. *Ann. Rev. Genet.* 22:421-477.
- Welter, M.E., D.S. Clayton, M.A. miler, and J.F. Petolino.** 1995. Morphotypes of friable embryogenic maize callus. *Plant Cell Rep.* 14:725-729.

- Westendorf A., D. Benndorf, R. Muller, W. Babel.** 2002. The two enantiospecific dichlorprop/ α -ketoglutarate-dioxygenases from *Delftia acidovorans* MC1-protein and sequence data of *RdpA* and *SdpA*. *Microbiol. Res.* 157:317-22.
- Westendorf, A., R.H. Muller, and W. Babel.** 2003. Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* MC1 initiating the degradation of phenoxypropionates and phenoxyacetate herbicides. *Acta Biotechnol.* 23: 3-17.
- WSSA.** 2002. *Herbicide Handbook* (8th ed). Weed Science Society of America. Lawrence, KS 492 pp.
- Zeng P, D. Vadnais, Z. Zhang, and J. Polacco.** 2004. Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merr.]. *Plant Cell Rep.* 22: 478 – 482.
- Zhang, Z., A. Xing, P.E. Staswick, and T. E. Clemente.** 1999. The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell, Tissue, and Organ Culture* 56:37-46.
- Zhao, Z.Y., T. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer, and D. Pierce.** 2000. *Agrobacterium*-mediated sorghum transformation, *Plant Mol. Biol.* 44:789-798.
- Zipper, C., M. Bunk, A. Zehnder, H. Kohler.** 1998. Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by *Sphingobium herbicidovorans* MH. *J. Bact.* 13:3368-3374.

Abstract

[00323]

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

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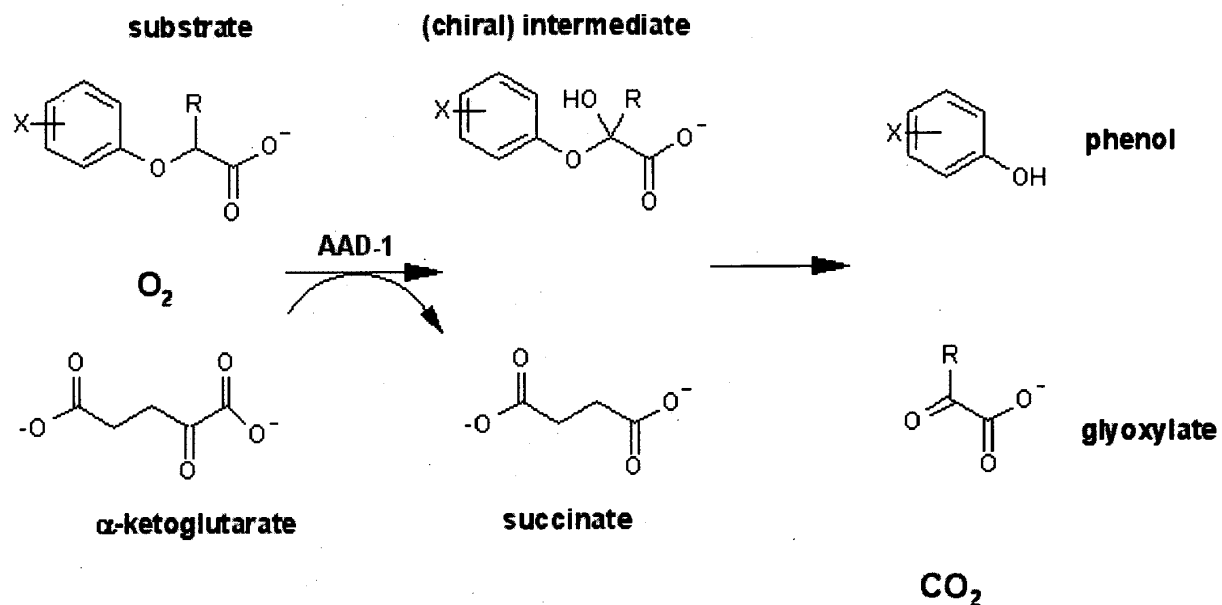
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Figure 1: General scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.



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

Figure 2: Representative data showing the loss of herbicidal activity from a 2,4-D solution treated with heterologously-expressed AAD-1 (v1) enzyme extract. EM2 is the background culture media and the pET vector control extract has no *AAD-1* (v1) gene to express.

Arabidopsis Plate Test Root Length Effect Elicited by 2,4-D + AAD1 v1 extract reaction solution

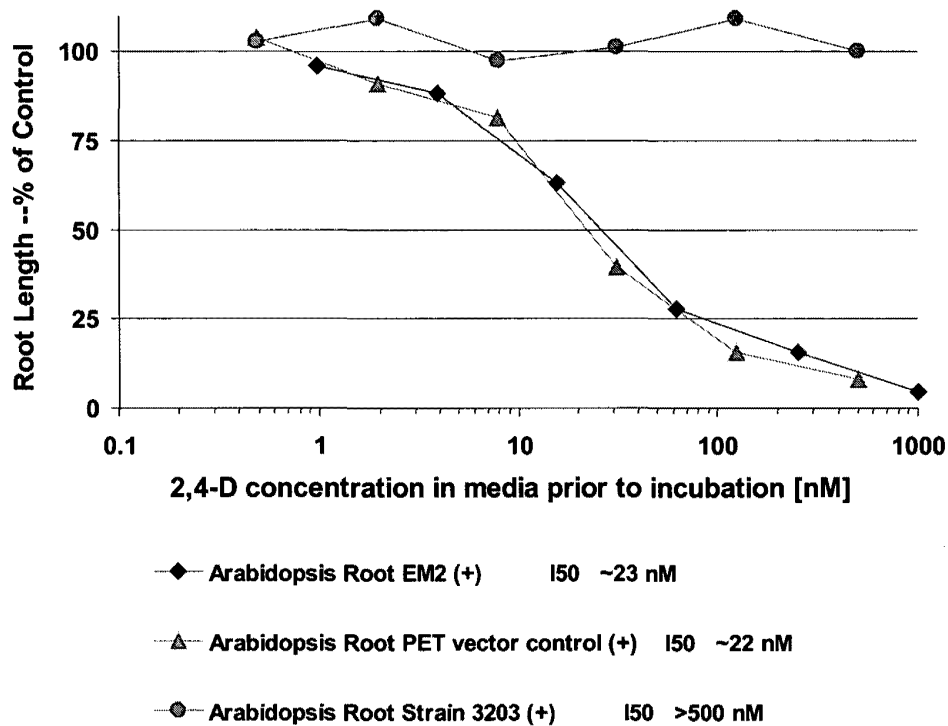


Figure 3: Representative data showing the loss of herbicidal activity from a haloxyfop solution treated with heterologously-expressed AAD-1 (v1) enzyme extract. pET vector control extract has no *AAD-1* (v1) gene to express.

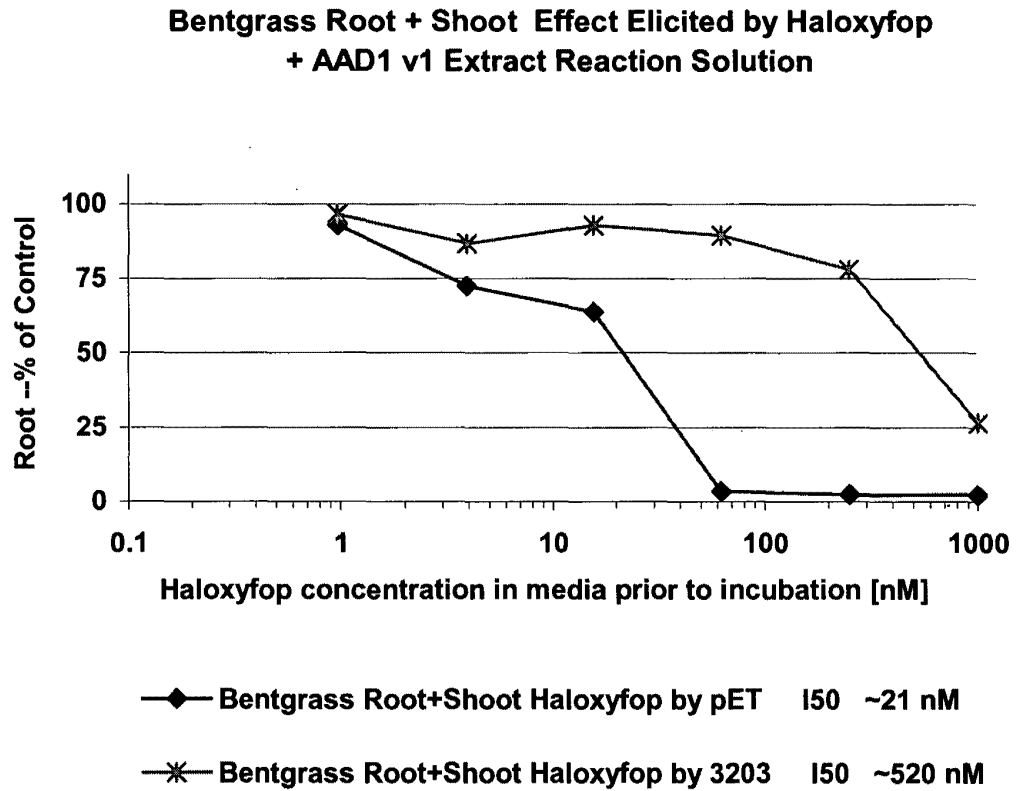


Figure 4: Anticipated phenols produced from various herbicides catalyzed by AAD-1.

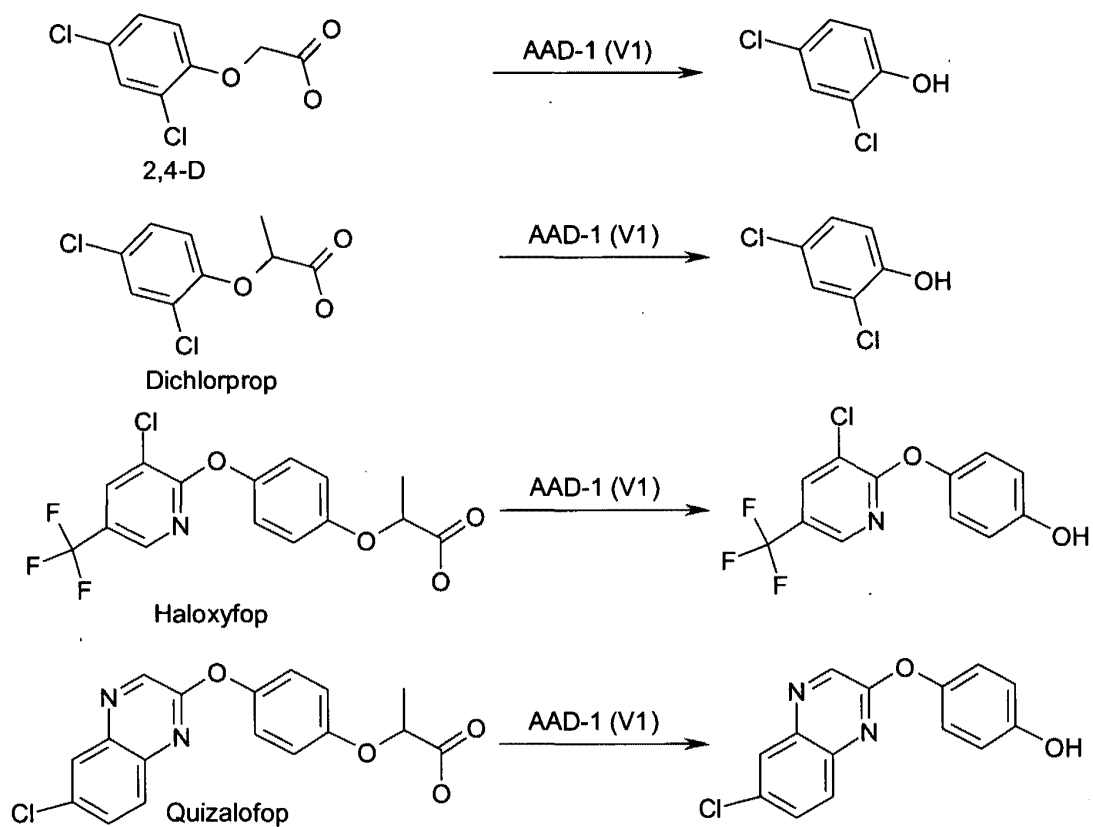


Figure 5: Activity of recombinant AAD-1 (v1) expressed in *E. coli*. **2,4-dichlorophenol production monitored by colorimetric detection at 510 nm.** Varying amounts of extracts from *E. coli* expressing a plasmid for *AAD-1* (v1) or a control plasmid were assayed using 1 mM 2,4-D as substrate.

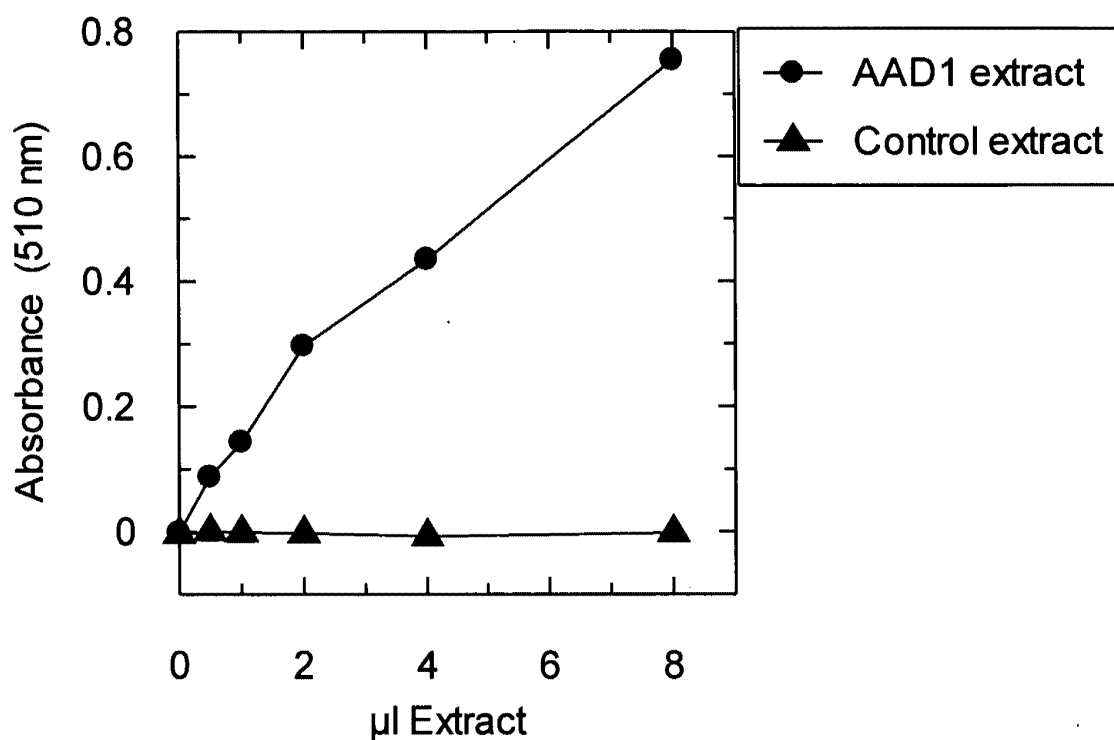
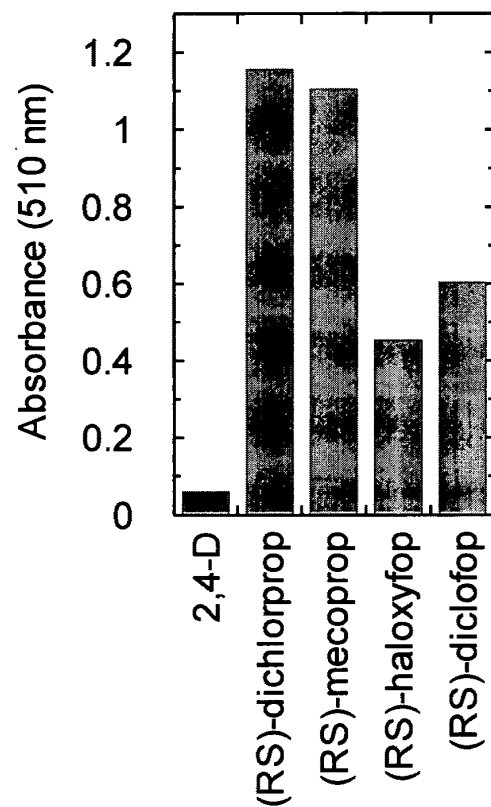


Figure 6: Activity of recombinant AAD-1 (v1) expressed in *E. coli*. **Phenol production by AAD-1 (v1) extract from various herbicide substrates:** Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μ M Fe²⁺, 200 μ M Na ascorbate, 1 mM α -ketoglutarate using 4 μ l *E. coli* extract.

6A.



6B.

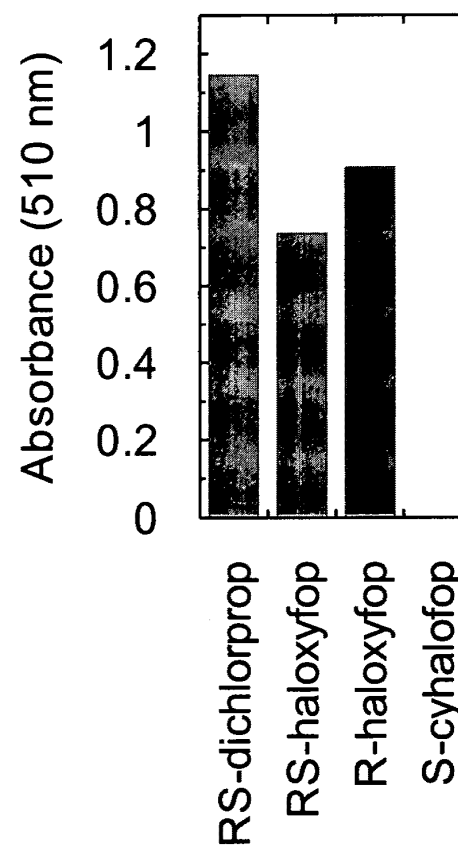


Figure 7: **Response of AAD-1 (v1) reaction rate to substrate concentration for four herbicide substrates.** Curves were fitted to the experimental data using Grafit and the Michaelis-Menten equation. The derived kinetic constants are shown in Table 9.

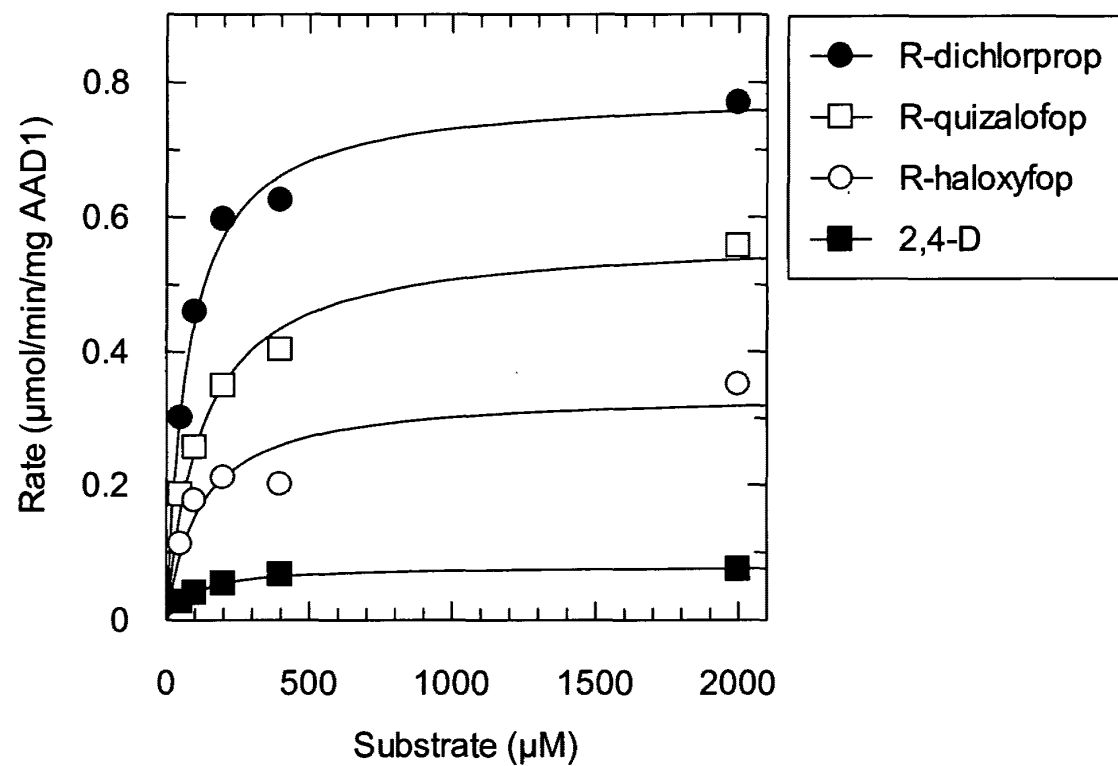
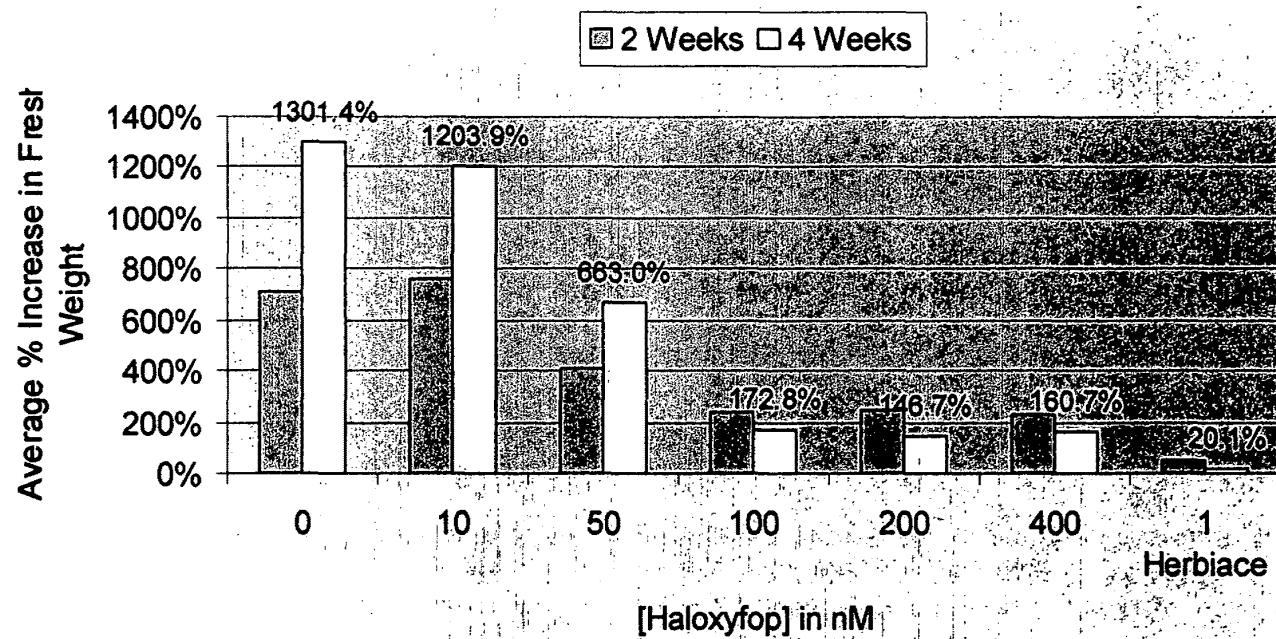
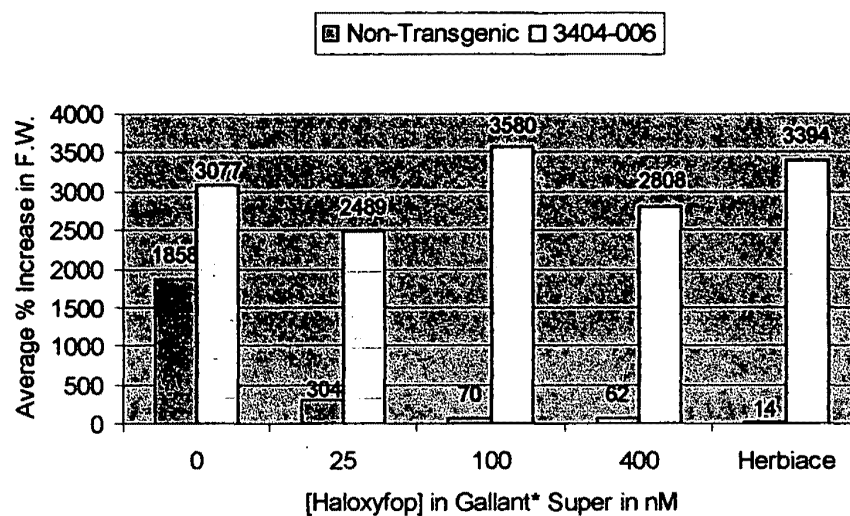


Figure 8: Dose response of callused maize suspensions to R-haloxyfop after 4 weeks.



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Figure 9. *AAD-1* (v3)-transformed corn callus response to haloxyfop.



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Figure 10. Response of several AAD-1 (v3) –transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

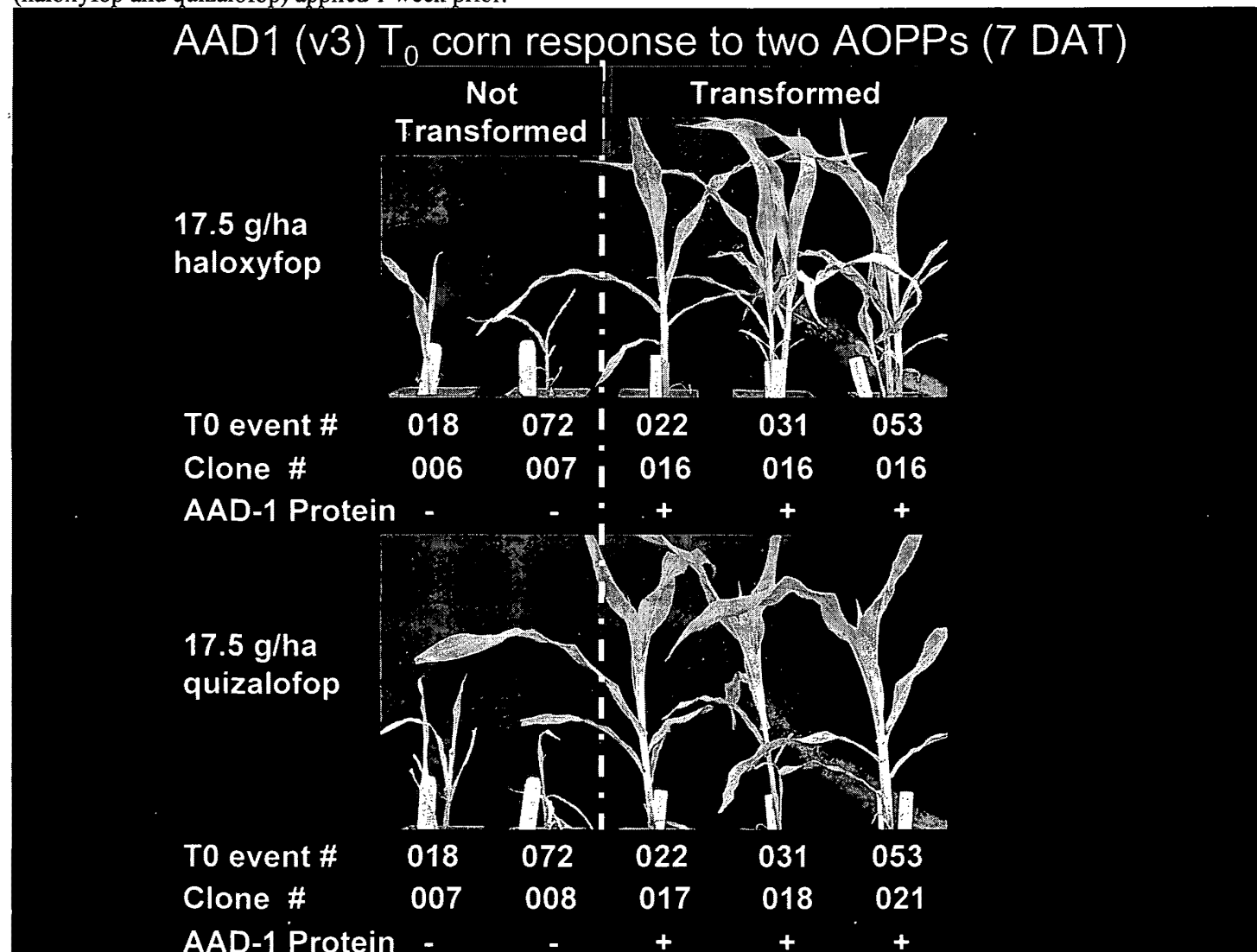


Figure 11. Fitted curves showing relative activities on 2,4-D by AAD-1 (v1) and AAD-2. Concentrations of AADs in each extract are approximately equivalent

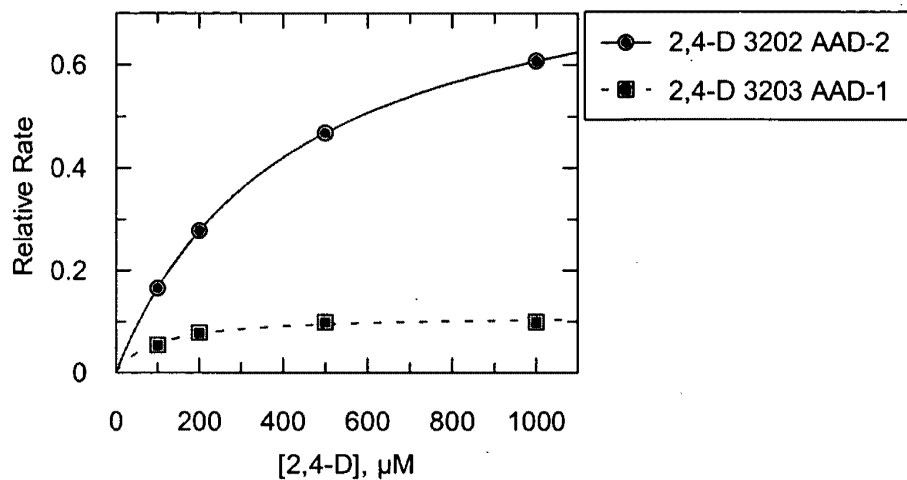


Figure 12. Dose response of AAD-1 v3 (plant optimized), AAD-1 v2 (native), or AAD-2 (native)-transformed T₁ Arabidopsis to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T₁ family. The relative protective capability from 2,4-D injury of individual genes is plant optimized *AAD-1* (v3) > native *AAD-1* (v2) > native *AAD-2*.

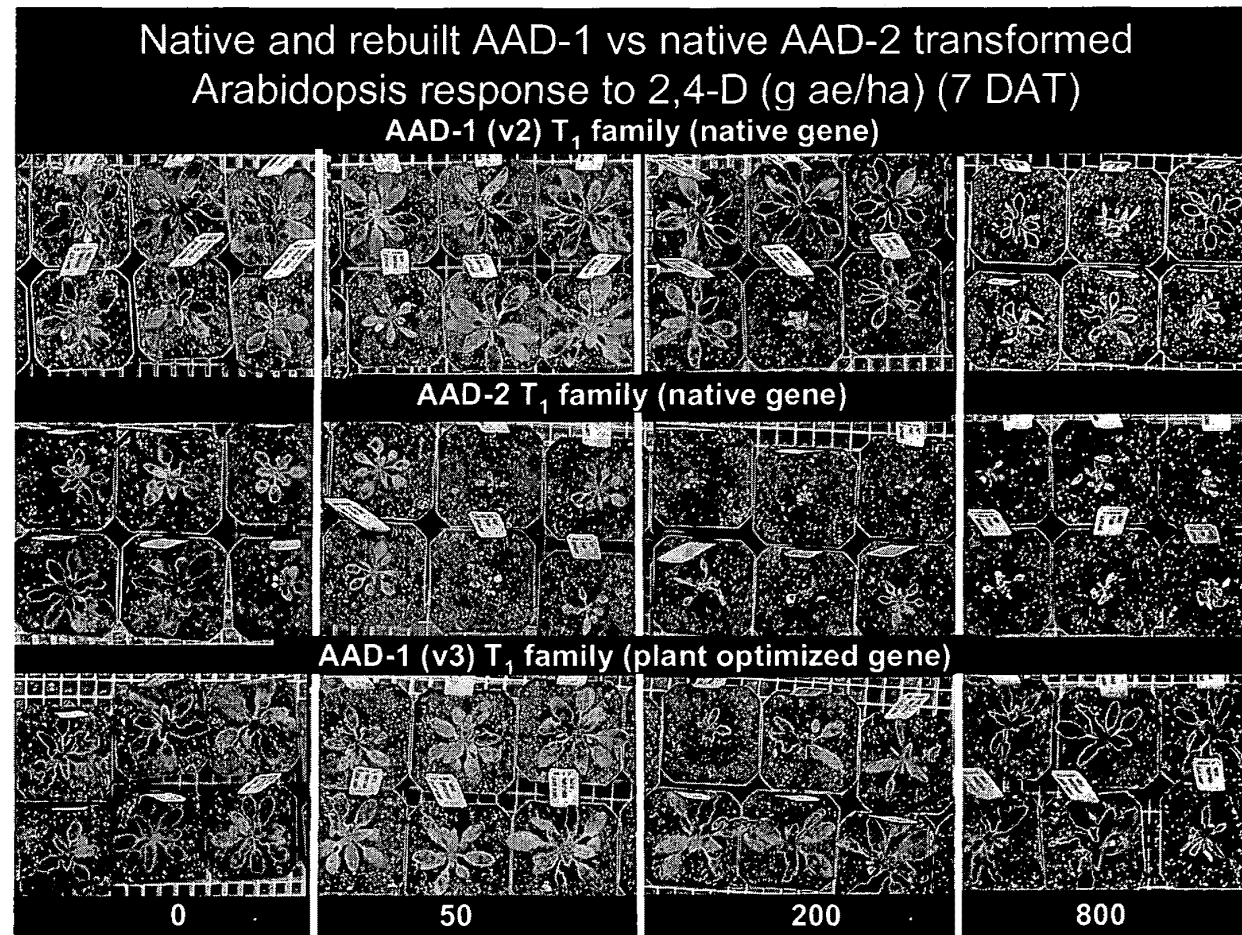
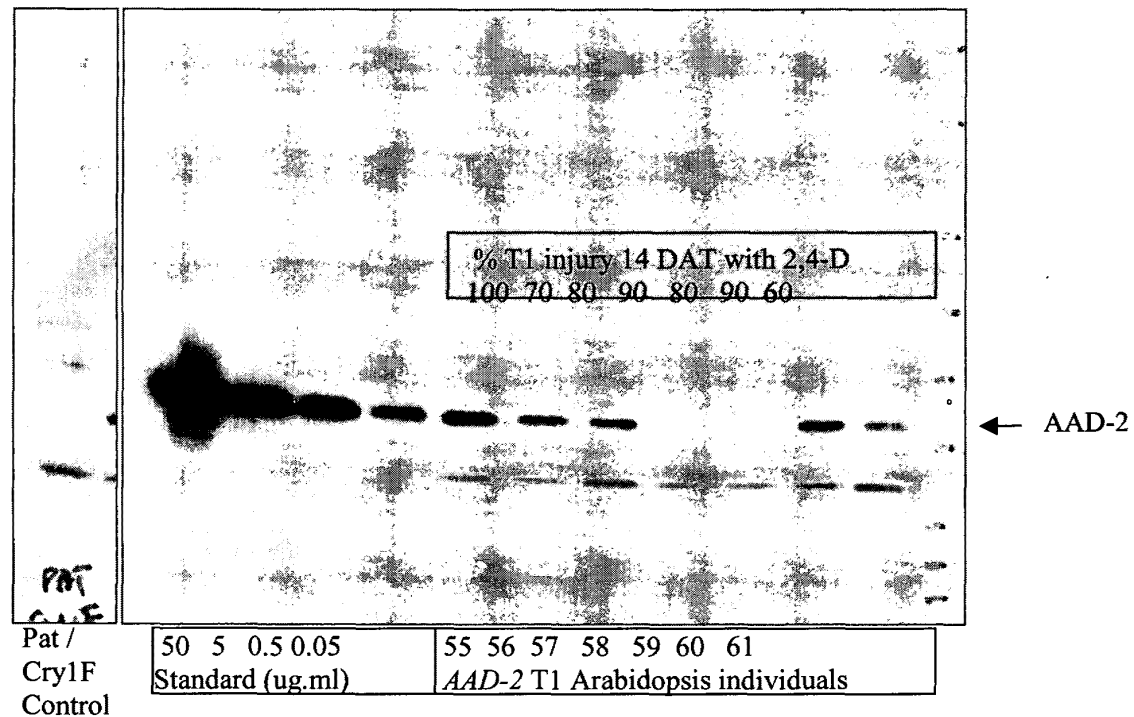


Figure 13. Western blot analysis of individual native *AAD-2*-transformed T1 Arabidopsis plants. This demonstrates plants expressing the *AAD-2* protein suffering severe injury from 200 g ae/ha 2,4-D treatments, which normally causes little injury to native *AAD-1* (v2) or plant optimized *AAD-1* (v3)-transformed Arabidopsis. *AAD-2* protein is identified on the gel. Several background bands were detected in *AAD-2* transformed and Pat/Cry1F transformed samples.



SEQ ID No. 1:

AAD-1 forward primer:

TCTAGAAGGAGATATACCATGCATGCTGCACTGTCCCCCTCTCCCAGCG

SEQ ID No. 2:

AAD-1 reverse primer:

CTCGAGTTACTAGCGCGCCGGGCGCACGCCACCGACCG

SEQ ID No. 3:

AAD-1 (v1) primary sequence: (primer linkers are highlighted and underlined)

TCTAGAAGGAGATATACCATGCATGCTGCACTGTCCCCCTCTCCCAGCGCTTTGAGCGCA
TCGCGGTCCAGCCGCTGACCGGCGTCCTGGGCGCCGAGATCACCGGCGTCGACCTGCGC
GAGCCGCTCGACGACAGCACCTGGAACGAAATCCTCGACGCGTTCCACACTTACCAGGTCA
TCTATTTTCCCGGCCAGGCGATCACCAACGAACAGCACATCGCCTTCAGCCGGCGCTTCGG
CCCCGTCGATCCCGTGCCCCCTGCTCAAGAGCATCGAAGGGTATCCAGAGGTGCAGATGAT
CCGCCGGAAGCCAACGAAAGCGGGCGTGTGATCGGTGAYGACTGGCACACCGACAGCAC
CTTCCTGGACGCACCGCCGCGCCGCTGGTGATGCGCGCGATCGACGTGCCCGAGCATG
GCGGCGACACCGGTTTCTGAGCATGTACACCGCGTGGGAGACGCTGTGCGCCACCATGC
AGGCCACCATCGAAGGGTTGAACGTAGTGACAGCGCCACGCGTGTGTTCGGCTCGCTCT
ACCAGGCCCAGAACC GGCGCTTCAGCAACACCAGCGTCAAGGTGATGGACGTGACGCGG
GCGACCGTGAACCGTGACCCCCCTGGTGGTGACCCATCCGGGCAGCGGCCGCAAGGGC
CTGTACGTGAACCAGGTCTATTGCCAGCGCATCGAGGGCATGACCGATGCCGAAAGCAAAC
CGCTGCTGCAGTTCCTGTACGAGCATGCGACACGGTTCGATTTACCTGCCGCGTGCGCTG
GAAGAAGGACCAGGTCTGGTCTGGGACAACCTGTGCACGATGCACCGGGCCGTACCCGA
CTACGCGGGCAAGTTCGGCTACCTGACGCGCACCACGGTCGGTGGCGTGCGCCCGGCGC
GCTAGTAACTCGAG

SEQ ID No. 4:

AAD-1 (v2) primary sequence:

CCATGGCTGCTGCACTGTCCCCCTCTCCCAGCGCTTTGAGCGCATCGCGGTCCAGCCGC
TGACCGGCGTCCTGGGCGCCGAGATCACCGGCGTCGACCTGCGCGAGCCGCTCGACGAC
AGCACCTGGAACGAAATCCTCGACGCGTTCCACACTTACCAGGTCACTATTTTCCCGGCCA
GGCGATCACCAACGAACAGCACATCGCCTTCAGCCGGCGCTTCGGCCCCGTCGATCCCGT
GCCCCTGCTCAAGAGCATCGAAGGGTATCCAGAGGTGCAGATGATCCGCCGCGAAGCCAA
CGAAAGCGGGCGTGTGATCGGTGATGACTGGCACACCGACAGCACCTTCCTGGACGCACC
GCCGGCCGCCGTGGTGATGCGCGCGATCGACGTGCCCGAGCATGGCGGCGACACCGGTT
TTCTGAGCATGTACACCGCGTGGGAGACGCTGTGCGCCACCATGCAGGCCACCATCGAAG
GGTTGAACGTAGTGACAGCGCCACGCGTGTGTTTCGGCTCGCTCTACCAGGCCCAGAACC
GGCGCTTCAGCAACACCAGCGTCAAGGTGATGGACGTGACGCGGGCGACCGTGAAACCG
TGACCCCCCTGGTGGTGACCCATCCGGGCAGCGGCTGCAAGGGCCTGTACGTGAACCAGG
TCTATTGCCAGCGCATCGAGGGCATGACCGATGCCGAAAGCAAACCGCTGCTGCAGTTCCT
GTACGAGCATGCGACACGGTTCGATTTACCTGCCGCGTGCGCTGGAAGAAGGACCAGGT

CCTGGTCTGGGACAACCTGTGCACGATGCACCGGGCCGTACCCGACTACGCGGGCAAGTT
CCGCTACCTGACGCGCACCACGGTCGGTGGCGTGCGCCCGGCGCGCTAGTTGAGCTC

SEQ ID No. 5:

AAD-1 (v3) primary sequence:

CCATGGCTCATGCTGCCCTCAGCCCTCTCTCCCAACGCTTTGAGAGAATAGCTGTCCAGCC
ACTCACTGGTGTCTTGGTGCTGAGATCACTGGAGTGGACTTGAGGGAACCACTTGATGAC
AGCACCTGGAATGAGATATTGGATGCCTTCCACACTTACCAAGTCATCTACTTTCCTGGCCA
AGCAATCACCAATGAGCAGCACATTGCATTCTCAAGAAGGTTTGGACCAGTTGATCCAGTGC
CTCTTCTCAAGAGCATTGAAGGCTATCCAGAGGTTTCTAGATGATCCGCAGAGAAGCCAATGA
GTCTGGAAGGGTGATTGGTGATGACTGGCACACAGACTCCACTTTCCTTGATGCACCTCCA
GCTGCTGTTGTGATGAGGGCCATAGATGTTCTGAGCATGGCGGAGACACTGGGTTTCCTTT
CAATGTACACAGCTTGGGAGACCTTGTCTCCAACCATGCAAGCCACCATCGAAGGGCTCAA
CGTTGTGCACTCTGCCACACGTGTGTTTCGGTTCCTCTACCAAGCACAGAACCGTCGCTTC
AGCAACACCTCAGTCAAGGTGATGGATGTTGATGCTGGTGACAGAGAGACAGTCCATCCCT
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CACCAGATTTGACTTCACTTGCCGTGTGAGGTGGAAGAAAGACCAAGTCCTTGTCTGGGAC
AACTTGTGCACCATGCACCGTGTGTTCTGACTATGCTGGCAAGTTCAGATACTTGACTCG
CACCACAGTTGGTGAGTTAGGCCTGCCCGCTGAGTAGTTAGCTTAATCACCTAGAGCTC

SEQ ID No. 6:

RdpA(ncol) 5" primer

CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC

SEQ ID No. 7:

3'saci primer

GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA

SEQ ID No. 8:

BstEII/ Del NotI 5' primer

TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC

SEQ ID No. 9:

AAD-1 v1 Translation (primary protein sequence)

MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFH
TYQVIYFPGQAITNEQHIAFSRRFGPVDPVPLLSIEGYPEVQMIRREANESGRV
IGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQ
ATIEGLNVVHSATRVFSGSLYQAQNRFSNTSVKVMVDVDAGDRETVHPLVVT
HPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 10:

AAD-1 v2 Translation (differences from v1 are highlighted)

M~~A~~AAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTY
QVIYFPGQAITNEQHIAFSRRFGPVDVPLLKSIEGYPEVQMIRREANESGRV
IGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQ
ATIEGLNVVHSATRVFGSLYQAQNRFSNTSVKVMVDVAGDRETVHPLVVT
HPGSG~~C~~KGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 11:

AAD-1 v3 Translation

M~~A~~~~T~~AAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHT
YQVIYFPGQAITNEQHIAFSRRFGPVDVPLLKSIEGYPEVQMIRREANESGR
VIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTM
QATIEGLNVVHSATRVFGSLYQAQNRFSNTSVKVMVDVAGDRETVHPLVV
THPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 12

AAD-2 nucleotide sequence

ATGACGATCGCCATCCGGCAGCTTCAGACGCATTTTGTCTGGCCAGGTTTC
CGGCCTCGATTTGCGAAAGCCGCTCACGCCGGGCGAGGCCCGCGAGGT
CGAGTCCGCCATGGACAAATACGCGGTGCTCGTTTTCCACGACCAGGACA
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SEQ ID No. 13

AAD-2 translation

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SEQ ID No. 14

brjap 5'(speI)

ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T

SEQ ID No. 15

br jap 3' (xhoI)

TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC

SEQ ID No. 16

M13 Forward

GTA AAA CGA CGG CCA GT

SEQ ID NO:17

M13 Reverse

CAG GAA ACA GCT ATG AC

SEQ ID No. 18

NcoI of Brady

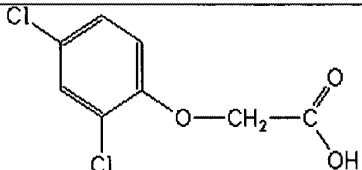
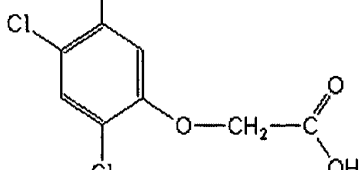
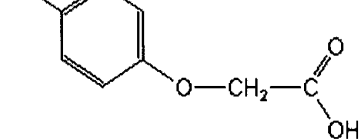
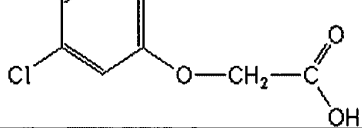
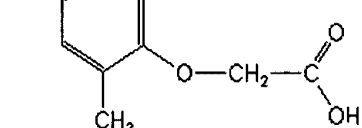
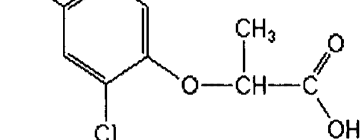
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Table 1. Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered 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.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	
Dichlorprop	120-36-5	25 – 12000	100 - 2240	

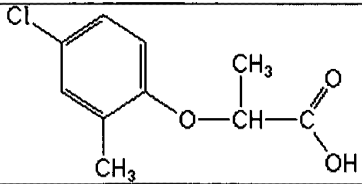
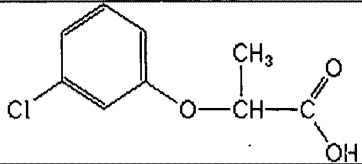
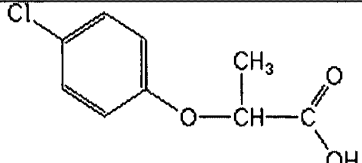
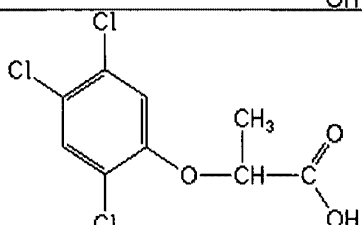
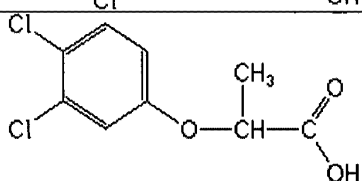
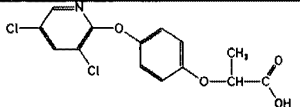
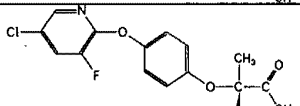
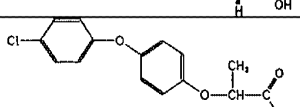
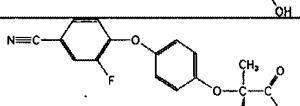
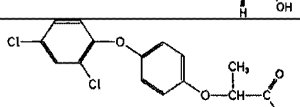
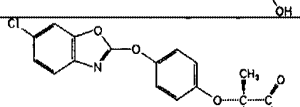
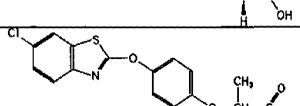
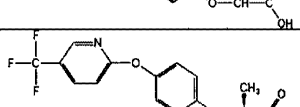
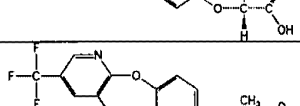
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Mecoprop	7085-19-0	25 – 4000	250 - 3360	
Cloprop	101-10-0	25 – 4000	25 – 4000	
4-CPP	3307-39-9	25 – 4000	25 – 4000	
Fenoprop	93-72-1	25 – 4000	25 – 4000	
3,4-DP	3307-41-3	25 – 4000	25 – 4000	

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.

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	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25 - 420	
Haloxifop	69806-40-2	10-2000	20 - 600	

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Isoxapyrifop	87757-18-4	10-2000	30 - 240	
Metamifop	256412-89-2	10-2000	35 - 280	
Propaquizafop	111479-05-1	10-2000	30 - 240	
Quizalofop	76578-14-8	10-2000	20 - 240	
Trifop	58597-74-4	10-2000	10-2000	

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.

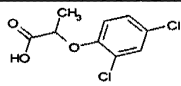
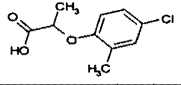
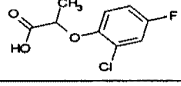
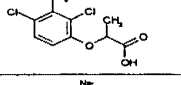
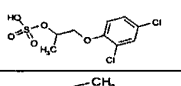
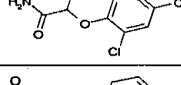
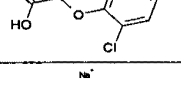
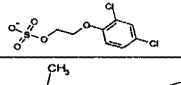
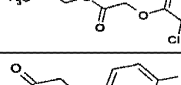
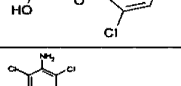
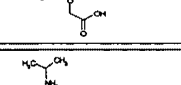
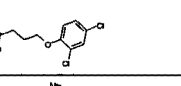
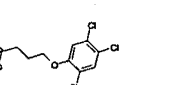
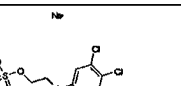
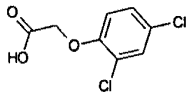
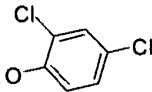
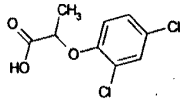
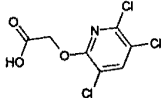
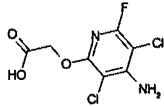
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichloroprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxypropionate	0.304	2.358
	11113675	(R,S)-3-aminodichloroprop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02
	188527		0.003	0.036

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalifop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinafop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiafop	0.199	1.055
	8671	haloxyfop	0.18	1.5
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663

	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

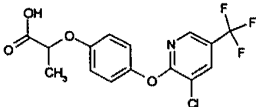
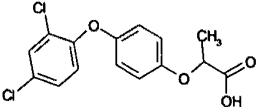
Table 6: *Arabidopsis* plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

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

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

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

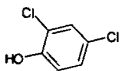
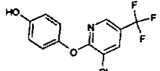
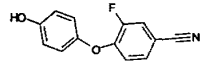
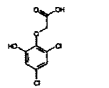
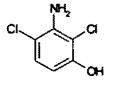
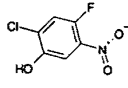
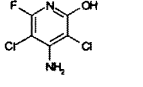
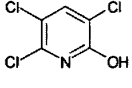
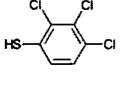
Table 7: Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR ₅₀ (nM)			GR ₅₀ ratio **	Structure
	Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1		
Haloxypop-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.

Table 8: Detection of various phenols derived from AAD-1 substrates. Phenols were added to assay buffer (25 mM MOPS pH 6.8, 200 μM Fe^{2+} , 200 μM Na ascorbate, 1 mM α -ketoglutarate) to give a final concentration of 100 μM , then treated with pH 10 buffer, 4-aminopyridine and potassium ferricyanide as described in the text. The absorbance at the peak wavelength was recorded.

Description	STRUCTURE	Registry ID	Absorbance	λ (nm)
Phenol from 2,4-D and dichlorprop		195116	0.45	510
Phenol from haloxyfop		53046	0.41	510
Phenol from cyhalofop		197928	0.43	510
6-hydroxy-2,4-D		11246087	0.40	580
3-amino-2,4-dichlorophenol		11263523	0.44	480
Dihalonitrophenol		411197	0.40	420
Pyridinol from fluroxypyr		61784	0.05	510
Pyridinol from triclopyr		159926	0.04	510
Thiophenol		84700	0.05	510

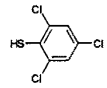
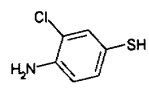
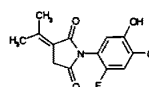
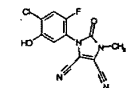
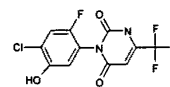
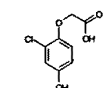
Thiophenol		84788	0.05	510
Aminothiophenol		488185	0.04	510
phenol of 208620		208596	0.16	510
phenol of 322183		254904	0.06	510
phenol of 323583		377704	0.17	510
phenol of 11220072 (cf. fluthiacet)		11213585	0.16	510
4-hydroxy-2-Cl phenoxyacetate		11241152	0.13	510

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

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

Table 10: 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*.

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

Table 11: 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

Table 12: Preferred amino acid codons for proteins expressed in maize

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

Table 13. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

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

Table 15: *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/CryIF*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

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

Table 16: T₁ *Arabidopsis* response to a range of dichlorprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T₁ is an independent transformation event, one can expect significant variation of individual T₁ responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/CryIF*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

AAD-1V3				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100

PAT/CryIF				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	23.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100

Wildtype				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

Table 17: 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.

AAD-1 v3				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100

PAT/Cry1F				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100

Wildtype				% Injury		
Averages	<20%	20-40%	>40%	Ave	Std dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

Table 18. Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

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

(lethal dose = 8.8 g ae/ha) (lethal dose = 62.5 g ae/ha) (lethal dose = 4.4 g ae/ha)

Note:

* Gallant super# + 1%COC (v/v)

**Clincher #+ 1%COC (v/v)

*** Assure II + 1%COC (v/v)

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