

[00477] 24.5 – Tissue harvesting, DNA isolation and quantification.

[00478] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00479] 24.6 – Southern blot analysis.

[00480] Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80°C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse - GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and 45 seconds followed by 72°C for 10 minutes.

[00481] The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it

RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65°C in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in **Table 36**.

Table 36. Southern Results.		
Event	Integration Southern data	PTU Southern data
	Number of bands	Expected size 3049bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

[00483] Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

[00484] 24.7 -- Western data

[00485] Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in **Table 37**.

Table 37.						
Lane	Sample Name	TSP (µg/mL)	ELISA		Western	
			[AAD1] (ng/mL)	Expression (ppm)		
1	Control	6719.58	0.00	0.00	-	
2	63-1A	8311.87	351.17	42.25	±	
3	63-1F	11453.31	2092.35	182.69	++	
4	63-4B	13835.09	216.00	15.61	+	
5	63-4D	13656.49	717.05	52.51	++	
6	63-6C	5343.63	0.00	0.00	-	
7	AAD1 Standard (0.5 µg/mL)				+++	
8	AAD1 Standard (5.0 µg/mL)				+++++	

Example 25 – Turf Grass Transformation Procedures

[00486] Genetic transformation, with *AAD-1* ($\nu 3$) substituted for the “bar” gene as described below, of creeping bentgrass mediated by *Agrobacterium tumefaciens* could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. See “Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation” (Luo *et. al.*, 2004).

[00487] Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T₁ generation. All independent transformation events carried one to three copies of the transgene, and a majority (60–65%) contained only a single copy of the foreign gene with no apparent rearrangements.

[00488] 25.1 – Seed preparation for embryogenic callus induction.

[00489] Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytigel. The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min).

[00490] 25.2 – Embryogenic callus induction.

[00491] The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

[00492] 25.3 – *Agrobacterium* infection and co-cultivation.

[00493] One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μ M acetosyringone. A 10- μ l aliquot of *Agrobacterium* (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C.

[00494] 25.4 – Resting stage and *Agrobacterium* control.

[00495] For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

[00496] 25.5 – Selection and potential transgenic colony identification.

[00497] Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

[00498] 25.6 – Regeneration of transgenic plants.

[00499] For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2–3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

[00500] 25.7 – Root induction and transfer to greenhouse.

[00501] Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3–5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

[00502] 25.8 – Vernalization and out-crossing of transgenic plants.

[00503] Transgenic plants were maintained out of doors in a containment nursery (3–6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3–4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25°C, and T1 plants were grown in the greenhouse for further analysis.

[00504] 25.9 – Other target grasses.

[00505] Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutata*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

Example 26 – AAD-1 (v3) in Canola

[00506] 26.1 – Canola transformation.

[00507] The AAD-1 (v3) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation. The construct contained AAD-1 (v3) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

[00508] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00509] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00510] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the

segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00511] Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of *AAD-1* (*v3*), whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the *AAD-1* protein as determined by ELISA (section on protein analysis).

[00512] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

[00513] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00514] The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the *AAD-1* (*v3*) gene.

[00515] 26.2 – “Molecular analysis”: canola materials and methods

[00516] 26.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

(Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00517] 26.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-1* (v3) were (Forward – ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse – CGGGCAGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-1* (v3) events tested positive. Three negative control samples tested negative.

[00518] 26.3 – ELISA.

[00519] Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

Table 38. Expression of AAD1 in Canola calli.					
Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	-
Control 2	61	2549.60	35.00	13.73	-
Control 3	59	2374.41	22.79	9.60	-

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ClaimsClaim 1.

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

Claim 2.

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

Claim 3.

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

Claim 4.

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

Claim 5.

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

Claim 6.

The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.

Claim 7.

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

Claim 8.

The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

Claim 9.

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

Claim 10.

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

Claim 11.

The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

Claim 12.

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

Claim 13.

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

Claim 14.

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

Claim 15.

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

Claim 16.

The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

Claim 17.

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

Claim 18.

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

Claim 19.

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

Claim 20.

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

Claim 21.

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

Claim 22.

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

Claim 23.

The method of claim 22 wherein said first herbicide and said second herbicide are applied sequentially.

Claim 24.

The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

Claim 25.

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

Claim 26.

The method of claim 13 wherein said plant is resistant to glyphosate.

Claim 27.

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

Claim 28.

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

Claim 29.

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

Claim 30.

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

Claim 31.

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

Claim 32.

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

Claim 33.

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

Claim 34.

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

Claim 35.

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

Claim 36.

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

Claim 37.

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

Claim 38.

The method of claim 28 wherein said method further comprises applying a third herbicide.

Claim 39.

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

Claim 40.

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

Claim 41.

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

Claim 42.

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

Claim 43.

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

Claim 44.

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

Claim 45.

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

Claim 46.

The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

Claim 47.

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

Claim 48.

The polynucleotide of claim 47 wherein said promoter is a plant promoter.

Claim 49.

The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

Claim 50.

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

Claim 51.

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

Claim 52.

A polynucleotide of claim 47 or 50 wherein said polynucleotide is used as a selectable marker in the method of claim 50.

Claim 53.

A method of detecting whether a plant comprises a polynucleotide of claim 47 or 50 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

Claim 54.

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

Claim 55.

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

Claim 56.

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

Claim 57.

A seed comprising a plant cell of claim 1.

Claim 58.

A plant grown from the seed of claim 57.

Claim 59.

A part, progeny, or asexual propagate of the plant of claim 5.

Claim 60.

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

Claim 61.

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

Claim 62.

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

Claim 63.

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

Claim 64.

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

Claim 65.

The method of claim 64 wherein said herbicide is a phenoxy auxin.

Claim 66.

The method of claim 64 wherein said herbicide is an aryloxy phenoxypropionate.

Claim 67.

The method of claim 64 wherein said plants are dicots.

Claim 68.

The method of claim 64 wherein said plants are monocots.

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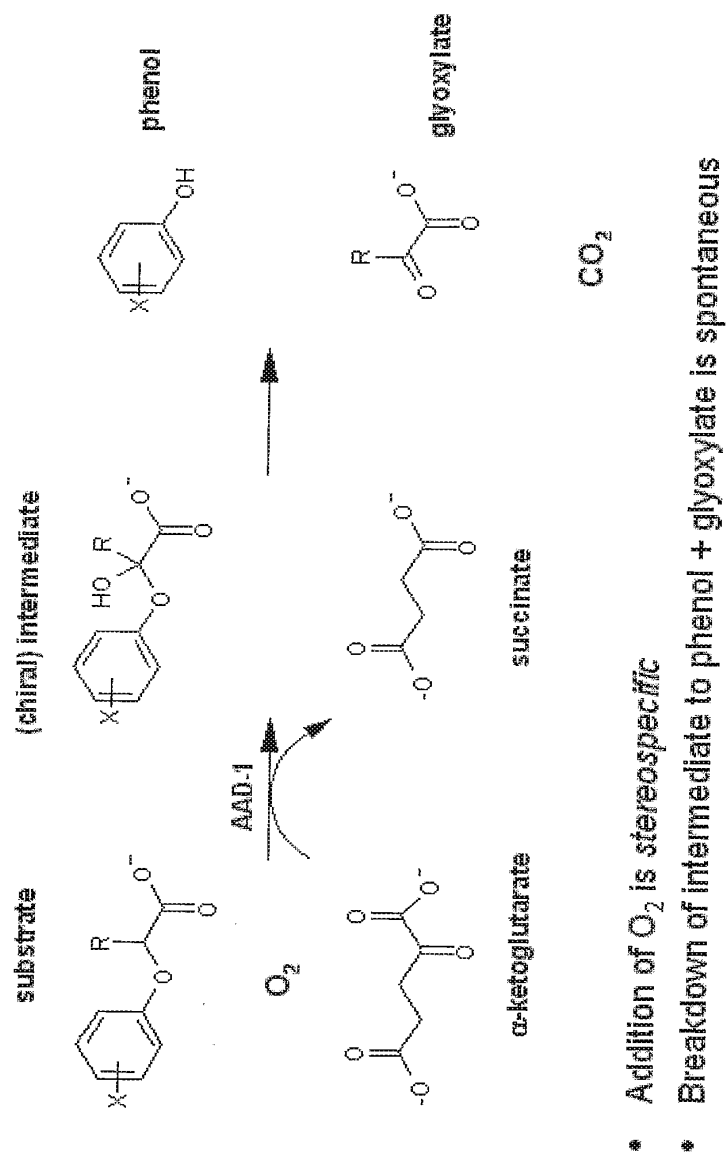


Fig. 1

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Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution

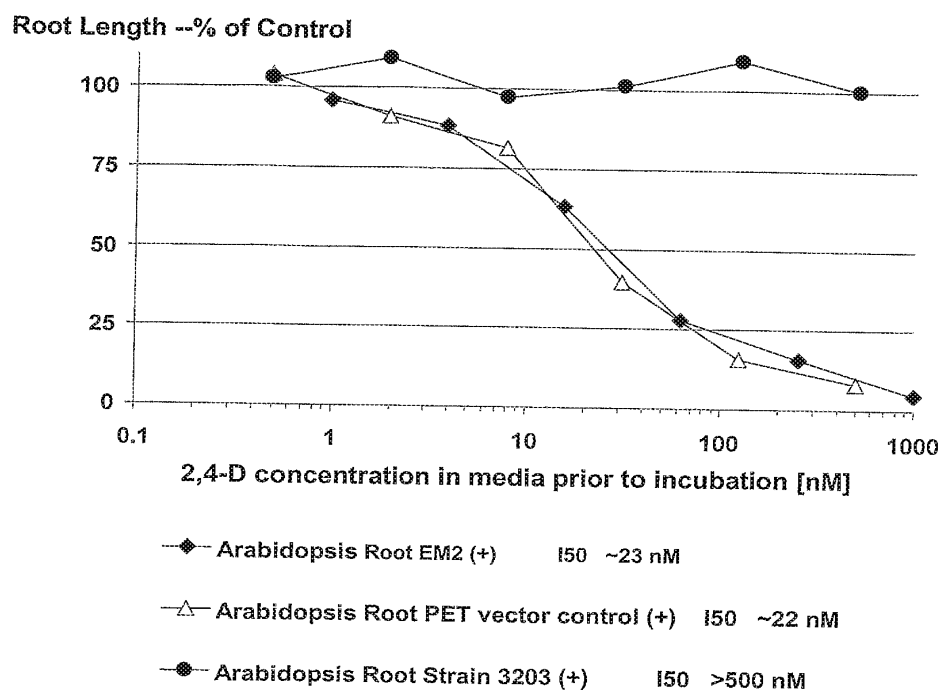


Fig. 2

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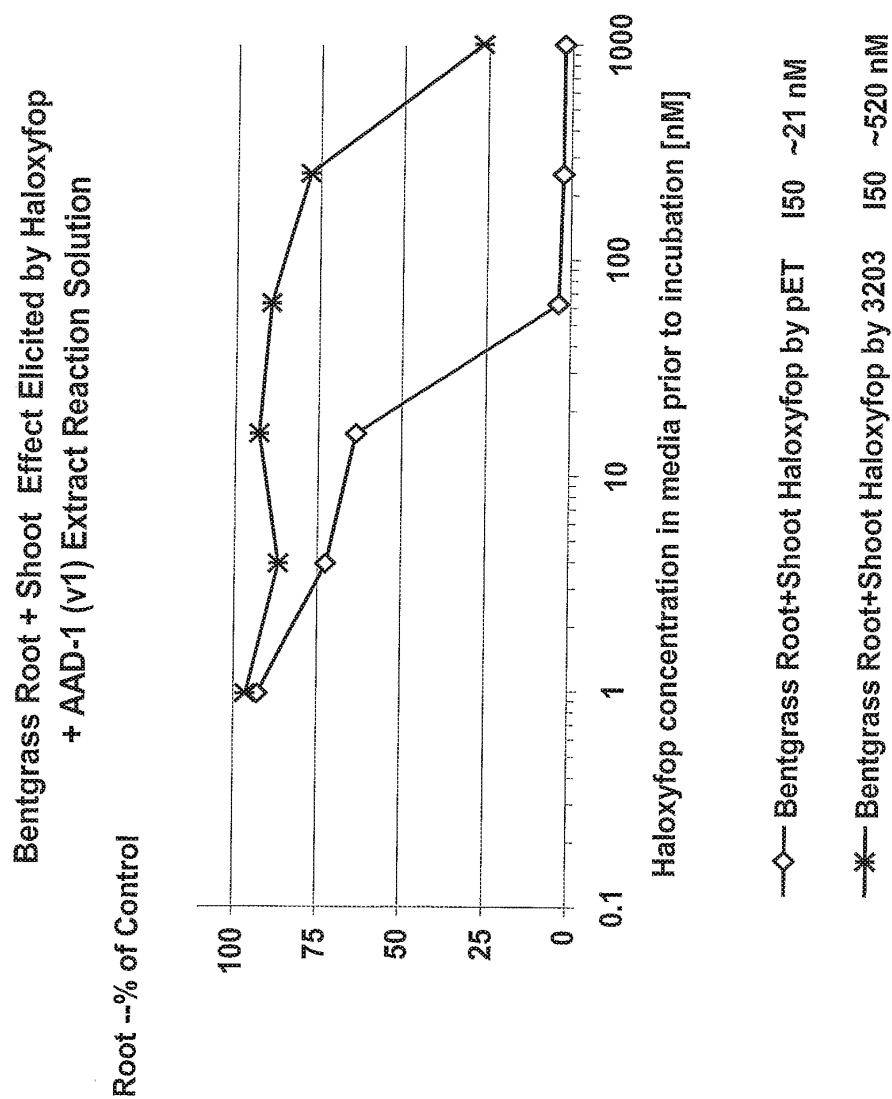


Fig. 3

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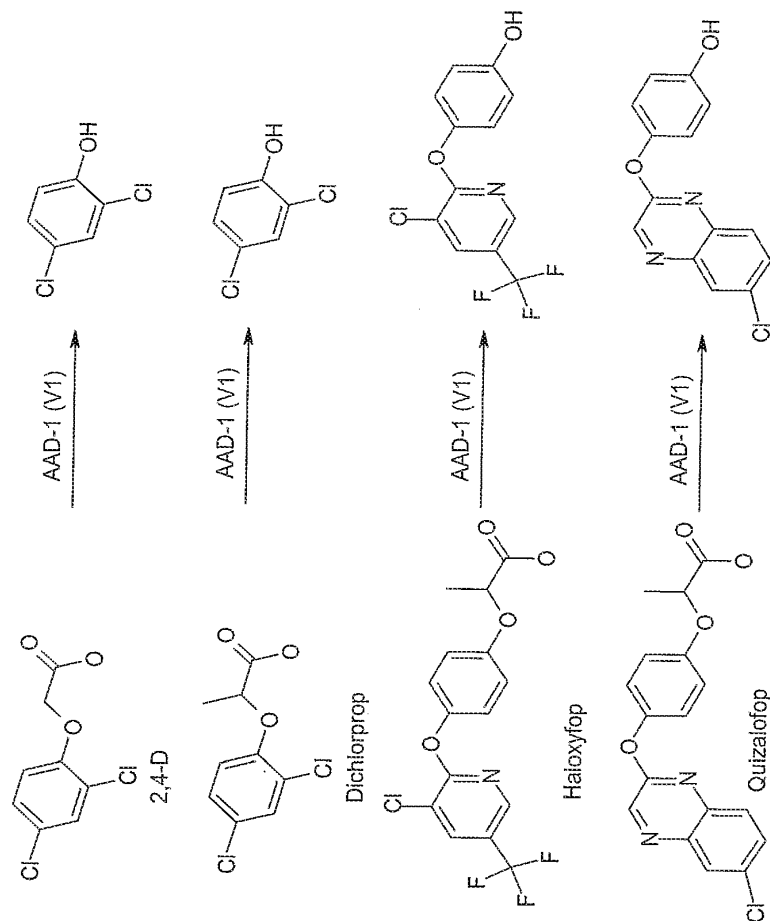


Fig. 4

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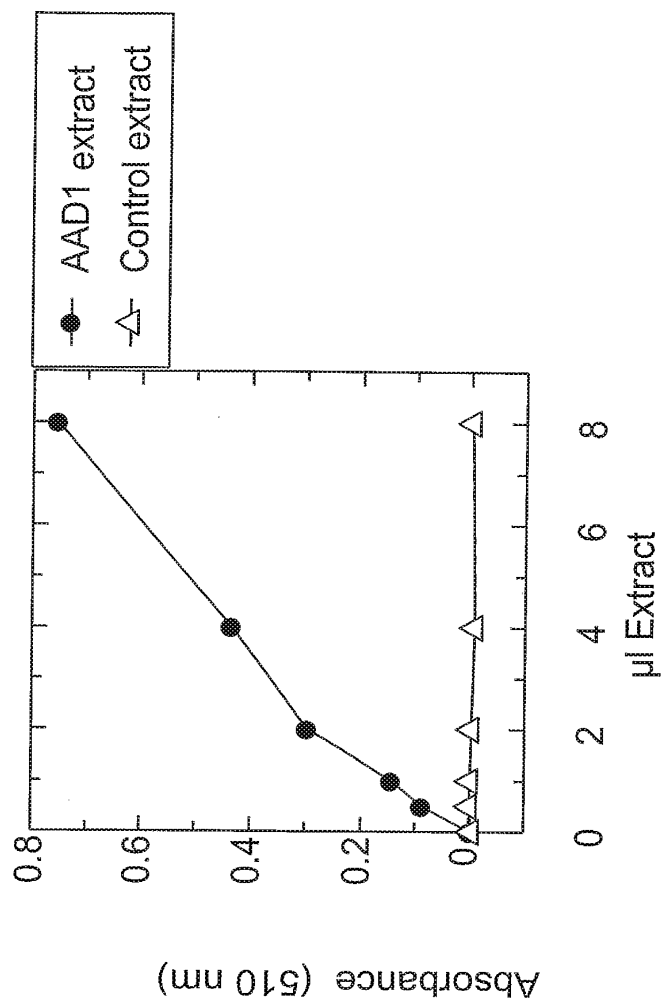


Fig. 5

Fig. 6A

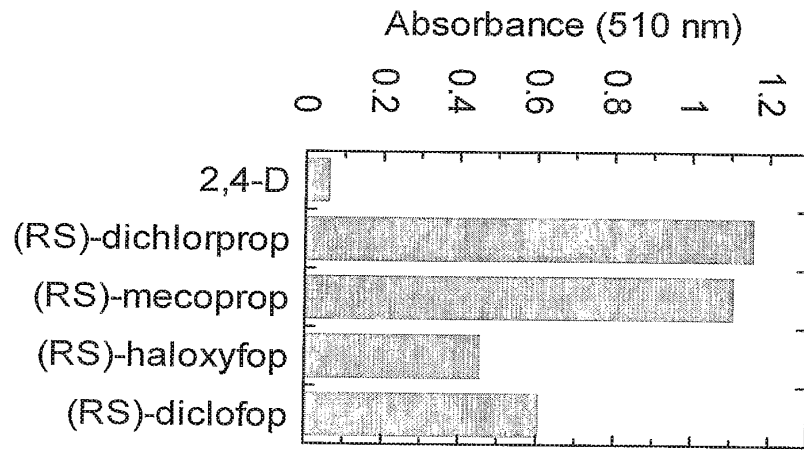
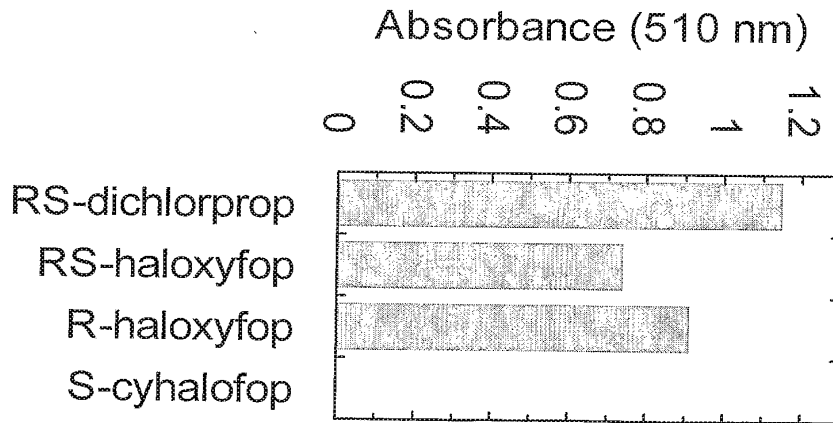


Fig. 6B



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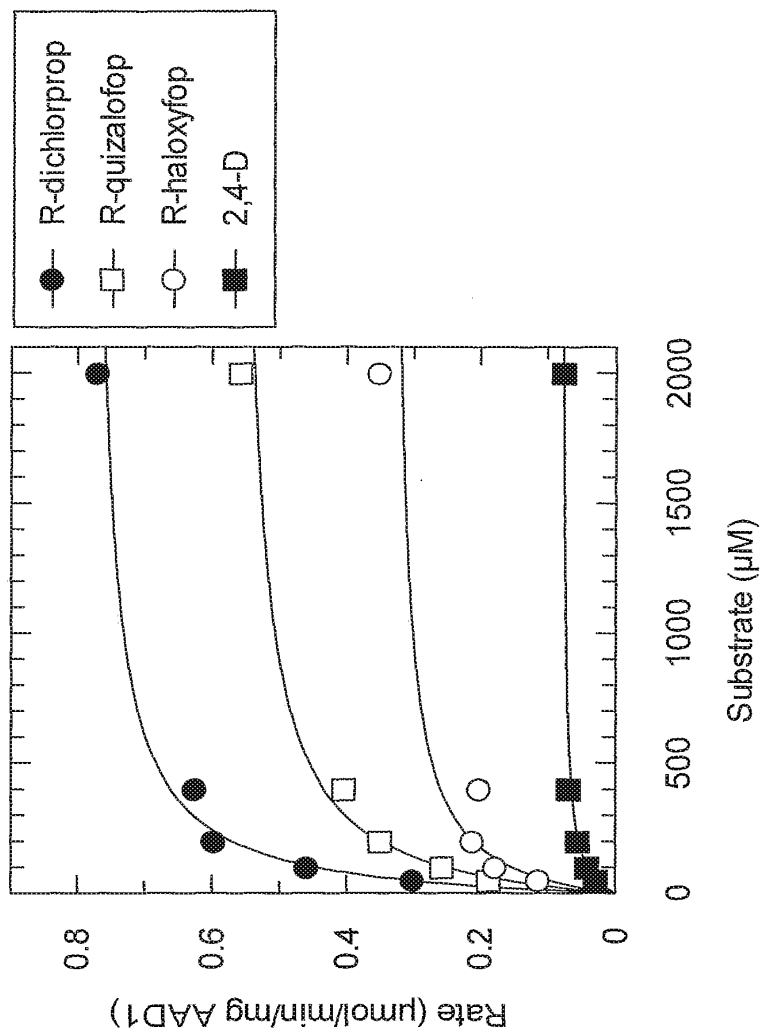


Fig. 7

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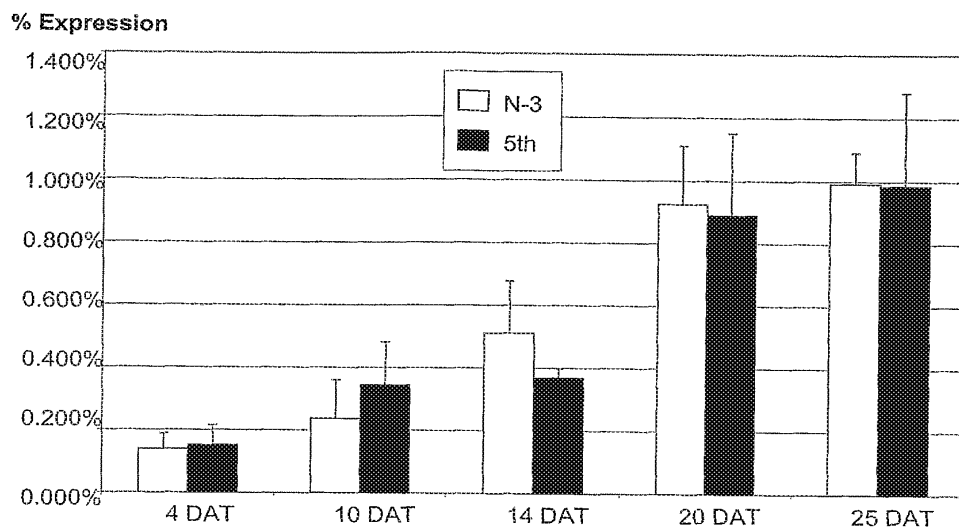


Fig. 8A

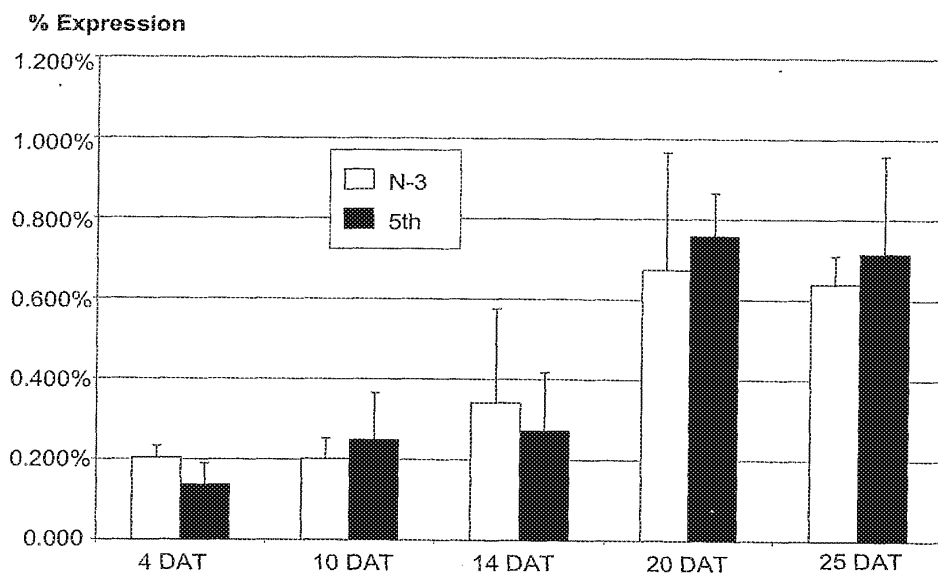


Fig. 8B

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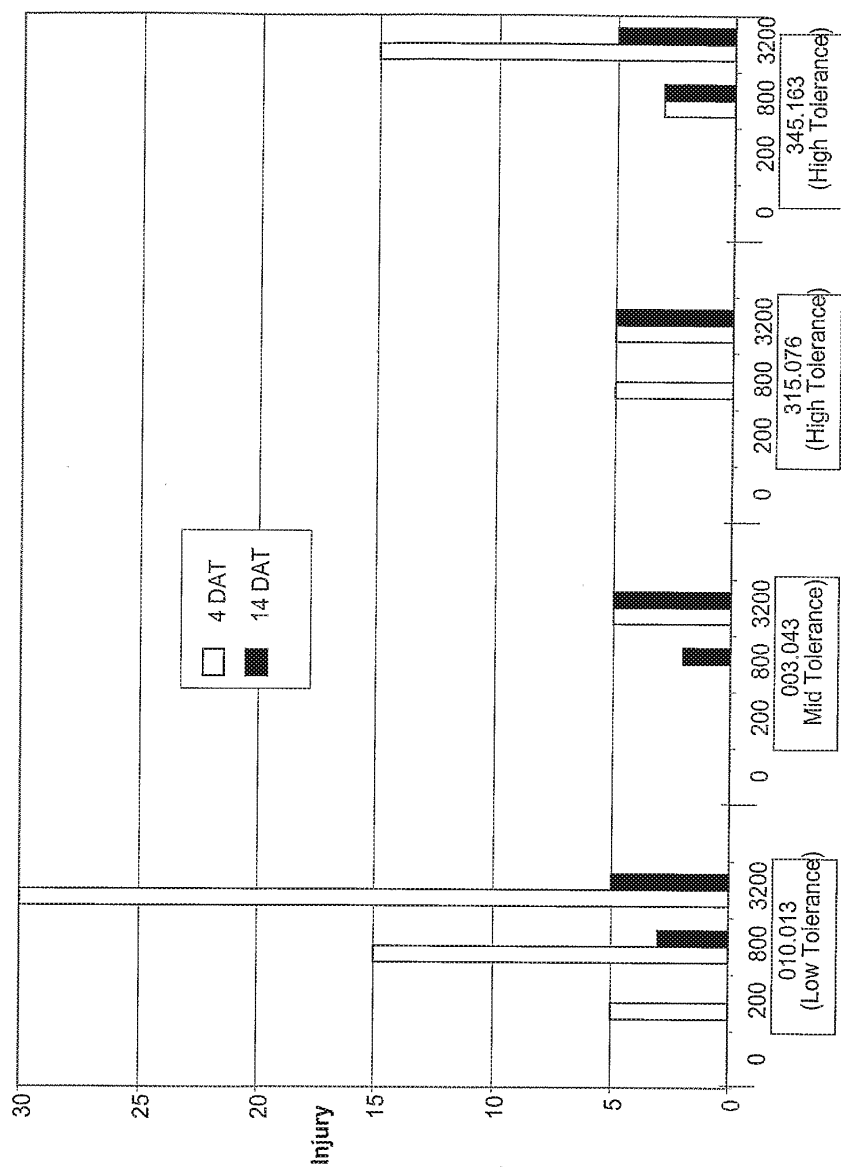


Fig. 9A

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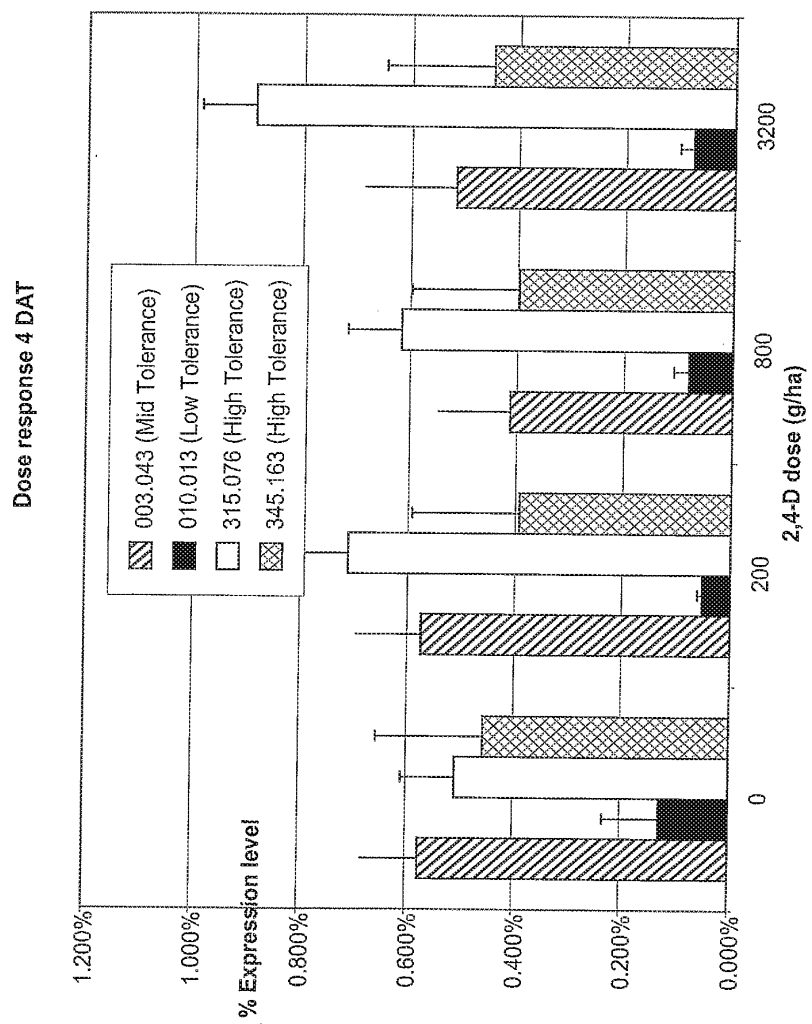


Fig. 9B

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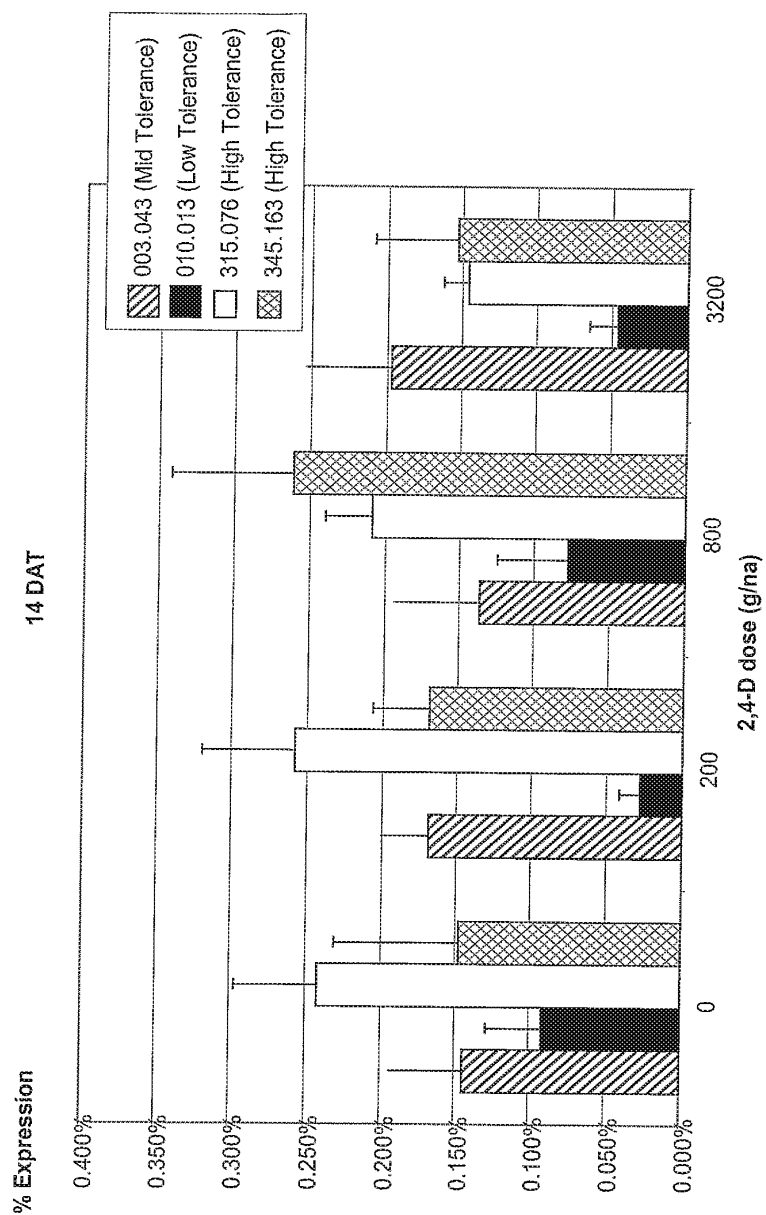


Fig. 9C

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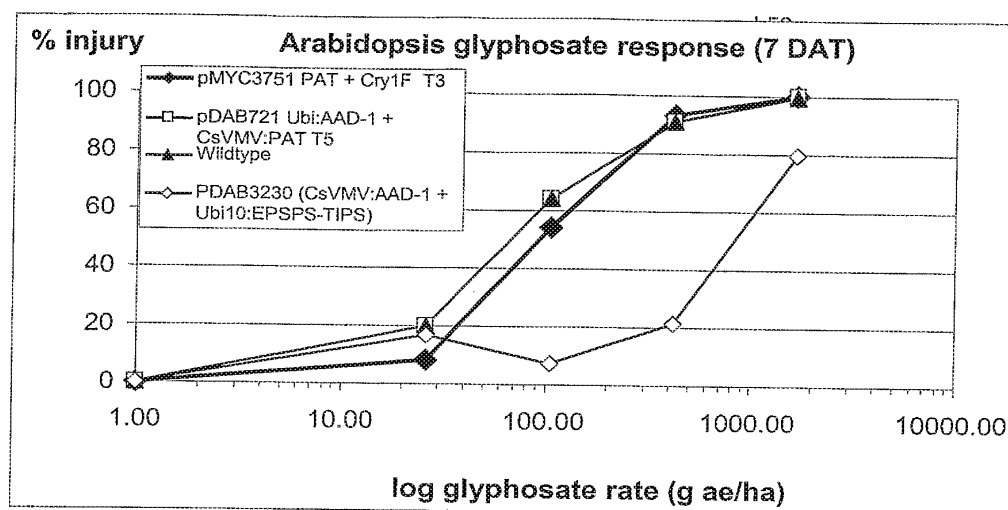


Fig. 10

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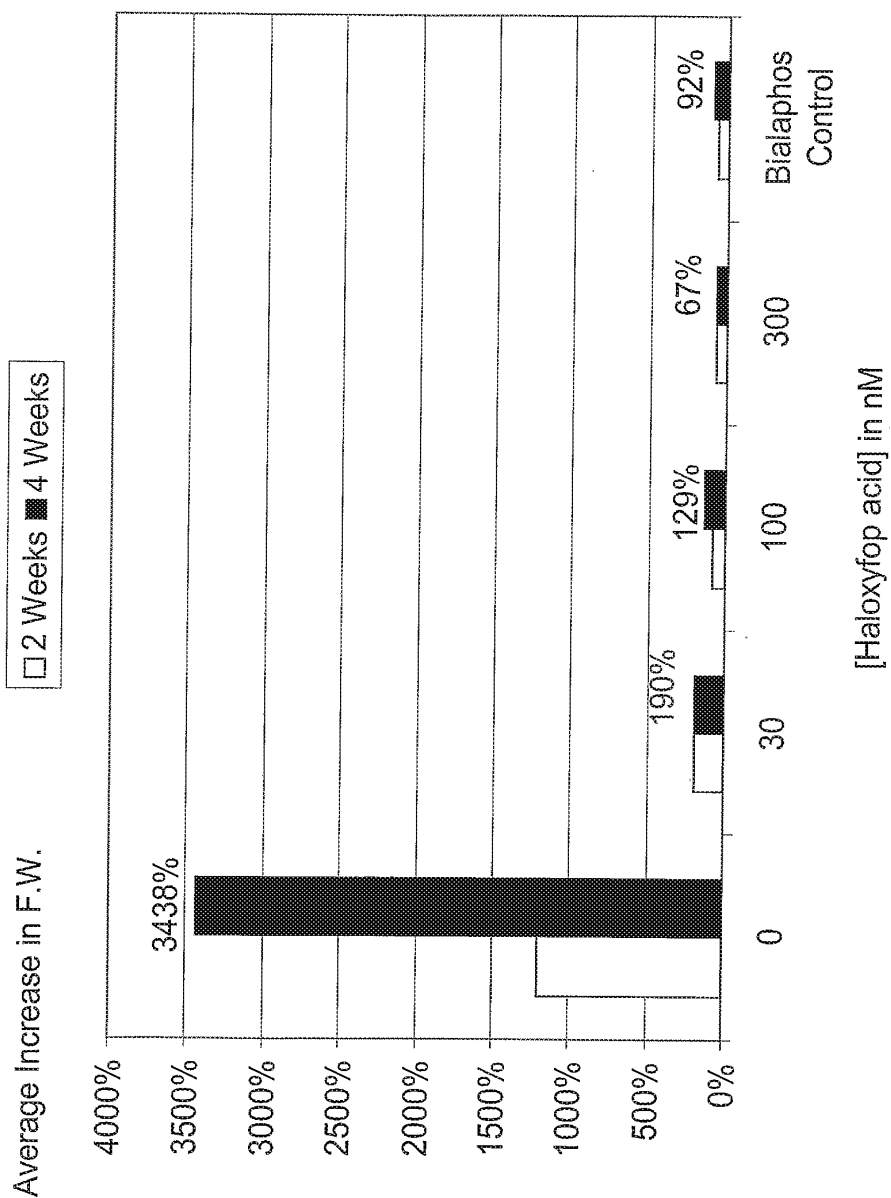


Fig. 11

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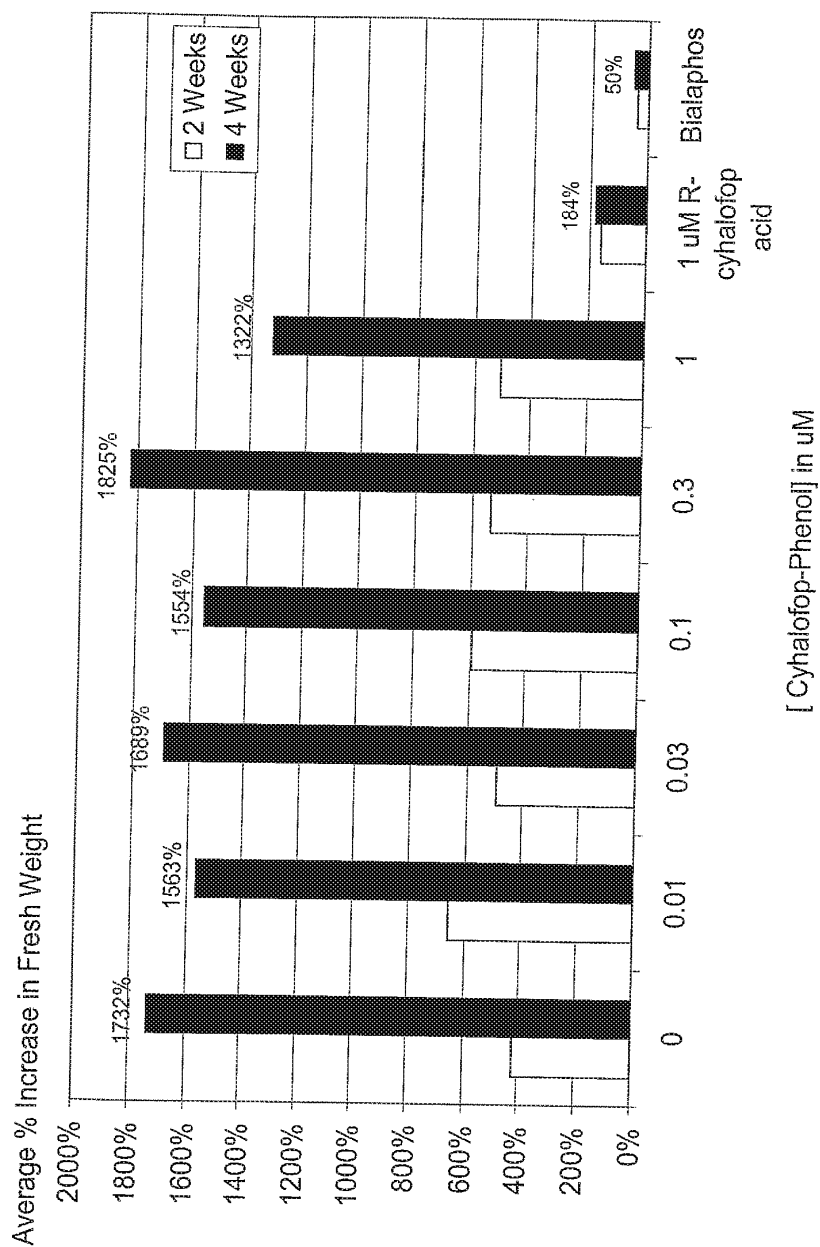


Fig. 12

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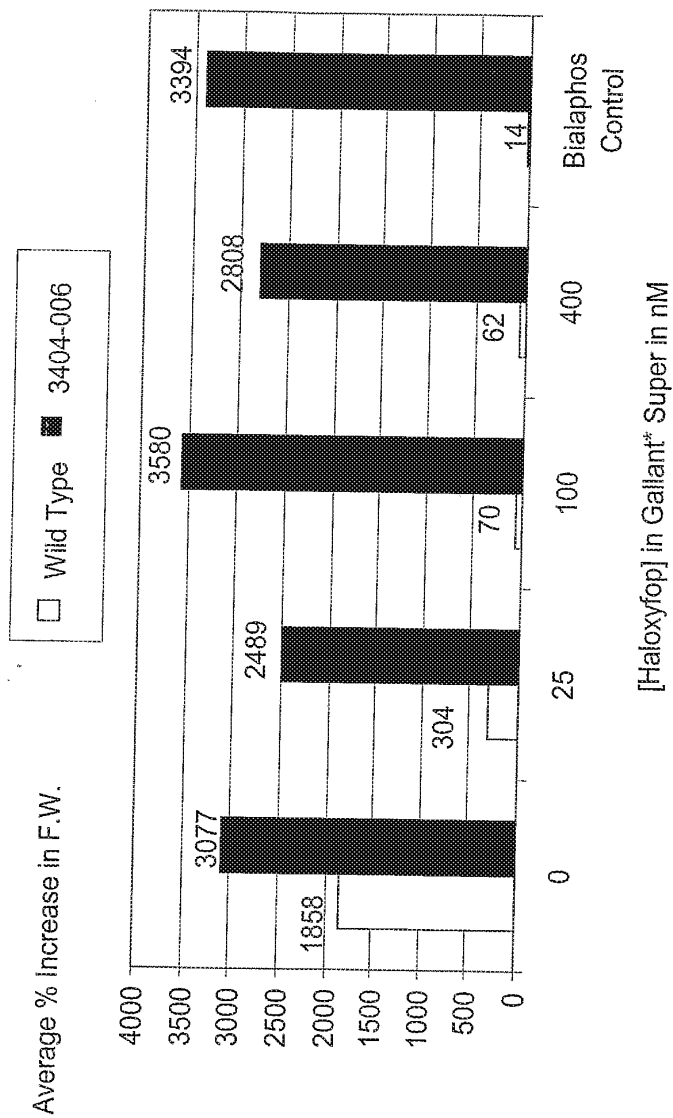


Fig. 13

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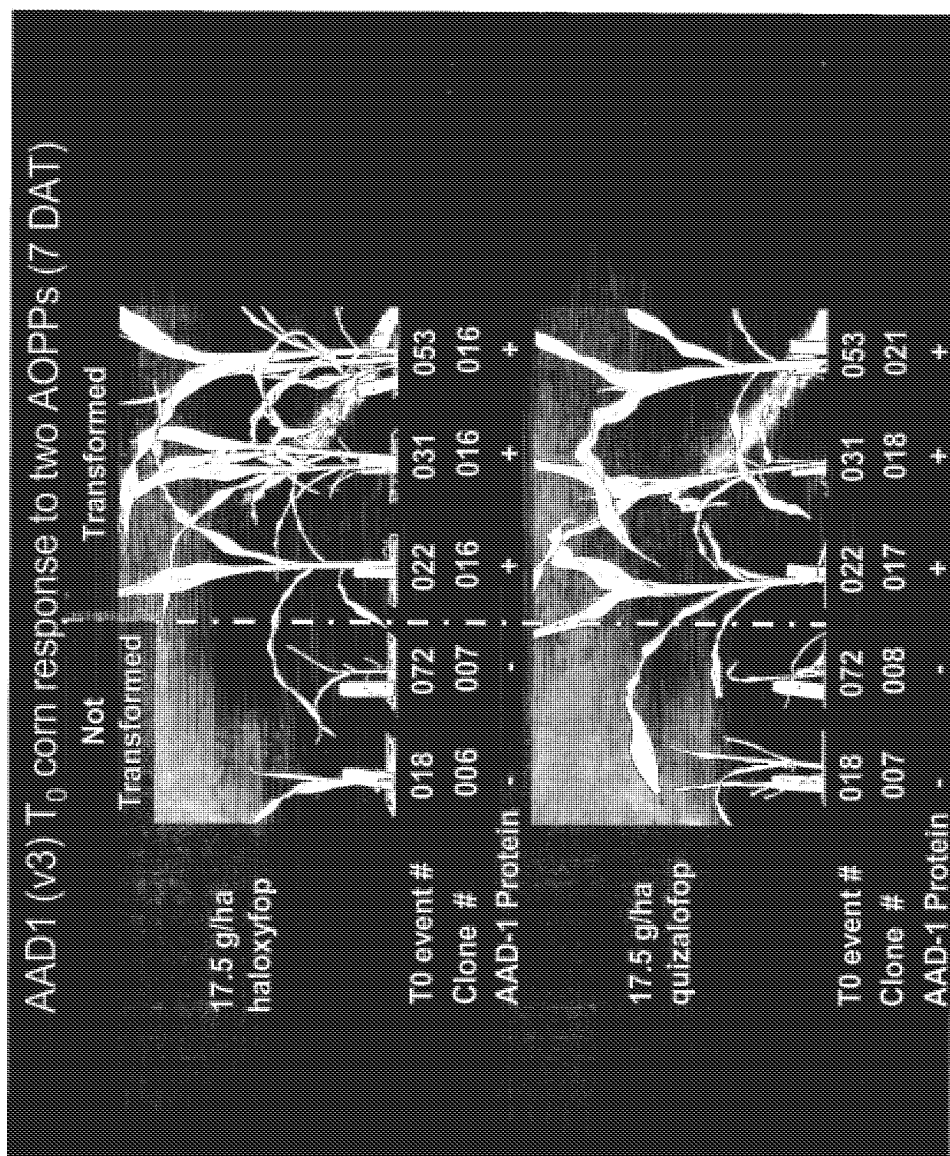


Fig. 14

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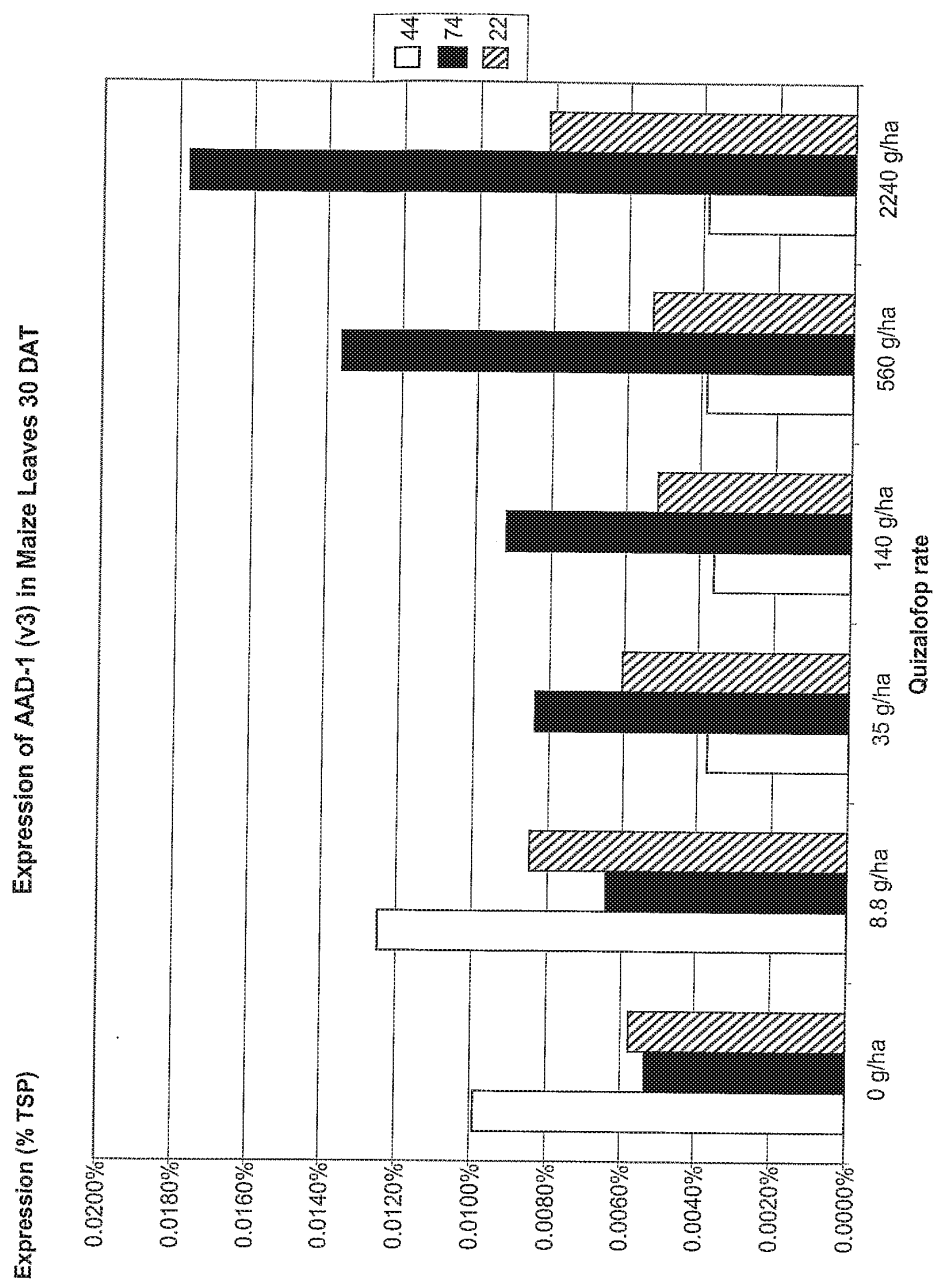


Fig. 15

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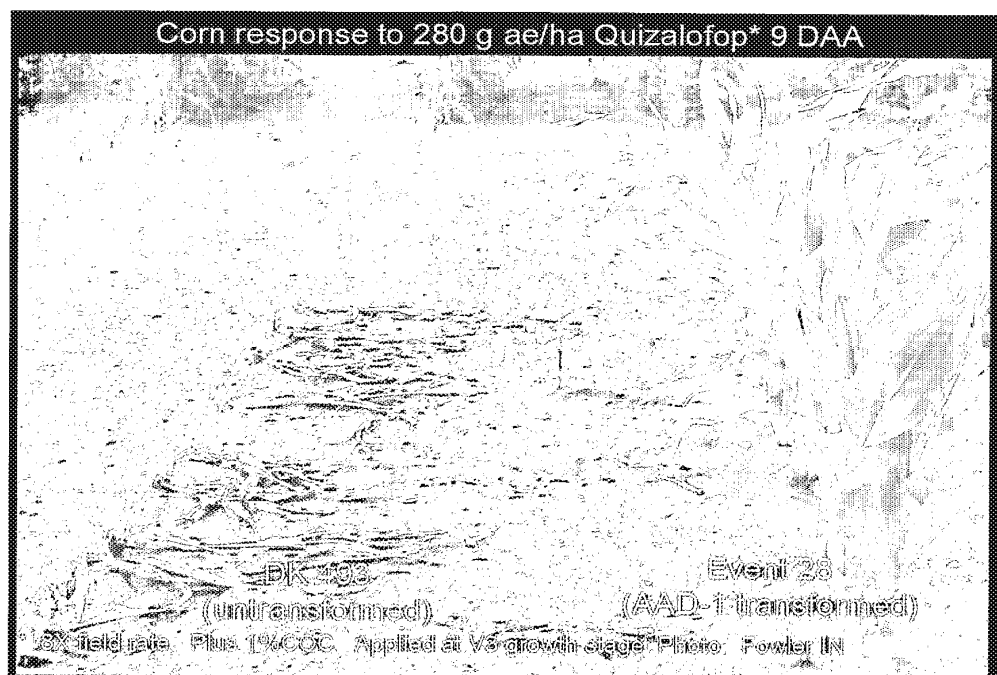


Fig. 16

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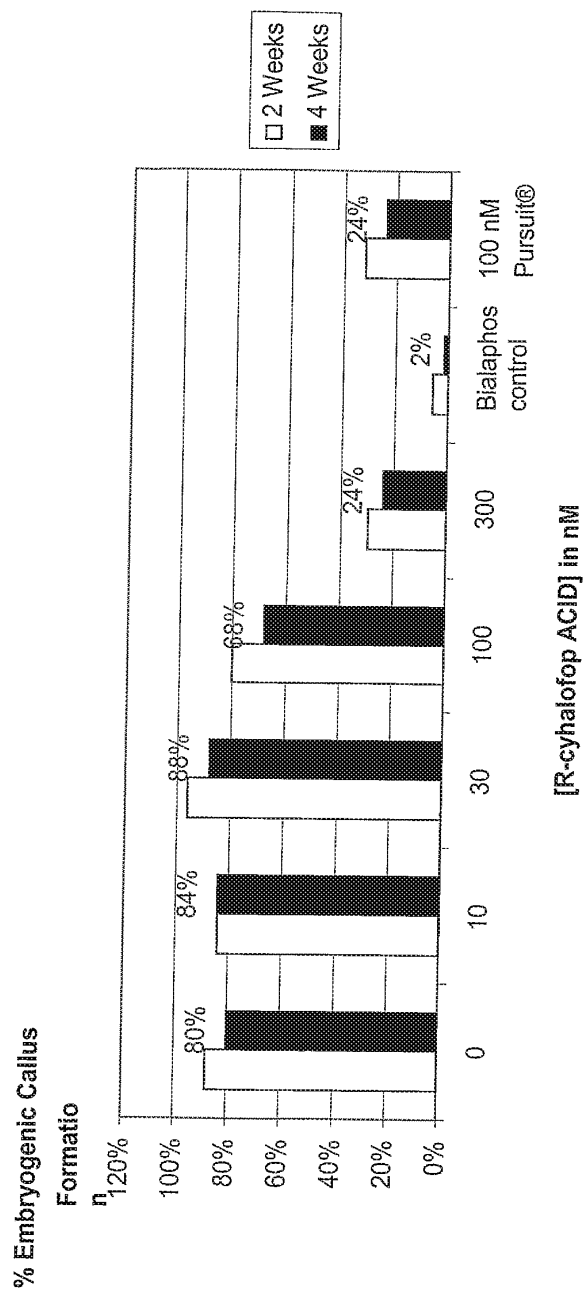
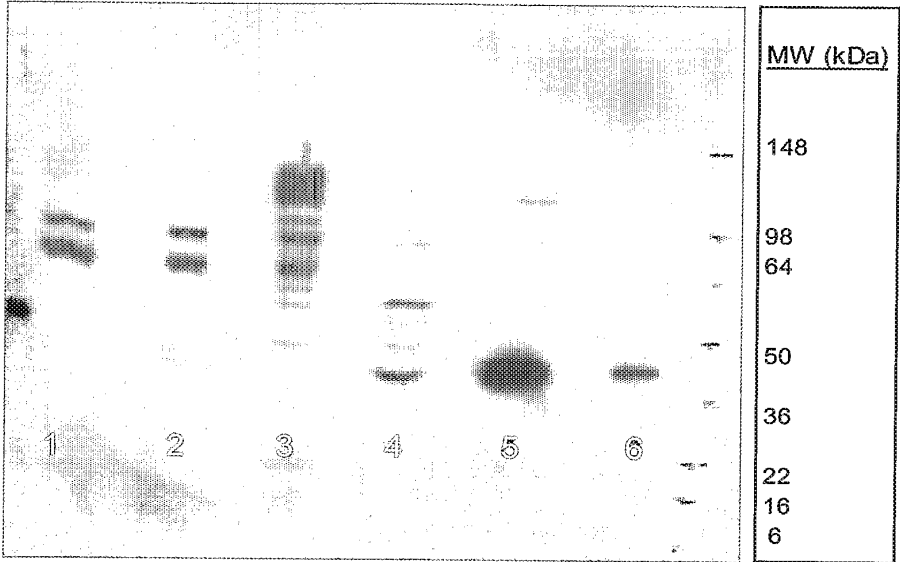


Fig. 17

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Western

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

Fig. 18

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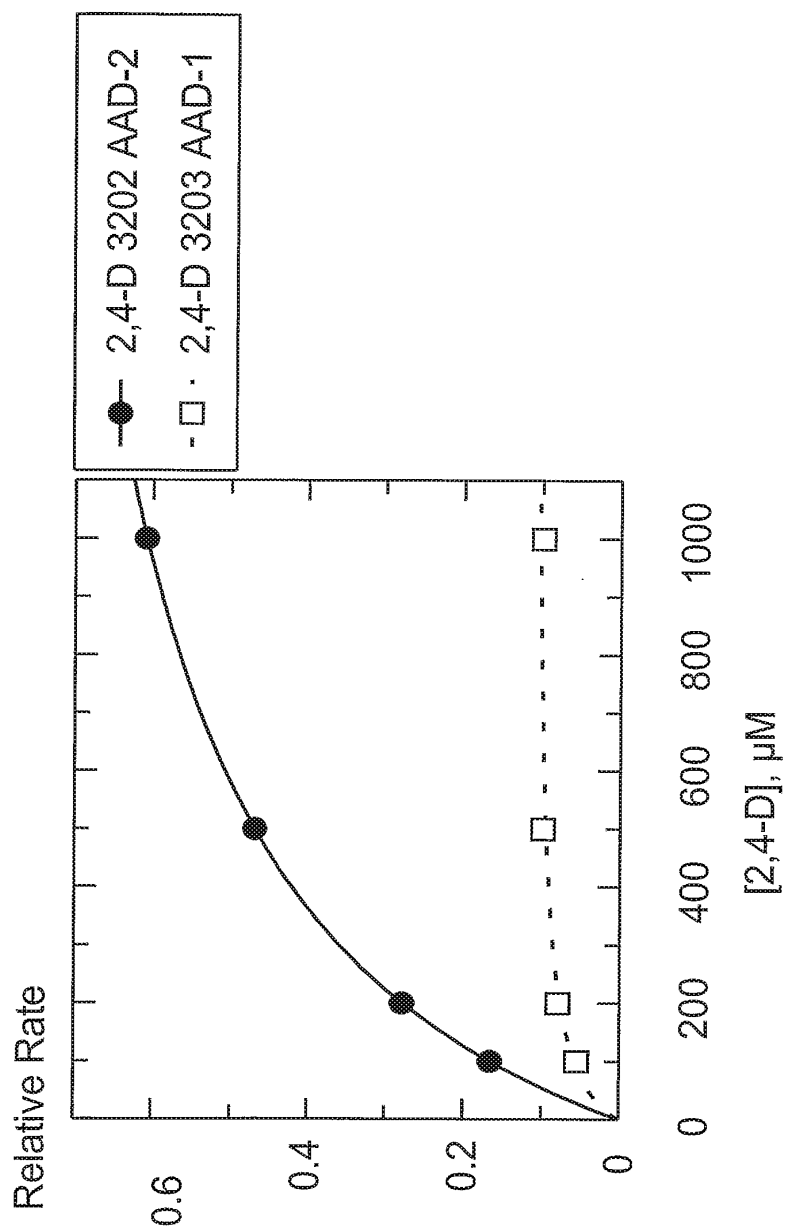


Fig. 19

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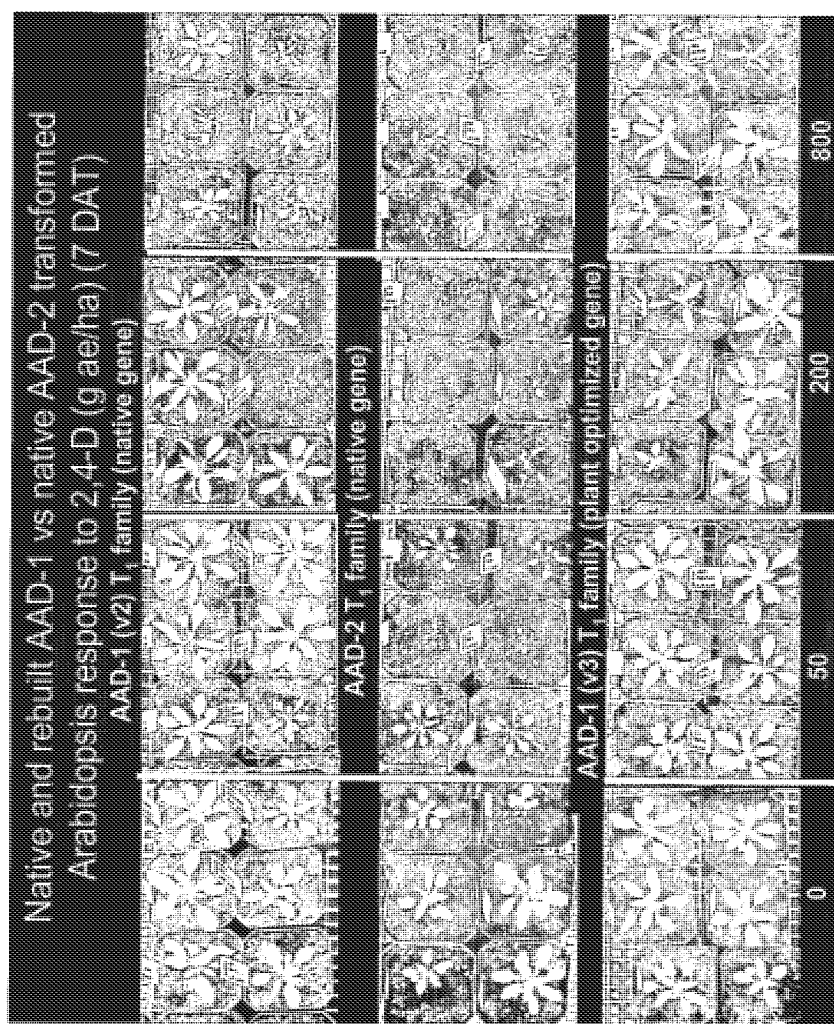


Fig. 20

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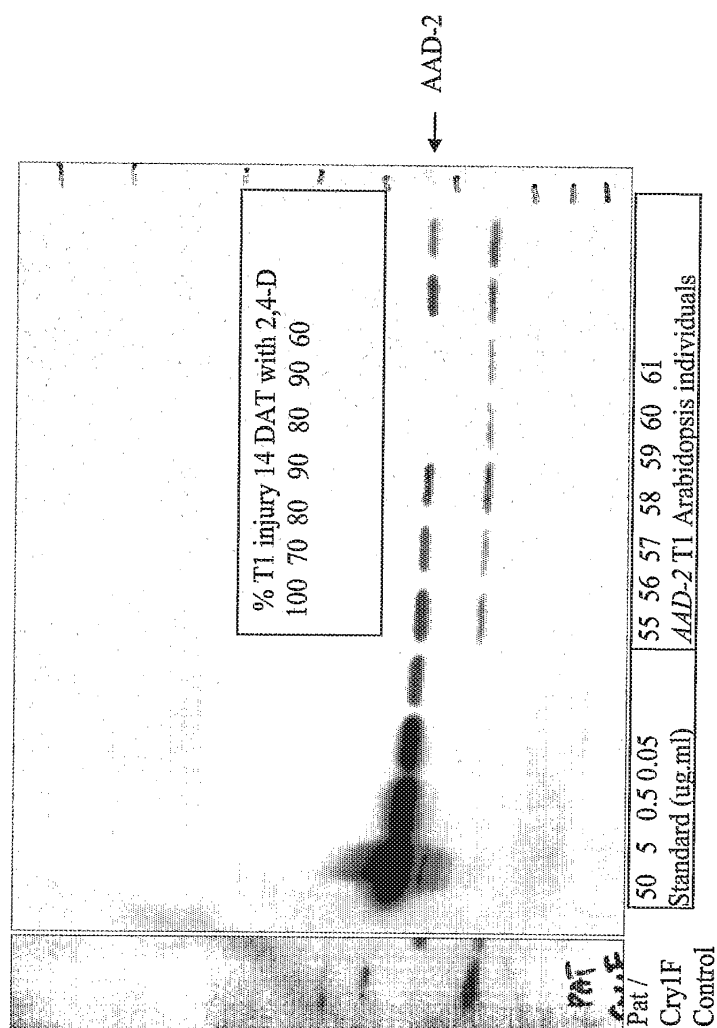


Fig. 21

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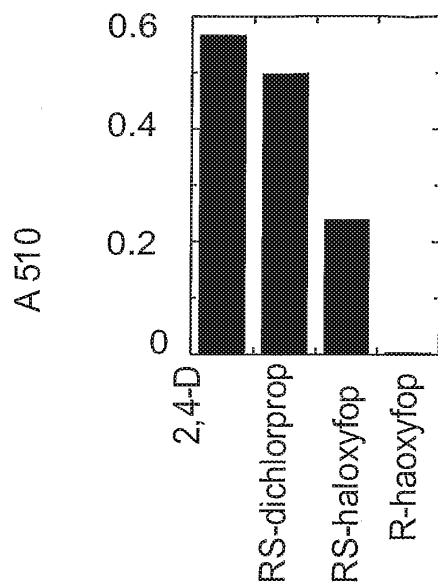


Fig. 22

SEQUENCE LISTING

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 <220>
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 <222> (907)..(915)
 <223> Primer linker
 <400> 3
 tctagaagga gatataccat gcatgctgca ctgtccccc tctcccagcg ctttgagcgc 60
 atcgcggtcc agccgctgac cggcgtcctg ggcgcgaga tcaccggcgt cgacctgcgc 120
 gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttcacac ttaccaggtc 180

```

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc      240
ggccccgtcg atcccggtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg      300
atccgccgcg aagccaacga aagcggggcgt gtgatcgggtg aygactggca caccgacagc      360
accttctctg acgcaccgcc ggccgcgctg gtgatgcgcg cgatcgacgt gcccgagcat      420
ggcggcgaca ccggttttct gagcatgtac acccgctggg agacgctgtc gccaccatg      480
caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggtcgcctc      540
taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg      600
ggcgaccgtg aaaccgtgca cccctggtg gtgacccatc cgggcagcgg ccgcaagggc      660
ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa      720
ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgctgctgc      780
tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtacct      840
gactacgcgg gcaagttccg ctacctgacg cgcaccacgg tcggtggcgt gcgcccggcg      900
cgctagtaac tcgag                                           915

```

```

<210> 4
<211> 897
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> AAD-1 (v2) primary sequence

```

```

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

```

```

<220>
<221> misc_feature
<222> (891)..(897)
<223> primer linker

```

```

<400> 4
ccatggctgc tgcactgtcc cccctctccc agcgttttga gcgcacgcgc gtccagccgc      60
tgaccggcgt cctgggcgcc gagatcaccg gcgtcgacct gcgcgagccg ctcgacgaca      120
gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccgccc      180
aggcgatcac caacgaacag cacatcgctt tcagccggcg cttcgccccc gtcgatcccc      240
tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca      300
acgaaagcgg gcgtgtgacg ggtgatgact ggcacaccga cagcaccttc ctggacgcac      360
cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccca gcatggcggc gacaccggtt      420

```

tctctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480
ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540
ggcgcttcag caacaccagc gtcaagggtg tggacgtcga cgcgggagac cgtgaaaccg 600
tgcacccccct ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660
tctattgccca gcgcacagag ggcattgacc atgccgaaag caaacgcctg ctgcagttcc 720
tgtacgagca tgcgacacgg ttcgatttca cctgcgcgtg gcgctggaag aaggaccagg 780
tcctggtctg ggacaacctg tgcacgatgc accggggcgt acccgactac gcgggcaagt 840
tccgctacct gacgcgcacc acggtcgggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> 5
<211> 919
<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v3) primary sequence

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (6)..(8)
<223> additional alanine codon (GCT)

<220>
<221> misc_feature
<222> (894)..(919)
<223> primer linker

<400> 5
ccatggctca tgctgcctc agccctctct cccaacgctt tgagagaata gctgtccagc 60
cactcactgg tgtccttggg gctgagatca ctggagtggg cttgagggaa ccacttgatg 120
acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcata tactttcctg 180
gccaagcaat caccaatgag cagcacattg cattctcaag aagggttggg ccagttgatc 240
cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgata cgcagagaag 300
ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttcccttgatg 360
cacctccagc tgctgttgtg atgagggccca tagatgttcc tgagcatggc ggagacactg 420
ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480
aagggtctaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

```

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga      600
cagtcocatcc cttgggtgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc      660
aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt      720
tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc      780
aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca      840
agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta      900
gcttaatcac ctagagctc                                          919

```

```

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> rdpA(ncoI) 5' primer

```

```

<400> 6
cccatggctg ctgcactgtc cccctctctc      30

```

```

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

```

```

<220>
<223> 3'saci primer

```

```

<400> 7
gagctcacta gcgcgccggg cgcacgccac cga      33

```

```

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

```

```

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

```

```

<400> 8
tggtggtgac ccatccgggc agcggctgca agggcc      36

```

```

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

```

```

<400> 9

```

```

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

```

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Gly Gly Val Arg Pro Ala Arg
290 295

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

<220>
<223> AAD-1 v2 Translation

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

<220>
<221> MISC_FEATURE
<222> (212)..(212)
<223> Different from v1

<400> 10

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Gly Gly Val Arg Pro Ala Arg
 290 295

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

<220>
 <223> AAD-1 v3 Translation

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

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<400> 11

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Val Gly Gly Val Arg Pro Ala Arg
 290 295

<210> 12

<211> 888

<212> DNA

<213> Bradyrhizobium japonicum USDA 110

<400> 12

```

atgacgatcg ccattccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat      60
ttgcgaaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac      120
gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg      180
aacttcggcc agcgcgagga cgcgcgcggc ggcacggcca ccaaggagaa ggactaccgg      240
ctgcaatccg gcctgaacga cgtctccaat ctccggcaagg acggcaagcc gctggccaag      300
gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc      360
cgtcccatte ccgcaaaatt ctgctgctg tggcgcgcg tggatgaacc gacgggcggc      420
aacaccgaat tcgcggacat gcgcgcgcgc tatgacgcgc tcgacgacga gaccaaggcc      480
gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc      540
accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc      600
acctatccgg tccaccgcgc caagtcgctg tatctctcgt cgcattgccg caagatcgcc      660
agcatgagcg tgccggaggg gcggctgctg ttgcgcgac tcaacgagca cgcgacgcag      720
ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc      780
cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgcgcgcgc      840
acgggtggcg ggacggagcc gacggtgcag cagcaggcgg cggagtag      888

```

<210> 13

<211> 289

<212> PRT

<213> Bradyrhizobium japonicum USDA 110

<400> 13

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Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
 1 5 10 15
 Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
 20 25 30
 Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
 35 40 45
 Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
 50 55 60
 Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
 65 70 75 80
 Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
 85 90 95
 Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
 100 105 110
 Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
 115 120 125
 Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
 130 135 140
 Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
 145 150 155 160
 Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
 165 170 175
 Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
 180 185 190
 Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
 195 200 205
 Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
 210 215 220
 Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
 225 230 235 240

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

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

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

Val

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

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

<400> 14
actagtaaca aagaaggaga tataccatga cgat

34

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

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

<400> 15
ttctcgagct atcactccgc cgcctgctgc tgc

33

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

<220>
<223> M13 forward primer

<400> 16
gtaaaacgac ggccagt

17

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

<220>
<223> M13 reverse primer

<400> 17

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caggaaacag ctatgac

17

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

<220>
<223> NcoI of Brady

<400> 18
tataccacat gtcgatcgcc atcgggcagc tt

32

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

<220>
<223> SacI of Brady

<400> 19
gagctcctat cactccgcgc cctgetgctg cac

33

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

<400> 20

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

<210> 21

<211> 525

<212> PRT

<213> Artificial sequence

<220>

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

<400> 21

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

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

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
 35 40 45
 His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
 50 55 60
 Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
 65 70 75 80
 Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
 85 90 95
 Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
 100 105 110
 Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
 115 120 125
 Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
 130 135 140
 Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
 145 150 155 160
 Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
 165 170 175
 Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
 180 185 190
 Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
 195 200 205
 Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
 210 215 220
 Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
 225 230 235 240
 Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
 245 250 255
 Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
 260 265 270

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

<220>
 <221> misc_feature
 <222> (1579)..(1604)
 <223> Sequence included to introduce translation stop codons in all six
 open reading frames, and to introduce a SacI restriction enzyme
 recognition site for cloning purposes

<400> 22
 atgggtcaag tctccgtgt tcacaatctt gctcagtc aa cccaaatctt tggacattca 60
 agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttgcccacg cctttgggga 120
 gcatccaaga gtgcataacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt 180
 gggaaaggca attctgggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc 240
 agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaa c aatcactctg 300
 cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc 360
 actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga 420
 actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt 480
 ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttggt tcttggcaat 540
 gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600
 tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660
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Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
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65           70           75           80

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Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
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Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
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Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
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21/22

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Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
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Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
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Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
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Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
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Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
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Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
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Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
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21

**TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING
REJECTION OVER A PENDING "REFERENCE" APPLICATION**

Docket Number (Optional)
14764-245772

In re Application of: Terry R. Wright

Application No.: 14/491,197

Filed: September 19, 2014

For: NOVEL HERBICIDE RESISTANCE GENES

The applicant, Dow AgroSciences LLC, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number 15/184,601, filed, June 16, 2016 as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said **reference** application, "as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application," in the event that: any such patent granted on the pending **reference** application expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. ☒ The undersigned is an attorney or agent of record. Reg. No. 46,535

<u>/Rebecca L. Ball/</u>	<u>12 April 2017</u>
Signature	Date
<u>Rebecca L. Ball</u>	
Typed or printed name	
<u>Attorney for Applicant</u>	<u>317-231-7511</u>
Title	Telephone Number

- ☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

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This collection of information is required by 37 CFR 1.321. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 14764-245772
<p>In re Application of: Terry R. Wright</p> <p>Application No.: 14/491,197</p> <p>Filed: September 19, 2014</p> <p>For: NOVEL HERBICIDE RESISTANCE GENES</p> <p>The applicant, <u>Dow AgroSciences LLC</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>9,062,284</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none">expires for failure to pay a maintenance fee;is held unenforceable;is found invalid by a court of competent jurisdiction;is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;has all claims canceled by a reexamination certificate;is reissued; oris in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. ☒ The undersigned is an attorney or agent of record. Reg. No. <u>46,535</u></p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"><div style="width: 60%; text-align: center;"><u>/Rebecca L. Ball/</u> Signature</div><div style="width: 35%; text-align: center;"><u>12 April 2017</u> Date</div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Rebecca L. Ball</u> Typed or printed name</div><div style="width: 35%;"></div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Attorney for Applicant</u> Title</div><div style="width: 35%; text-align: center;"><u>317-231-7511</u> Telephone Number</div></div> <p>☒ Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 14764-245772
<p>In re Application of: Terry R. Wright</p> <p>Application No.: 14/491,197</p> <p>Filed: September 19, 2014</p> <p>For: NOVEL HERBICIDE RESISTANCE GENES</p> <p>The applicant, <u>Dow AgroSciences LLC</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>8,916,752</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none">expires for failure to pay a maintenance fee;is held unenforceable;is found invalid by a court of competent jurisdiction;is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;has all claims canceled by a reexamination certificate;is reissued; oris in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. ☒ The undersigned is an attorney or agent of record. Reg. No. <u>46,535</u></p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"><div style="width: 60%; text-align: center;"><u>/Rebecca L. Ball/</u> Signature</div><div style="width: 35%; text-align: center;"><u>12 April 2017</u> Date</div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Rebecca L. Ball</u> Typed or printed name</div><div style="width: 35%;"></div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Attorney for Applicant</u> Title</div><div style="width: 35%; text-align: center;"><u>317-231-7511</u> Telephone Number</div></div> <p>☒ Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 14764-245772
<p>In re Application of: Terry R. Wright</p> <p>Application No.: 14/491,197</p> <p>Filed: September 19, 2014</p> <p>For: NOVEL HERBICIDE RESISTANCE GENES</p> <p>The applicant, <u>Dow AgroSciences LLC</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>8,283,522</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none">expires for failure to pay a maintenance fee;is held unenforceable;is found invalid by a court of competent jurisdiction;is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;has all claims canceled by a reexamination certificate;is reissued; oris in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. ☒ The undersigned is an attorney or agent of record. Reg. No. <u>46,535</u></p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"><div style="width: 60%; text-align: center;"><u>/Rebecca L. Ball/</u> Signature</div><div style="width: 35%; text-align: center;"><u>12 April 2017</u> Date</div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Rebecca L. Ball</u> Typed or printed name</div><div style="width: 35%;"></div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Attorney for Applicant</u> Title</div><div style="width: 35%; text-align: center;"><u>317-231-7511</u> Telephone Number</div></div> <p>☒ Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

This collection of information is required by 37 CFR 1.321. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 14764-245772
<p>In re Application of: Terry R. Wright</p> <p>Application No.: 14/491,197</p> <p>Filed: September 19, 2014</p> <p>For: NOVEL HERBICIDE RESISTANCE GENES</p> <p>The applicant, <u>Dow AgroSciences LLC</u>, owner of <u>100</u> percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent No. <u>8,278,505</u> as the term of said prior patent is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.</p> <p>In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:</p> <ul style="list-style-type: none">expires for failure to pay a maintenance fee;is held unenforceable;is found invalid by a court of competent jurisdiction;is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;has all claims canceled by a reexamination certificate;is reissued; oris in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer. <p>Check either box 1 or 2 below, if appropriate.</p> <p>1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.</p> <p>I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p>2. ☒ The undersigned is an attorney or agent of record. Reg. No. <u>46,535</u></p> <div style="display: flex; justify-content: space-between; margin-top: 20px;"><div style="width: 60%; text-align: center;"><u>/Rebecca L. Ball/</u> Signature</div><div style="width: 35%; text-align: center;"><u>12 April 2017</u> Date</div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Rebecca L. Ball</u> Typed or printed name</div><div style="width: 35%;"></div></div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"><div style="width: 60%; text-align: center;"><u>Attorney for Applicant</u> Title</div><div style="width: 35%; text-align: center;"><u>317-231-7511</u> Telephone Number</div></div> <p>☒ Terminal disclaimer fee under 37 CFR 1.20(d) included.</p> <p style="text-align: center;">WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.</p>	

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Electronic Patent Application Fee Transmittal				
Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Rebecca Ball/Garla Twyman		
Attorney Docket Number:		DAS-P0129-04-US-E		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
STATUTORY OR TERMINAL DISCLAIMER	1814	5	160	800
Total in USD (\$)				980

Electronic Acknowledgement Receipt	
EFS ID:	28906199
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Rebecca Ball/Garla Twyman
Filer Authorized By:	Rebecca Ball
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	12-APR-2017
Filing Date:	19-SEP-2014
Time Stamp:	14:44:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$980
RAM confirmation Number	041317INTEFSW00001024100435
Deposit Account	100435
Authorized User	Garla Twyman
<p>The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:</p> <p>37 CFR 1.16 (National application filing, search, and examination fees)</p> <p>37 CFR 1.17 (Patent application and reexamination processing fees)</p>	

37 CFR 1.19 (Document supply fees)					
37 CFR 1.20 (Post Issuance fees)					
37 CFR 1.21 (Miscellaneous fees and charges)					
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Response_and_Amendment_under_37_C_F_R_1_111.pdf	68379	yes	19
			3fda4882c66c15f9f65384538306e19e099521f3		
	Multipart Description/PDF files in .zip description				
	Document Description		Start		End
	Amendment/Req. Reconsideration-After Non-Final Reject		1		2
	Specification		3		3
	Claims		4		8
	Applicant Arguments/Remarks Made in an Amendment		9		19
Warnings:					
Information:					
2	Transmittal Letter	Supplemental_INFORMATION_Disclosure_Statement.pdf	23549	no	3
			7c607537bcf68842fd389fca0bc33d4ffdbcfb75		
Warnings:					
Information:					
3	Foreign Reference	WO2007053482.pdf	9685796	no	164
			e0f3885260158ed15bf0069cbd5f2501a6b60aa9		
Warnings:					
Information:					
4	Non Patent Literature	Schleinitz_et_al_AEM2004.pdf	943100	no	10
			589f70ef745bef3100ea4802d7be35bd651d63a7		
Warnings:					
Information:					

5	Non Patent Literature	GRESSEL_HerbicidesWeed.pdf	547023	no	28
			aed73470dbff89be48abcaa196a39e3afe82babe		
Warnings:					
Information:					
6	Non Patent Literature	Hegg.pdf	380476	no	13
			e072c082cbc7aeb3f637e64492cdab61556c3640		
Warnings:					
Information:					
7	Non Patent Literature	Perlack_3324_full.pdf	3625348	no	5
			d461752c9bbf780829bab971eb06ff755caf4e07		
Warnings:					
Information:					
8	Non Patent Literature	Kasuga.pdf	402563	no	5
			80096f5b912edd8aa83dac7359f2bc4580bbe369		
Warnings:					
Information:					
9	Non Patent Literature	Verdaguer_010007740.pdf	2571367	no	11
			4738e1c4a7d4296db937895c1c5625beb9a724b6		
Warnings:					
Information:					
10	Non Patent Literature	Westendorf_et_al-2003-Acta_Biotechnologica.pdf	629064	no	15
			472a668f44fb69c87ec05d0a613988a48a47f9cc		
Warnings:					
Information:					
11	Non Patent Literature	Fourgoux-Nicol.pdf	567024	no	16
			19c7e8706c666e96f0abd31850e17e2e66c674f4		
Warnings:					
Information:					

12	Non Patent Literature	2005_PreSoyHerb.pdf	159847	no	23
			56d9122681e2debe3fcd76431aaa6682459d6124		
Warnings:					
Information:					
13	Non Patent Literature	Halford.pdf	223148	no	9
			fd189867fd9ee9bd9eb40c35d939f82329869d33		
Warnings:					
Information:					
14	Foreign Reference	WO2005107437_Part_1.pdf	16324176	no	101
			6a98896ff9699a14dbd6eefce97e6b3c99d875dd		
Warnings:					
Information:					
15	Foreign Reference	WO2005107437_Part_2.pdf	14612256	no	114
			66fbca060330e479077ab64f857619a662c11831		
Warnings:					
Information:					
16	Terminal Disclaimer Filed	15-184601_TD.pdf	246893	no	2
			3727767763385395a635804f09a7d0b555e97114		
Warnings:					
Information:					
17	Terminal Disclaimer Filed	9062284_TD.pdf	160596	no	2
			8b1d92d3e2184bbac0365821b24acc4d5287d20d		
Warnings:					
Information:					
18	Terminal Disclaimer Filed	8916752_TD.pdf	160728	no	2
			efc8e2b9d4888126644a969394b7ebd961511343		
Warnings:					
Information:					

19	Terminal Disclaimer Filed	8283522_TD.pdf	160686	no	2
			36b7b6a446bccb8a9c5a1ca6f8b0fd8806cd316b		
Warnings:					
Information:					
20	Terminal Disclaimer Filed	8278505_TD.pdf	160659	no	2
			6e1eb1260640106b18eea654aa9738205e45198a		
Warnings:					
Information:					
21	Fee Worksheet (SB06)	fee-info.pdf	32563	no	2
			ac09c9c9f8056f2036000520c759116fa8145268		
Warnings:					
Information:					
Total Files Size (in bytes):			51685241		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p>New Applications Under 35 U.S.C. 111 If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p>National Stage of an International Application under 35 U.S.C. 371 If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p>New International Application Filed with the USPTO as a Receiving Office If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875					Application or Docket Number 14/491,197		Filing Date 09/19/2014		☐ To be Mailed	
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO										
APPLICATION AS FILED – PART I										
(Column 1)		(Column 2)								
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)		
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A		280		
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))		N/A		N/A		N/A				
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A				
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		X \$ =				
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		X \$ =				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).								
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))										
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL		280		
APPLICATION AS AMENDED – PART II										
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT	04/12/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 24	Minus	** 25	= 0	X \$80 =		0		
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	X \$420 =		0		
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
TOTAL ADD'L FEE						0				
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =				
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =				
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
TOTAL ADD'L FEE										
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.										

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LIE
GWENDOLYN MYERS

Application Number 	Application/Control No. 14/491,197	Applicant(s)/Patent under Reexamination WRIGHT ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL
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TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 4/12/17	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
5/Tds approved. Lawana Hixon

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
107540	7590	06/23/2017	EXAMINER	
Barnes & Thornburg LLP (Dow)			KRUSE, DAVID H	
11 South Meridian Street			ART UNIT	
Indianapolis, IN 46204			PAPER NUMBER	
			1663	
			NOTIFICATION DATE	
			DELIVERY MODE	
			06/23/2017	
			ELECTRONIC	

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Office Action Summary	Application No. 14/491,197	Applicant(s) WRIGHT ET AL.	
	Examiner DAVID H. KRUSE	Art Unit 1663	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 12 April 2017.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 1-24 and 26 is/are pending in the application.
5a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 6) ☐ Claim(s) ____ is/are allowed.
- 7) ☒ Claim(s) 1-8, 10-24 and 26 is/are rejected.
- 8) ☒ Claim(s) 9 is/are objected to.
- 9) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some** c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
Paper No(s)/Mail Date ____.
- 3) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date ____.
- 4) ☐ Other: ____.

The present application is being examined under the pre-AIA first to invent provisions.

STATUS OF THE APPLICATION

1. This Office action is in response to the Amendment and Remarks filed on 12 April 2017.
2. The objection to the Specification is withdrawn in view of Applicant's amendment.
3. The objections to claims 4, 10 and 19 are withdrawn in view of Applicant's amendments to the claims.
4. Claim 25 has been cancelled, any objection or rejection thereof is now moot.
5. The rejection under 35 USC 101 of claims 1, 2 and 21-24 is withdrawn in view of Applicant's amendments to the claims.
6. The rejection of claims 21-24 and 26 under 35 USC 112, 1st paragraph for lack of written description is withdrawn in view of Applicant's amendments to the claims.
7. The rejection of claims 8 and 9 under 35 USC 112, 2nd paragraph is withdrawn in view of Applicant's amendments to the claims.
8. The rejection of claims 1, 2 and 21-24 under pre-AIA 35 U.S.C. 102(b) is withdrawn in view of Applicant's amendments to the claims.

Terminal Disclaimer

9. The terminal disclaimers filed on 12 April 2017 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of U.S. Patents 9,062,284 or 8,278,505 or 8,283,522 or 8,916,752 have been reviewed and are accepted. The terminal disclaimers have been recorded.

Art Unit: 1663

10. The terminal disclaimer filed on 12 April 2017 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of any patent granted on Application Number 15/184,601 has been reviewed and is accepted. The terminal disclaimer has been recorded.

Claim Rejections - 35 USC § 103

11. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

12. Claims 1-8, 11-24 and 26 remain rejected and claims 8 and 10 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz et al (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365), Pallett et al (U.S. Patent 7,250,561 B1, § 371(c)(1) date of 15 June 1998) and Xu et al (U.S. Patent 6,665,384). This rejection is repeated for the reason of record as set forth in the last Office action mailed 12 January 2017. Applicant's arguments filed 12 April 2017 have been fully considered but they are not persuasive.

Claims 8 and 10 are included in this rejection based on Applicant's amendments to said claims. As used herein, SdpA and AAD-12 are used interchangeably in this rejection as they are synonyms of the same enzyme.

Kaphammer teaches a plant cell comprising a polynucleotide operably linked to a CAMV 35S promoter (Figure 3) that encodes a protein having aryloxyalkanoate

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dioxygenase activity, resistance to 2,4-dichlorophenoxyacetate (2,4-D) at claim 11, and a plant regenerated therefrom at claim 15. Kaphammer teaches an isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity at SEQ ID NO: 1. Kaphammer teaches transforming a soybean cell at Example 11, columns 21-22. Kaphammer teaches a method of controlling weeds by applying 2,4-D herbicide to a crop field at column 2, lines 26-28. Kaphammer teaches that a transgene that confers resistance to glyphosate can also be introduced into the plant cell (column 3, lines 11-6).

Kaphammer does not teach an isolated nucleic acid encoding instant SEQ ID NO: 2 or having the nucleotide sequence of instant SEQ ID NO: 1. Kaphammer does not teach introducing a polynucleotide that confers glufosinate resistance. Kaphammer does not teach using a cassava vein mosaic virus promoter.

Schleinitz et al teach an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5th paragraph). The encoded SdpA protein has aryloxyalkanoate dioxygenase activity and has greatest activity of degrading S-enantiomers of mecoprop and dichlorprop and has some activity toward 2,4-D (page 5363, left column, 2nd paragraph).

Pallett et al teach introducing polynucleotides to a transformed plant cell that renders said plant cell resistant to multiple herbicides including glyphosate (column 1, lines 62-64) and glufosinate (column 2, lines 27-29).

Xu et al teach a cassava vein mosaic virus promoter operably linked to a sequence of interest at Figure 3. Xu et al teach that genes encoding herbicide

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resistance can be used as selectable markers (column 7, lines 5-12) and that said sequence of interest can be a selectable marker at claim 27.

It would have been prima face obvious to one of ordinary skill in the art at the time of Applicants' invention to modify the teachings of Kaphammer to introduce a polynucleotide encoding instant SEQ ID NO: 2 as taught by Schleinitz et al in addition to a polynucleotide encoding resistance to glyphosate and glufosinate as suggested Pallett et al. Given the success of Pallett et al, and Kaphammer one of ordinary skill in the art would have had a reasonable expectation of success. Given dichlorprop was a known herbicide as taught by Schleinitz et al (page 5357, left column, 1st paragraph), one of ordinary skill in the art would have been motivated to introduce said polynucleotide encoding instant SEQ ID NO: 2 into a plant cell for the purposes of making an herbicide tolerant plant from said plant cell. The SdpA enzyme taught by Schleinitz *et al* is taught was being capable of degrading S-enantiomers of mecoprop and dichlorprop and has some activity toward 2,4-D (page 5364, left column, 2nd paragraph). The method of instant claim 5 is essentially the same as a method of controlling weeds wherein the herbicide tolerant plant is selected by applying an herbicide, such as those methods taught by Kaphammer and Pallett et al. It would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to substitute functionally similar/equivalent transgenes to produce a phenoxy auxin herbicide resistant plant.

Applicant argues that Kaphammer discloses the *tfdA* gene which only shares about 31% identity with the aad-12 polynucleotide sequence. Applicant argues that Schleinitz *et al* provides information on two genes encoding dioxygenases capable of

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degrading the phenoxypropionate derivatives dichlorprop and mecoprop (see page 5357, left column, first paragraph). Applicant argues that the activities of the proteins encoded by these genes were only assayed in bacteria. Applicant argues that Schleinitz *et al* does not teach that any of the proteins described in Schleinitz *et al* can be used in the context of conferring herbicide tolerance to plants. Pallett *et al* and Xu *et al* do not cure the deficiencies of Kaphammer or Schleinitz *et al* (paragraph spanning pages 13-14 of the Remarks).

Applicant argues that the present invention relates to the use of polynucleotides, encoding an aryloxyalkanoate dioxygenase (AAD-12) enzyme, to provide tolerance in plants to more than one class of herbicide. Applicant argues that the *aad-12* nucleic acid sequences (SEQ ID NOS: 1, 3, and 5) provide plants transformed with these polynucleotides tolerance to at least two classes of herbicides: phenoxyacetate auxin and pyridyloxyacetate auxin herbicides. Applicant argues that no aryloxyalkanoate dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of these two different chemical classes, phenoxyacetate and pyridyloxyacetate auxin herbicides, in plants. Applicant argues that the polynucleotides specified in the claims for plant expression, possess properties that are not obvious over Schleinitz *et al* and Kaphammer.

Applicant argues that Kaphammer does nothing to make obvious the polynucleotides specified in the claims conferring tolerance to plants to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides. Applicant argues that Kaphammer relates to *tfdA*, which shares only about

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31% identity with AAD-12. Applicant argues that the AAD-12 polynucleotide sequences, specified in the claims, are not obvious over Kaphammer. Applicant argues that Kaphammer mentions only tolerance to phenoxyacetate auxin herbicides, and not tolerance to pyridyloxyacetate auxin herbicides. Applicant argues that Kaphammer provides no teaching that an aad-12 polynucleotide could be used to provide a plant tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides (page 14 of the Remarks).

Applicant argues that Schleinitz *et al* provides nothing further to make the claimed methods and compositions obvious. Applicant argues that Schleinitz *et al* provides information on two genes encoding dioxygenases capable of degrading the phenoxypropionate derivatives, dichlorprop and mecoprop. Applicant argues that the activities of the proteins encoded by these genes were assayed in bacteria. Applicant argues that Schleinitz *et al* does not have a teaching that any of the proteins described in Schleinitz *et al* can be used in the context of conferring herbicide tolerance to plants, let alone tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides (page 15, 1st paragraph of the Remarks).

Applicant argues that not only does *aad-12* provide surprisingly strong herbicide tolerance to plants, but the expression of the claimed *aad-12* polynucleotides renders plants not only tolerant to phenoxyacetate auxins, such as 2,4-D, but also to pyridyloxyacetate herbicides, such as triclopyr and fluroxypyr (see page 4, paragraph [0015]) (page 16, 3rd paragraph of the Remarks).

Applicant's arguments are not found to be persuasive. The differences between the tfdA taught by Kaphammer and the SdpA enzyme was acknowledged by Schleinitz *et al.* Schleinitz *et al.* had taught that tfdA and SdpA share substantial structural similarity with group II α KG-dependent dioxygenases (page 5362, right column, 3rd paragraph). Applicant's arguments are not commensurate in scope with what is claimed. While there is nothing in the prior art to suggest that the SdpA enzyme would degrade pyridyloxy auxin herbicides it was well recognized that SdpA does degrade phenoxy auxin herbicides just like tfdA. The claimed expression cassette (instant claims 1-3) and the polynucleotide (instant claims 21-24 and 26) would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to try in view of Kaphammer, Schleinitz *et al.* and Xu *et al.* As addressed in the rejection, 2,4-D, mecoprop and dichlorprop are herbicides that were well known in the art at the time of Applicant's invention and so the methods of controlling weeds (instant claims 4-8 and 10-20) would have been obvious to try in view of the teachings of Kaphammer and Schleinitz *et al.*

Allowable Subject Matter

13. Claim 9 is objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Conclusion

14. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP

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§ 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

15. No claims are allowed.

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

BARNES & THORNBURG LLP

11 South Meridian Street
 Indianapolis, Indiana 46204
 (317) 236-1313
 (317) 231-7433 Fax

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Group:</i>	1663	}	
		}	
<i>Confirmation No.:</i>	3336	}	
		}	
<i>Application No.:</i>	14/491,197	}	
		}	
<i>Invention:</i>	NOVEL HERBICIDE RESISTANCE GENES	}	
		}	ELECTRONICALLY FILED:
<i>Inventors:</i>	Terry R. Wright et al.	}	<u>April 12, 2017</u>
		}	
<i>Filed:</i>	September 19, 2014	}	
		}	
<i>Attorney</i>		}	
<i>Docket:</i>	14764-245772	}	
		}	
<i>Examiner:</i>	KRUSE, DAVID H.	}	

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

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

Sir:

This Statement is filed in the captioned application pursuant to 37 C.F.R. §1.56. The filing of this Statement shall not be construed to be an admission that the information cited in the Statement is, or is considered to be, material to patentability as defined in §1.56(b).

No representation is intended that a complete search has been made of the prior art or that no better art references than listed below are available. Pursuant to 37 C.F.R. § 1.98 (a)(2)(i) and 37 C.F.R. § 1.98 (a)(2)(ii) copies of foreign patent applications and non-patent-literature, or their abstracts, are provided with this paper for review by the Examiner.

Applicant hereby authorizes the Commissioner to charge any fees for a large entity to the Barnes & Thornburg Deposit Account No. 10-0435, with reference to our Matter No. 14764-245772.

Respectfully Submitted,
BARNES & THORNBURG LLP

/Rebecca L. Ball/

Rebecca L. Ball
Registration No. 46,535

Indianapolis, IN
(317) 231-7511

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

Sheet 1 of 1

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT				ATTY. DOCKET NO. 14764-245772		APPLICATION NO. 14/491,197	
				APPLICANT Terry R. Wright et al.			
				FILING DATE September 19, 2014		GROUP 1663	
U.S. PATENT DOCUMENTS							
*Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate
	AA	5,608,147	03-1997	Bryan J. Kaphammer			
	AB	7,205,561	6-1998	Pallet, et al.			
	AC	6,645,497	11-2003	Malvar et al.			
	AD	8,283,522	10-2012	Wright, Terry R.			
	AE	8,916,752	12-2014	Wright, Terry R.			
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						
FOREIGN PATENT DOCUMENTS							
		Document Number	Date	Country	Class	Subclass	Translation Yes No
	AL	WO 2005/107437	11-2005	WO			
	AM	WO 2007/053482	05-2007	WO			
	AN						
	AO						
OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)							
	AP	SCHLEINITZ, et al., Localization and Characterization of two Novel Genes Encoding Stereospecific Diosxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in <i>Delftia acidovorans</i> MC1, Applied and Environmental Microbiology, Sept. 2004, Vol. 70, p. 5357-5365					
	AR	GRESSEL, Jonathan, Molecular biology of weed control, Transgenic Research 2000, Vol. 9, p. 355-382					
	AS	HEGG, Eric L. et al., Herbicide-Degrading α -Keto Acid-Dependent Enzyme TfdA: Metal Coordination Environment and Mechanistic Insights, Biochemistry, 1999, Vol. 38, p. 16714-16726					
	AT	PERLACK, Frederick J., Modification of the Coding Sequence Enhances Plant Expression of Insect Control Protein Genes, <i>Proc. Natl. Acad. Sci.</i> , USA, Biochemistry, April 1991, Vol. 88, p. 3324-3328					
	AU	KASUGA, Mie, Improving Plant Drought, Salt, and Freezing Tolerance by Gene Transfer of a Single Stress-Inducible Transcription Factor, Nature Biotechnology, March 1999, Vol. 17, p. 287-291					
	AV	VERDAGUER, Bertrand, Isolation and Expression in Transgenic Tobacco and Rice Plants, of the Cassava Vein Mosaic Virus (CVMV) Promoter, Plant Molecular Biology, 1996, Vol. 31, p. 1129-1139.					
	AW	WESTENDORF, et al., Purification and Characterisation of the Enantiospecific Dioxygenases from <i>Delftia acidovorans</i> MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides, Acta Biotechnol. 2003, Vol. 23, p. 3-17					
	AX	FOURGOUX-NICOL et al., Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte, Plant Biology, 1999, Vol. 40, p. 857-872					
	AY	Iowa State University Extension 2005 Herbicide manual for Agricultural Professionals, p. 50-72					
	AZ	Halford et al., Weed Technology, 2001, Vol. 15, p. 737-744					
Examiner /DAVID H KRUSE/					Date Considered 06/15/2017		
*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.							

BASED ON FORM PTO 1449

Search Notes 	Application/Control No. 14491197	Applicant(s)/Patent Under Reexamination WRIGHT ET AL.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor Name Search	12/29/2016	/DK/
EAST: USPAT, USPGPU, EPO, JPO, DERWENT	12/29/2016	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	12/29/2016	/DK/
Sequence Search of SEQ I DNO: 1-5 included interference search	12/9/2016	/DK/
Update EAST	6/15/2017	/DK/

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/DAVID H KRUSE/ Primary Examiner.Art Unit 1663
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EAST Search History**EAST Search History (Prior Art)**

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	261	aryloxyalkanoate adj dioxygenase	USPAT	OR	OFF	2017/06/15 13:54
L2	359	cassava vein mosaic virus promoter herbicide	USPAT	AND	OFF	2017/06/15 13:54
L3	85	L2 and herbicide.clm.	USPAT	AND	OFF	2017/06/15 13:54
L4	66	cassava adj vein adj mosaic adj virus adj promoter	USPAT	OR	OFF	2017/06/15 13:54
L5	19	L4 and herbicide.clm.	USPAT	OR	OFF	2017/06/15 13:54
L6	30	l1 and "AAD-12"	USPAT	OR	OFF	2017/06/15 13:55

EAST Search History (Interference)

< This search history is empty >

6/ 15/ 2017 1:56:38 PM**C:\Users\dkruse\Documents\EAST\Workspaces\14491197.wsp**

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Jun 23, 2017 03:42:16 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	CTFR	06/23/2017	14764-245772
	1449	06/23/2017	14764-245772

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

BARNES & THORNBURG LLP

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Indianapolis, Indiana 46204
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Customer No.: 107540 }
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Group: 1663 }
 }
Confirmation No.: 3336 }
 }
Application No.: 14/491,197 }
 }
Invention: **NOVEL HERBICIDE** }
 RESISTANCE GENES }
 }
Inventors: Wright, Terry R., et al. }
 }
Filed: September 19, 2014 }
 }
Attorney Docket: 14764-245772 }
 }
Examiner: KRUSE, DAVID H. }

FILED ELECTRONICALLY:
August 23, 2017

RESPONSE AND AMENDMENT UNDER 37 C.F.R. § 1.116

Mail Stop AF
Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed June 23, 2017, Applicant respectfully requests entry of the following amendments and consideration of the accompanying remarks.

Applicant believes that no fees are required with this response. However, if any fees are required, the Commissioner is hereby authorized to charge those fees to the account of

U.S. Application Serial No. 14/491,197

Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

A complete listing of the pending claims is provided pursuant to 37 C.F.R. § 1.121(c)(1). Please add new claim 27. Please amend claims 1, 8, 10, 21, 22, and 24 as follows.

In the Claims

1. (Currently Amended) An expression cassette for expression in a plant cell, comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter,[[,]] wherein said polynucleotide encodes a protein that catalyzes degradation of a ~~herbicide selected from the group consisting of a phenoxy auxin and [[a]] pyridyloxy auxin herbicides~~, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

2. (Original) The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.

3. (Previously Presented) The expression cassette of claim 1, wherein said plant promoter or said plant virus promoter is selected from a cassava vein mosaic virus promoter, a CaMV 35S promoter, a Figwort Mosaic Virus promoter, a rice actin promoter, a phaseolin promoter, an Arabidopsis thaliana Ubiquitin 10 promoter, a maize ubiquitin promoter, an Arabidopsis thaliana Act2 promoter, an Arabidopsis thaliana Ubiquitin 11 promoter, and an Arabidopsis thaliana Ubiquitin 3 promoter.

4. (Previously Presented) A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising the expression cassette of claim 1.

5. (Previously Presented) A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of an aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprises at least one plant that is stably transformed with the expression cassette of claim 1 and is able to grow in said effective amount of the aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette.

6. (Original) The method of claim 4, wherein said plurality of crop plants are dicots.

7. (Previously Presented) The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar beet plants, tomato plants, and sunflower plants.

8. (Currently Amended) The method of claim 4, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, and wherein said pyridyloxy auxin herbicide comprises one or more pyridyloxyacetate herbicides.

9. (Previously Presented) The method of claim 8, wherein said pyridyloxyacetate herbicide(s) is selected from the group consisting of triclopyr and fluroxypyr.

10. (Currently Amended) The method of claim 4, wherein said applying step comprises applying said herbicide to said crop plants within 14 days of planting a seed in said field, wherein said seed comprises an expression cassette comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of ~~a herbicide selected from the group consisting of a~~ phenoxy auxin and ~~[[a]]~~ pyridyloxy auxin herbicides, wherein said protein has at least 95%

amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

11. (Previously Presented) The method of claim 4, wherein said plurality of crop plants further comprises a second polynucleotide encoding a second protein that confers said crop plants with resistance to at least one other herbicide, wherein said second polynucleotide is heterologous, and said method further comprises applying said at least one other herbicide to at least a portion of said field.

12. (Original) The method of claim 11, wherein said herbicides are applied sequentially or concurrently.

13. (Original) The method of claim 11, wherein said herbicides are applied from a tank mix.

14. (Original) The method of claim 11, wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispyribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyriithiobac, pyroxasulfone, pyroxsulam,

quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr, thidiazuron, thiencazone, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflurosulfuron, and tritosulfuron.

15. (Original) The method of claim 4, wherein said weeds are glyphosate-resistant.

16. (Original) The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.

17. (Previously Presented) The method of claim 4, wherein said herbicide is the phenoxy auxin herbicide.

18. (Original) The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.

19. (Previously Presented) A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising the expression cassette of claim 1.

20. (Previously Presented) The method of claim 19 wherein said volunteer plants are glyphosate-resistant and of a different species than said transgenic crop plants.

21. (Currently Amended) A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy

auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

22. (Currently Amended) A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter, and wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has SEQ ID NO:3.

23. (Original) The polynucleotide of claim 22 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

24. (Currently Amended) An isolated polynucleotide that is operably linked to a heterologous plant promoter or a plant virus promoter, and wherein the polynucleotide encodes a protein that enzymatically degrades ~~a herbicide selected from the group consisting of a~~ phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

25. (Cancelled)

26. (Previously Presented) The polynucleotide of claim 24 wherein said promoter is a cassava vein mosaic virus promoter.

27. (New) The polynucleotide of claim 24 wherein the polynucleotide has SEQ ID NO:3.

REMARKS

(I) CLAIM AMENDMENTS

Claims 1, 8, 10, 21, 22, and 24 have been amended. Claim 8 has been amended to add the word “said.” Claims 1, 10, and 24 have been amended to remove the Markush group language. These claims now require that the “polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides.” Claims 21 and 22 have been amended to require that “the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides.” New claim 27 has been added. Support for the amendments can be found in the originally filed claims and throughout the specification as filed. Applicant respectfully requests entry of the amendments.

(II) ALLOWABLE SUBJECT MATTER

Applicant thanks the Examiner for acknowledging that claim 9 is allowable if rewritten in independent form.

(III) REJECTION UNDER 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-8, 10-24, and 26 under 35 U.S.C. § 103(a), asserting that the claims are obvious over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al.*, Pallet *et al.* (U.S. Patent 7,250,561 B1), and Xu *et al.*, (U.S. Patent 6,664,384). Applicant notes the Office Action states the Xu *et al.* U.S. Patent number is 6,665,384, but Applicant believes the Examiner meant 6,664,384 as evidenced by the 892 Form provided January 12, 2017.

Applicant respectfully traverses the Examiner's rejections under 35 U.S.C. §103(a). Claims 1, 10, 21, 22, and 24 have been amended, and as a result these claims, and their dependent claims 2-8, 11-20, 23, and 26, are not obvious over the combined teachings of Kaphammer, Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.*

The present invention relates to the use of polynucleotides, encoding an aryloxyalkanoate dioxygenase (AAD-12) enzyme, to provide tolerance in plants to more than one class of herbicide. The *aad-12* nucleic acid sequences (SEQ ID NOS: 1, 3, and 5) provide plants transformed with these polynucleotides tolerance to at least two classes of herbicides: phenoxyacetate auxin and pyridyloxyacetate auxin herbicides. No aryloxyalkanoate dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of ***these two different chemical classes, phenoxyacetate and pyridyloxyacetate auxin herbicides, in plants.*** Therefore, the polynucleotides specified in the claims for plant expression, possess properties that are not obvious over Schleinitz *et al.* and Kaphammer in combination with Pallet *et al.* and Xu *et al.*

Kaphammer does nothing to make obvious the polynucleotides specified in the claims conferring plants with tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides. First, Kaphammer relates to the *tfdA* gene, which shares only about 31% identity with *aad-12*. So, the *aad-12* polynucleotide sequences and the corresponding amino acid sequences, specified in the claims, are not obvious over Kaphammer. Also, Kaphammer mentions only tolerance to phenoxyacetate auxin herbicides, and not tolerance to pyridyloxyacetate auxin herbicides. Thus, Kaphammer provides no teaching that an *aad-12* polynucleotide or protein could be used to provide a plant tolerance to

two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

Schleinitz *et al.* provides nothing further to make the claimed methods and compositions obvious. Schleinitz *et al.* provides information on two genes, *rdpA* and *sdpA*, encoding dioxygenases capable of degrading the phenoxypropionate derivatives, dichlorprop and mecoprop. The activities of the proteins encoded by these genes were only assayed in bacteria. Thus, Schleinitz *et al.* does not have a teaching that any of the proteins described in Schleinitz *et al.* can be used in the context of conferring herbicide tolerance to plants, let alone tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

Pallet *et al.* describes polynucleotides and methods for transforming Maize cells with genes that provide some level of resistance to dihalohydroxybenzonitriles, hydroxylphenyl pyruvate dioxygenase (HPPD), EPSPS-inhibiting herbicides, and acetolactate synthase (ALS) inhibiting herbicide, altogether different herbicide classes than those specified in the claims. Xu *et al.* describes designing promoters for expressing genes using pieces of a viral promoter. Thus, the Pallet *et al.* and Xu *et al.* references do nothing to overcome the insufficiencies of Kaphammer and Schleinitz *et al.* Pallet *et al.* and Xu *et al.* do not provide any suggestion that the claimed *aad-12* polynucleotides could be used in plants to provide tolerance to both phenoxyacetate and pyridyloxyacetate auxin herbicides. Thus, the above-discussed unexpected suitability of *aad-12* polynucleotides to confer strong herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer, Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.*

Beginning at the bottom of page 5 through page 7 of the June 23, 2017 Office Action, the Examiner reiterates Applicant's arguments discussed above. The Examiner then provides a response to Applicant's arguments in the first paragraph on page 8 of the June 23, 2017 Office Action. On page 8, first paragraph, lines 5-8, the Examiner states the following,

Applicant's arguments are not commensurate in scope with what is claimed. While *there is nothing in the prior art to suggest that the SdpA enzyme would degrade pyridyloxy auxin herbicides* it was well recognized that SdpA does degrade phenoxy auxin herbicides just like tfdA.

See Page 8, lines 5-8 of the June 23, 2017 Office Action. (Emphasis Added). Thus, the Examiner conceded that "there is nothing in the prior art to suggest that the SdpA enzyme would degrade pyridyloxy auxin herbicides." On the other hand, the Examiner indicates it was known that the SdpA enzyme (described in Schleinitz *et al.*) and the enzyme encoded by *tfdA* (described in Kaphammer) degrade phenoxy auxin herbicides.

As indicated by the Examiner, there is nothing in the prior art to suggest that the enzymes described in the prior art would degrade pyridyloxy auxin herbicides. Accordingly, in response to the Examiner's statements on page 8 of the Office Action, Applicant has amended claim 1 to remove the Markush group language. Thus, claim 1 now requires that the polynucleotide encodes a protein that catalyzes degradation of **both** phenoxy auxin and pyridyloxy auxin herbicides. Claim 10 has been amended consistent with claim 1. Claims 21, 22, and 24 have also been amended to require that the claimed polynucleotide encodes a protein that enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides. Therefore, all of the claims require that the polynucleotide encodes a protein that catalyzes degradation of **both**

phenoxy auxin and pyridyloxy auxin herbicides, and Applicant's arguments are commensurate in scope with what is claimed.

Although the arguments presented in the April 12, 2017 response regarding unexpectedly strong herbicide tolerance of the plants expressing the *aad-12* polynucleotides still apply, the above discussed unexpected suitability of *aad-12* polynucleotides to confer herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is alone sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer, Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.* Withdrawal of the rejection of claims 1-8, 10-24, and 26 under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

The foregoing amendments and remarks are believed to fully respond to the Examiner's rejections.

Respectfully submitted,
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Registration No. 46,535

Indianapolis, Indiana 46204
317-231-7511

DMS 4860790v1

Electronic Acknowledgement Receipt	
EFS ID:	30164402
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Rebecca Ball/Garla Twyman
Filer Authorized By:	Rebecca Ball
Attorney Docket Number:	14764-245772
Receipt Date:	23-AUG-2017
Filing Date:	19-SEP-2014
Time Stamp:	16:25:50
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Response_and_Amendment_under_37_C_F_R_1_116.pdf	68964 bd8675ddcd261d857ae3a2adccd50e977982cbb9	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	Start	End
	Response After Final Action	1	2
	Claims	3	7
	Applicant Arguments/Remarks Made in an Amendment	8	12
Warnings:			
Information:			
Total Files Size (in bytes):		68964	
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>			

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875					Application or Docket Number 14/491,197		Filing Date 09/19/2014		☒ To be Mailed	
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO										
APPLICATION AS FILED – PART I										
(Column 1)		(Column 2)								
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)		
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A				
☐ SEARCH FEE (37 CFR 1.16(k), (j), or (m))		N/A		N/A		N/A				
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A				
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		X \$ =				
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		X \$ =				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).								
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))										
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL				
APPLICATION AS AMENDED – PART II										
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT	08/23/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 26	Minus	** 26	= 0	x \$80 =		0		
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	x \$420 =		0		
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE		0		
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =				
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =				
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE				
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.										

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
107540	7590	09/01/2017	EXAMINER	
Barnes & Thornburg LLP (Dow)			KRUSE, DAVID H	
11 South Meridian Street			ART UNIT	
Indianapolis, IN 46204			PAPER NUMBER	
			1663	
			NOTIFICATION DATE	
			DELIVERY MODE	
			09/01/2017	
			ELECTRONIC	

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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Advisory Action Before the Filing of an Appeal Brief	Application No. 14/491,197	Applicant(s) WRIGHT ET AL.	
	Examiner DAVID H. KRUSE	Art Unit 1663	AIA (First Inventor to File) Status No

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 23 August 2017 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.
NO NOTICE OF APPEAL FILED

1. ☒ The reply was filed after a final rejection. No Notice of Appeal has been filed. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114 if this is a utility or plant application. Note that RCEs are not permitted in design applications. The reply must be filed within one of the following time periods:

a) ☐ The period for reply expires _____ months from the mailing date of the final rejection.

b) ☒ The period for reply expires on: (1) the mailing date of this Advisory Action; or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

c) ☐ A prior Advisory Action was mailed more than 3 months after the mailing date of the final rejection in response to a first after-final reply filed within 2 months of the mailing date of the final rejection. The current period for reply expires _____ months from the mailing date of the prior Advisory Action or SIX MONTHS from the mailing date of the final rejection, whichever is earlier.

Examiner Note: If box 1 is checked, check either box (a), (b) or (c). ONLY CHECK BOX (b) WHEN THIS ADVISORY ACTION IS THE FIRST RESPONSE TO APPLICANT'S FIRST AFTER-FINAL REPLY WHICH WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. ONLY CHECK BOX (c) IN THE LIMITED SITUATION SET FORTH UNDER BOX (c). See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) or (c) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendments filed after a final rejection, but prior to the date of filing a brief, will not be entered because

a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);

b) ☐ They raise the issue of new matter (see NOTE below);

c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or

d) ☒ They present additional claims without canceling a corresponding number of finally rejected claims.

NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).

4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).

5. ☐ Applicant's reply has overcome the following rejection(s): _____.

6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

7. ☐ For purposes of appeal, the proposed amendment(s): (a) ☐ will not be entered, or (b) ☐ will be entered, and an explanation of how the new or amended claims would be rejected is provided below or appended.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

9. ☐ The affidavit or other evidence filed after final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).

10. ☐ The affidavit or other evidence filed after the date of filing the Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing of good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).

11. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

12. ☐ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: _____.

13. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____

14. ☐ Other: _____.

STATUS OF CLAIMS

15. The status of the claim(s) is (or will be) as follows:

Claim(s) allowed: _____.

Claim(s) objected to: 9.

Claim(s) rejected: 1-8,10-24 and 26.

Claim(s) withdrawn from consideration: _____.

	/DAVID H KRUSE/ Primary Examiner, Art Unit 1663
--	--

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Customer No.: 107540 }
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Group: 1663 }
 }
Confirmation No.: 3336 }
 }
Application No.: 14/491,197 }
 }
Invention: **NOVEL HERBICIDE** }
 RESISTANCE GENES }
 }
Inventors: Wright, Terry R., et al. }
 }
Filed: September 19, 2014 }
 }
Attorney Docket: 14764-245772 }
 }
Examiner: KRUSE, DAVID H. }

FILED ELECTRONICALLY:
August 23, 2017

RESPONSE AND AMENDMENT UNDER 37 C.F.R. § 1.116

Mail Stop AF
Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed June 23, 2017, Applicant respectfully requests entry of the following amendments and consideration of the accompanying remarks.

Applicant believes that no fees are required with this response. However, if any fees are required, the Commissioner is hereby authorized to charge those fees to the account of

U.S. Application Serial No. 14/491,197

Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

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Sep 01, 2017 04:17:03 AM

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	CTAV	09/01/2017	14764-245772

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Customer No.:</i>	107540	}
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<i>Group:</i>	1663	}
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<i>Confirmation No.:</i>	3336	}
		}
<i>Application No.:</i>	14/491,197	}
		}
<i>Invention:</i>	NOVEL HERBICIDE RESISTANCE GENES	}
		}
<i>Inventors:</i>	Wright, Terry R., et al.	}
		}
<i>Filed:</i>	September 19, 2014	}
		}
<i>Attorney Docket:</i>	14764-245772	}
		}
<i>Examiner:</i>	KRUSE, DAVID H.	}

**FILED ELECTRONICALLY:
September 15, 2017**

SUPPLEMENTAL RESPONSE AND AMENDMENT UNDER 37 C.F.R. § 1.116

Mail Stop AF
Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed June 23, 2017, Applicant respectfully requests entry of the following amendments and consideration of the accompanying remarks. Applicant is filing this Supplemental Response in response to the phone conversation between Applicant's undersigned attorney and the Examiner on September 8, 2017 during which the

Examiner indicated that Applicant should file a supplemental response to Applicant's August 23, 2017 response canceling one claim to be replaced by new claim 27 (see also item 3 of the Advisory Action issued on September 1, 2017). The Examiner indicated he would then consider Applicant's response. Applicant has canceled claim 23 and retained claim 27 in this response.

Applicant believes that no fees are required with this response. However, if any fees are required, the Commissioner is hereby authorized to charge those fees to the account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

A complete listing of the pending claims is provided pursuant to 37 C.F.R. § 1.121(c)(1). Please add new claim 27. Please cancel claim 23 without prejudice. Please amend claims 1, 8, 10, 21, 22, and 24 as follows.

In the Claims

1. (Currently Amended) An expression cassette for expression in a plant cell, comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter,[[,]] wherein said polynucleotide encodes a protein that catalyzes degradation of a ~~herbicide selected from the group consisting of a phenoxy auxin and [[a]] pyridyloxy auxin herbicides~~, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.
2. (Original) The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.
3. (Previously Presented) The expression cassette of claim 1, wherein said plant promoter or said plant virus promoter is selected from a cassava vein mosaic virus promoter, a CaMV 35S promoter, a Figwort Mosaic Virus promoter, a rice actin promoter, a phaseolin promoter, an Arabidopsis thaliana Ubiquitin 10 promoter, a maize ubiquitin promoter, an Arabidopsis thaliana Act2 promoter, an Arabidopsis thaliana Ubiquitin 11 promoter, and an Arabidopsis thaliana Ubiquitin 3 promoter.
4. (Previously Presented) A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising the expression cassette of claim 1.

5. (Previously Presented) A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of an aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprises at least one plant that is stably transformed with the expression cassette of claim 1 and is able to grow in said effective amount of the aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette.

6. (Original) The method of claim 4, wherein said plurality of crop plants are dicots.

7. (Previously Presented) The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar beet plants, tomato plants, and sunflower plants.

8. (Currently Amended) The method of claim 4, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, and wherein said pyridyloxy auxin herbicide comprises one or more pyridyloxyacetate herbicides.

9. (Previously Presented) The method of claim 8, wherein said pyridyloxyacetate herbicide(s) is selected from the group consisting of triclopyr and fluroxypyr.

10. (Currently Amended) The method of claim 4, wherein said applying step comprises applying said herbicide to said crop plants within 14 days of planting a seed in said field, wherein said seed comprises an expression cassette comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of ~~a herbicide selected from the group consisting of~~ a-phenoxy auxin and ~~[[a]]~~ pyridyloxy auxin herbicides, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

11. (Previously Presented) The method of claim 4, wherein said plurality of crop plants further comprises a second polynucleotide encoding a second protein that confers said crop plants with resistance to at least one other herbicide, wherein said second polynucleotide is heterologous, and said method further comprises applying said at least one other herbicide to least a portion of said field.

12. (Original) The method of claim 11, wherein said herbicides are applied sequentially or concurrently.

13. (Original) The method of claim 11, wherein said herbicides are applied from a tank mix.

14. (Original) The method of claim 11, wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispyribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyriithiobac, pyroxasulfone, pyroxsulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr,

thidiazuron, thien carbazole, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflusulfuron, and tritosulfuron.

15. (Original) The method of claim 4, wherein said weeds are glyphosate-resistant.

16. (Original) The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.

17. (Previously Presented) The method of claim 4, wherein said herbicide is the phenoxy auxin herbicide.

18. (Original) The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.

19. (Previously Presented) A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising the expression cassette of claim 1.

20. (Previously Presented) The method of claim 19 wherein said volunteer plants are glyphosate-resistant and of a different species than said transgenic crop plants.

21. (Currently Amended) A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

22. (Currently Amended) A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter,[[and]] wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has SEQ ID NO:3.

23. (Cancelled)

24. (Currently Amended) An isolated polynucleotide that is operably linked to a heterologous plant promoter or a plant virus promoter, [[and]] wherein the polynucleotide encodes a protein that enzymatically degrades ~~a herbicide selected from the group consisting of a~~ phenoxy auxin and [[a]] pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

25. (Cancelled)

26. (Previously Presented) The polynucleotide of claim 24 wherein said promoter is a cassava vein mosaic virus promoter.

27. (New) The polynucleotide of claim 24 wherein the polynucleotide has SEQ ID NO:3.

REMARKS

(I) CLAIM AMENDMENTS

Claims 1, 8, 10, 21, 22, and 24 have been amended. Claim 8 has been amended to add the word “said.” Claims 1, 10, and 24 have been amended to remove the Markush group language. These claims now require that the “polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides.” Claims 21 and 22 have been amended to require that “the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides.” New claim 27 has been added. Claim 23 has been canceled without prejudice. Support for the amendments can be found in the originally filed claims and throughout the specification as filed. Applicant respectfully requests entry of the amendments.

(II) ALLOWABLE SUBJECT MATTER

Applicant thanks the Examiner for acknowledging that claim 9 is allowable if rewritten in independent form.

(III) REJECTION UNDER 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-8, 10-24, and 26 under 35 U.S.C. § 103(a), asserting that the claims are obvious over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al.*, Pallet *et al.* (U.S. Patent 7,250,561 B1), and Xu *et al.*, (U.S. Patent 6,664,384). Applicant notes the Office Action states the Xu *et al.* U.S. Patent number is 6,665,384, but Applicant believes the Examiner meant 6,664,384 as evidenced by the 892 Form provided January 12, 2017.

Applicant respectfully traverses the Examiner's rejections under 35 U.S.C. §103(a). Claim 23 has been canceled without prejudice. Claims 1, 10, 21, 22, and 24 have been amended, and as a result these claims, and their dependent claims 2-8, 11-20, and 26, are not obvious over the combined teachings of Kaphammer, Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.*

The present invention relates to the use of polynucleotides, encoding an aryloxyalkanoate dioxygenase (AAD-12) enzyme, to provide tolerance in plants to more than one class of herbicide. The *aad-12* nucleic acid sequences (SEQ ID NOS: 1, 3, and 5) provide plants transformed with these polynucleotides tolerance to at least two classes of herbicides: phenoxyacetate auxin and pyridyloxyacetate auxin herbicides. No aryloxyalkanoate dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of ***these two different chemical classes, phenoxyacetate and pyridyloxyacetate auxin herbicides, in plants.*** Therefore, the polynucleotides specified in the claims for plant expression, possess properties that are not obvious over Schleinitz *et al.* and Kaphammer in combination with Pallet *et al.* and Xu *et al.*

Kaphammer does nothing to make obvious the polynucleotides specified in the claims conferring plants with tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides. First, Kaphammer relates to the *tfdA* gene, which shares only about 31% identity with *aad-12*. So, the *aad-12* polynucleotide sequences and the corresponding amino acid sequences, specified in the claims, are not obvious over Kaphammer. Also, Kaphammer mentions only tolerance to phenoxyacetate auxin herbicides, and not tolerance to pyridyloxyacetate auxin herbicides. Thus, Kaphammer provides no teaching that an *aad-12* polynucleotide or protein could be used to provide a plant tolerance to

two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

Schleinitz *et al.* provides nothing further to make the claimed methods and compositions obvious. Schleinitz *et al.* provides information on two genes, *rdpA* and *sdpA*, encoding dioxygenases capable of degrading the phenoxypropionate derivatives, dichlorprop and mecoprop. The activities of the proteins encoded by these genes were only assayed in bacteria. Thus, Schleinitz *et al.* does not have a teaching that any of the proteins described in Schleinitz *et al.* can be used in the context of conferring herbicide tolerance to plants, let alone tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

Pallet *et al.* describes polynucleotides and methods for transforming Maize cells with genes that provide some level of resistance to dihalohydroxybenzonitriles, hydroxylphenyl pyruvate dioxygenase (HPPD), EPSPS-inhibiting herbicides, and acetolactatesynthase (ALS) inhibiting herbicide, altogether different herbicide classes than those specified in the claims. Xu *et al.* describes designing promoters for expressing genes using pieces of a viral promoter. Thus, the Pallet *et al.* and Xu *et al.* references do nothing to overcome the insufficiencies of Kaphammer and Schleinitz *et al.* Pallet *et al.* and Xu *et al.* do not provide any suggestion that the claimed *aad-12* polynucleotides could be used in plants to provide tolerance to both phenoxyacetate and pyridyloxyacetate auxin herbicides. Thus, the above-discussed unexpected suitability of *aad-12* polynucleotides to confer strong herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer, Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.*

Beginning at the bottom of page 5 through page 7 of the June 23, 2017 Office Action, the Examiner reiterates Applicant's arguments discussed above. The Examiner then provides a response to Applicant's arguments in the first paragraph on page 8 of the June 23, 2017 Office Action. On page 8, first paragraph, lines 5-8, the Examiner states the following,

Applicant's arguments are not commensurate in scope with what is claimed. While *there is nothing in the prior art to suggest that the SdpA enzyme would degrade pyridyloxy auxin herbicides* it was well recognized that SdpA does degrade phenoxy auxin herbicides just like tfdA.

See Page 8, lines 5-8 of the June 23, 2017 Office Action. (Emphasis Added). Thus, the Examiner conceded that "there is nothing in the prior art to suggest that the SdpA enzyme would degrade pyridyloxy auxin herbicides." On the other hand, the Examiner indicates it was known that the SdpA enzyme (described in Schleinitz *et al.*) and the enzyme encoded by *tfdA* (described in Kaphammer) degrade phenoxy auxin herbicides.

As indicated by the Examiner, there is nothing in the prior art to suggest that the enzymes described in the prior art would degrade pyridyloxy auxin herbicides. Accordingly, in response to the Examiner's statements on page 8 of the Office Action, Applicant has amended claim 1 to remove the Markush group language. Thus, claim 1 now requires that the polynucleotide encodes a protein that catalyzes degradation of **both** phenoxy auxin and pyridyloxy auxin herbicides. Claim 10 has been amended consistent with claim 1. Claims 21, 22, and 24 have also been amended to require that the claimed polynucleotide encodes a protein that enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides. Therefore, all of the claims require that the polynucleotide encodes a protein that catalyzes degradation of **both** phenoxy auxin and pyridyloxy auxin herbicides, and Applicant's arguments are commensurate in scope with what is claimed.

Although the arguments presented in the April 12, 2017 response regarding unexpectedly strong herbicide tolerance of the plants expressing the *aad-12* polynucleotides still apply, the above discussed unexpected suitability of *aad-12* polynucleotides to confer herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is alone sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer , Schleinitz *et al.*, Pallet *et al.*, and Xu *et al.* Withdrawal of the rejection of claims 1-8, 10-22, 24, and 26 under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

The foregoing amendments and remarks are believed to fully respond to the Examiner's rejections.

Respectfully submitted,
BARNES & THORNBURG LLP

/Rebecca L. Ball/
Rebecca L. Ball
Registration No. 46,535

Indianapolis, Indiana 46204
317-231-7511

DMS 10906188v1

Electronic Acknowledgement Receipt	
EFS ID:	30376099
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Rebecca Ball/Sherri Cozart
Filer Authorized By:	Rebecca Ball
Attorney Docket Number:	14764-245772
Receipt Date:	15-SEP-2017
Filing Date:	19-SEP-2014
Time Stamp:	12:14:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Supplemental_Response_and_Amendment.pdf	485604 37be49a68f15bc0aeaf2a5c1a2f62a5d82577594	yes	12

Multipart Description/PDF files in .zip description			
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	Applicant Arguments/Remarks Made in an Amendment	8	12
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875				Application or Docket Number 14/491,197		Filing Date 09/19/2014		☐ To be Mailed	
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO									
APPLICATION AS FILED – PART I									
(Column 1)		(Column 2)							
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)	
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A			
☐ SEARCH FEE (37 CFR 1.16(k), (j), or (m))		N/A		N/A		N/A			
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A			
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		X \$ =			
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		X \$ =			
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).							
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))									
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL			
APPLICATION AS AMENDED – PART II									
(Column 1)		(Column 2)		(Column 3)					
AMENDMENT	09/15/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)	
	Total (37 CFR 1.16(i))	* 25	Minus	** 26	= 0	x \$80 =		0	
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	x \$420 =		0	
	☐ Application Size Fee (37 CFR 1.16(s))								
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))								
						TOTAL ADD'L FEE		0	
(Column 1)		(Column 2)		(Column 3)					
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)	
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =			
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =			
	☐ Application Size Fee (37 CFR 1.16(s))								
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))								
						TOTAL ADD'L FEE			
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.									

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LIE
ANTJUAN RIVERA

Doc code: RCEX

Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (07-14)

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**REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)**

Application Number	14/491,197	Filing Date	2014-09-19	Docket Number (if applicable)	14764-245772	Art Unit	1663
First Named Inventor	Terry R. Wright			Examiner Name	KRUSE, DAVID H		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C. 371, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV.

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☒ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____☒ Other Consider the Response After Final Action previously filed on 8/23/2017 and the Supplemental Response and Amendment previously filed on 9/15/2017☐ Enclosed☐ Amendment/Reply☐ Information Disclosure Statement (IDS)☐ Affidavit(s)/ Declaration(s)☐ Other _____**MISCELLANEOUS**☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)☐ Other _____**FEES**☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to
Deposit Account No 100435**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED**☒ Patent Practitioner Signature

Applicant Signature

Doc code: RCEX

Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (07-14)

Approved for use through 07/31/2016. OMB 0651-0031

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Signature of Registered U.S. Patent Practitioner			
Signature	Elizabeth Lehr Stetzer/	Date (YYYY-MM-DD)	2017-12-21
Name	Elizabeth Lehr Stetzer	Registration Number	63496

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

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Electronic Patent Application Fee Transmittal				
Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Elizabeth Lehr Stetzer/Stephanie Switzer		
Attorney Docket Number:		14764-245772		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
RCE- 1st Request	1801	1	1200	1200
Total in USD (\$)				1200

Electronic Acknowledgement Receipt	
EFS ID:	31301674
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Elizabeth Lehr Stetzer/Stephanie Switzer
Filer Authorized By:	Elizabeth Lehr Stetzer
Attorney Docket Number:	14764-245772
Receipt Date:	21-DEC-2017
Filing Date:	19-SEP-2014
Time Stamp:	13:41:27
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1200
RAM confirmation Number	122117INTEFSW00016346100435
Deposit Account	100435
Authorized User	Stephanie Switzer
<p>The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:</p> <p>37 CFR 1.16 (National application filing, search, and examination fees)</p> <p>37 CFR 1.17 (Patent application and reexamination processing fees)</p>	

37 CFR 1.19 (Document supply fees) 37 CFR 1.20 (Post Issuance fees) 37 CFR 1.21 (Miscellaneous fees and charges)					
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	sb0030efs.pdf	1363889	no	3
			a116154dae5347d77708804804e6610a18b2ae2		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30424	no	2
			84bfb1b197a0b657d5e3c3a63a40a7c9a1801c5ea		
Warnings:					
Information:					
Total Files Size (in bytes):			1394313		
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875					Application or Docket Number 14/491,197		Filing Date 09/19/2014		☐ To be Mailed	
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO										
APPLICATION AS FILED – PART I										
(Column 1)		(Column 2)								
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)		
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A				
☐ SEARCH FEE (37 CFR 1.16(k), (j), or (m))		N/A		N/A		N/A				
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A				
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		X \$ =				
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		X \$ =				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).								
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))										
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL				
APPLICATION AS AMENDED – PART II										
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT	12/21/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 25	Minus	** 26	= 0	x \$80 =		0		
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	x \$420 =		0		
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE		0		
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =				
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =				
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE				
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.										

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LIE
LINDA HUMES

EAST Search History**EAST Search History (Prior Art)**

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	310	aryloxyalkanoate adj dioxygenase	USPAT	OR	OFF	2018/01/24 09:46
L2	42	L1 and "AAD-12"	USPAT	OR	OFF	2018/01/24 09:46
L3	13	l1 and (rdpA or sdpA)	USPAT	OR	OFF	2018/01/24 09:47

1/24/2018 10:05:21 AM**C:\Users\dkruse\Documents\EAST\Workspaces\14491197.wsp**



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
107540	7590	01/30/2018		
Barnes & Thornburg LLP (Dow)			EXAMINER	
11 South Meridian Street			KRUSE, DAVID H	
Indianapolis, INDIANA 46204				
			ART UNIT	PAPER NUMBER
			1663	
			NOTIFICATION DATE	DELIVERY MODE
			01/30/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

STATUS OF THE APPLICATION

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 21 December 2017 has been entered.

Allowable Subject Matter

2. Claims 9 and 27 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

At instant claim 9, the prior art does not teach or suggest that the AAD-12 enzyme would be capable to degrading a pyridyloxy auxin herbicide, only that it is capable of degrading phenoxy auxin herbicides.

At instant claim 27, the specific codon optimized nucleotide sequence of SEQ ID NO: 3 would not have been obvious because based on the degeneracy of codon sequences encoding a specific polypeptide sequence there would be a substantially large number of coding sequences possible.

Claim Rejections - 35 USC § 103

3. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

4. Claims 1-8, 10-22, 24 and 26 remain rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz et al (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365), Pallett et al (U.S. Patent 7,250,561 B1, § 371(c)(1) date of 15 June 1998) and Xu et al (U.S. Patent 6,665,384). This rejection is repeated for the reason of record as set forth in the last Office action mailed 23 June 2017. Applicant's arguments filed 15 September 2017 have been fully considered but they are not persuasive.

As used herein, SdpA and AAD-12 are used interchangeably in this rejection as they are synonyms of the same enzyme.

Kaphammer teaches a plant cell comprising a polynucleotide operably linked to a CAMV 35S promoter (Figure 3) that encodes a protein having aryloxyalkanoate dioxygenase activity, resistance to 2,4-dichlorophenoxyacetate (2,4-D) at claim 11, and a plant regenerated therefrom at claim 15. Kaphammer teaches an isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity at SEQ ID NO: 1. Kaphammer teaches transforming a soybean cell at Example 11, columns 21-22. Kaphammer teaches a method of controlling weeds by applying 2,4-D herbicide to a crop field at column 2, lines 26-28. Kaphammer teaches that a transgene

that confers resistance to glyphosate can also be introduced into the plant cell (column 3, lines 11-6).

Kaphammer does not teach an isolated nucleic acid encoding instant SEQ ID NO: 2 or having the nucleotide sequence of instant SEQ ID NO: 1. Kaphammer does not teach introducing a polynucleotide that confers glufosinate resistance. Kaphammer does not teach using a cassava vein mosaic virus promoter.

Schleinitz et al teach an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5th paragraph). The encoded SdpA protein has aryloxyalkanoate dioxygenase activity and has greatest activity of degrading S-enantiomers of mecoprop and dichlorprop and has some activity toward 2,4-D (page 5363, left column, 2nd paragraph).

Pallett et al teach introducing polynucleotides to a transformed plant cell that renders said plant cell resistant to multiple herbicides including glyphosate (column 1, lines 62-64) and glufosinate (column 2, lines 27-29).

Xu et al teach a cassava vein mosaic virus promoter operably linked to a sequence of interest at Figure 3. Xu et al teach that genes encoding herbicide resistance can be used as selectable markers (column 7, lines 5-12) and that said sequence of interest can be a selectable marker at claim 27.

It would have been prima face obvious to one of ordinary skill in the art at the time of Applicants' invention to modify the teachings of Kaphammer to introduce a polynucleotide encoding instant SEQ ID NO: 2 as taught by Schleinitz et al in addition to a polynucleotide encoding resistance to glyphosate and glufosinate as suggested Pallett et al. Given the success of Pallett et al, and Kaphammer one of ordinary skill in the art

would have had a reasonable expectation of success. Given dichlorprop was a known herbicide as taught by Schleinitz et al (page 5357, left column, 1st paragraph), one of ordinary skill in the art would have been motivated to introduce said polynucleotide encoding instant SEQ ID NO: 2 into a plant cell for the purposes of making an herbicide tolerant plant from said plant cell. The SdpA enzyme taught by Schleinitz *et al* is taught as being capable of degrading S-enantiomers of mecoprop and dichlorprop and has some activity toward 2,4-D (page 5364, left column, 2nd paragraph). The method of instant claim 5 is essentially the same as a method of controlling weeds wherein the herbicide tolerant plant is selected by applying an herbicide, such as those methods taught by Kaphammer and Pallett et al. It would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to substitute functionally similar/equivalent transgenes to produce a phenoxy auxin herbicide resistant plant.

Applicant argues that no aryloxyalkanoate dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of these two different chemical classes, phenoxyacetate and pyridyloxyacetate auxin herbicides, in plants, therefore, the polynucleotides specified in the claims for plant expression, possess properties that are not obvious (2nd page, 2nd paragraph of the Remarks).

Applicant argues that Kaphammer does nothing to make obvious the polynucleotides specified in the claims conferring plants with tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides. Applicant argues that Kaphammer relates to the *tfdA* gene, which shares only about 31% identity with *aad-12*. Applicant argues that the *aad-12* polynucleotide sequences and the corresponding amino acid sequences, specified in the claims, are

not obvious over Kaphammer. Applicant argues that Kaphammer mentions only tolerance to phenoxyacetate auxin herbicides, and not tolerance to pyridyloxyacetate auxin herbicides. Applicant argues that Kaphammer provides no teaching that an aad-12 polynucleotide or protein could be used to provide a plant tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides (2nd page, 3rd paragraph of the Remarks).

Applicant argues that Schleinitz et al. does not have a teaching that any of the proteins described in Schleinitz et al. can be used in the context of conferring herbicide tolerance to plants, let alone tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides (3rd page, 2nd paragraph of the Remarks).

Applicant argues that Pallet et al. and Xu et al. do not provide any suggestion that the claimed aad-12 polynucleotides could be used in plants to provide tolerance to both phenoxyacetate and pyridyloxyacetate auxin herbicides. Applicant argues that the discussed unexpected suitability of aad-12 polynucleotides to confer strong herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer, Schleinitz et al., Pallet et al., and Xu et al. (3rd page, 3rd paragraph of the Remarks).

Applicant argues that in response to the Examiner's statements on page 8 of the Office Action (mailed 23 June 2017), Applicant has amended claim 1 to remove the Markush group language. Applicant argues that claim 1 now requires that the polynucleotide encodes a protein that catalyzes degradation of both phenoxy auxin and

pyridyloxy auxin herbicides. Applicant argues that claim 10 has been amended consistent with claim 1. Applicant argues that claims 21, 22, and 24 have also been amended to require that the claimed polynucleotide encodes a protein that enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides. Applicant argues that all of the claims require that the polynucleotide encodes a protein that catalyzes degradation of both phenoxy auxin and pyridyloxy auxin herbicides, and Applicant's arguments are commensurate in scope with what is claimed (4th page, last paragraph of the Remarks).

Applicant's arguments have been fully considered but are not found to be persuasive. The Examiner has acknowledged that the prior art does not suggest that the SdpA (syn. ADD-12) enzyme can be used to degrade pyridyloxy auxin herbicides, just phenoxy auxin herbicides. The fact that the SdpA enzyme can degrade both phenoxy auxin and pyridyloxy auxin herbicides is inherent in the structure of the enzyme and not a consequence of Applicant's invention. The fact that applicant has recognized another advantage which would flow naturally from following the suggestion of the prior art cannot be the basis for patentability **when the differences would otherwise be obvious**. See *Ex parte Obiaya*, 227 USPQ 58, 60 (Bd. Pat. App. & Inter. 1985).

Schleinitz *et al* actually suggest that the SdpA enzyme is a functional equivalent to the TfdA enzyme used in the prior art to degrade 2,4-D (a phenoxy auxin herbicide) on page 5363, left column, 2nd paragraph. It would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to try other functional equivalents to the TfdA enzyme encoding gene to make phenoxy auxin herbicide resistant plant and to use such plant in a method of controlling weeds.

What would not have been obvious is a method of using such a plant by applying a pyridyloxy auxin herbicide alone or with another herbicide such as a phenoxy auxin herbicide.

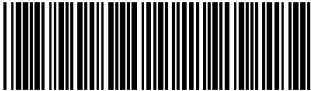
Conclusion

5. No claims are allowed.
6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Search Notes 	Application/Control No. 14/491,197	Applicant(s)/Patent Under Reexamination Wright et al.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	12/29/2016	/DK/
EAST: USPAT, USPGPU, EPO, JPO, DERWENT	12/29/2016	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	12/29/2016	/DK/
Sequence Search of SEQ I DNO: 1-5 included interference search	12/9/2016	/DK/
Update EAST	6/15/2017	/DK/
Update EAST	01/24/2018	/DK/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/DAVID H KRUSE/ Primary Examiner.Art Unit 1663	
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Application	Document	Mailroom Date	Attorney Docket No.
14491197	CTNF	01/30/2018	14764-245772

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<i>Customer No.:</i>	107540	}	
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<i>Group No.:</i>	1663	}	
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<i>Confirmation No.:</i>	3336	}	
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<i>Application No.:</i>	14/491,197	}	ELECTRONICALLY FILED:
		}	<u>May 2, 2018</u>
<i>Title:</i>	NOVEL HERBICIDE RESISTANCE GENES	}	
		}	
<i>Inventors:</i>	Wright, Terry R., et al.	}	
		}	
<i>Filed:</i>	September 19, 2014	}	
		}	
<i>Attorney Docket:</i>	14764-245772	}	
		}	
<i>Examiner:</i>	KRUSE, DAVID H	}	

**TRANSMITTAL OF DECLARATIONS FOR A UTILITY OR DESIGN
PATENT APPLICATION**

Director for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant, through their subscribing agents, hereby submit the executed
Declarations for a Utility or Design Patent Application (12 sheets).

Applicant believes no other fees are due at this time. If any additional fees are
due with this response, the Commissioner is hereby authorized to charge those fees to the

U.S. Application Serial No. 14/491,197

account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Respectfully submitted,
BARNES & THORNBURG LLP
/Adam S. Bobel/
Adam S. Bobel
Registration No. 70602
Attorney for Applicant

Indianapolis, Indiana
(317) 261-7942
adam.bobel@btlaw.com

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

Title of Invention	NOVEL HERBICIDE RESISTANCE GENES
<p>As the below named inventor, I hereby declare that:</p> <p>This declaration is directed to: ☐ The attached application, or ☒ United States application or PCT international application number <u>14/491,197</u> filed on <u>09/19/2014</u></p> <p>The above-identified application was made or authorized to be made by me.</p> <p>I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p style="text-align: center;">WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p> <p>LEGAL NAME OF INVENTOR</p> <p>Inventor: <u>Terry R. Wright</u> Date (Optional): <u>4/24/18</u></p> <p>Signature: <u>Terry R. Wright</u></p> <p>Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.</p>	

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

Privacy Act Statement

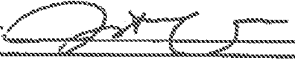
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LEGAL NAME OF INVENTOR	
Inventor: <u>Justin M. Lira</u> Date (Optional): <u>04/24/18</u> Signature: 	
<p>Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.</p>	

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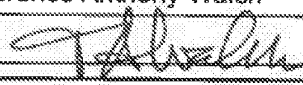
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<p>LEGAL NAME OF INVENTOR</p> <p>Inventor: <u>Terence Anthony Walsh</u> Date (Optional): <u>4/26/16</u></p> <p>Signature: <u></u></p>	
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LEGAL NAME OF INVENTOR	
<p>Inventor: <u>Donald Merio</u> Date (Optional) <u>April 28, 2018</u></p> <p>Signature: <u>Donald Merio</u></p>	
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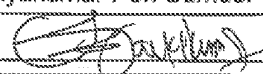
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	
<p>As the below named inventor, I hereby declare that:</p> <p>This declaration is directed to: ☐ The attached application, or ☒ United States application or PCT International application number <u>14/491,197</u> filed on <u>09/19/2014</u>.</p> <p>The above-identified application was made or authorized to be made by me.</p> <p>I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p> <p style="text-align: center;">WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p>		
LEGAL NAME OF INVENTOR		
Inventor:	Jayakumar Pon Samuel	Date (Optional):
Signature:		
<p>Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.</p>		

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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

Privacy Act Statement

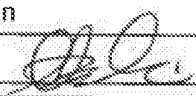
The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

Title of Invention	NOVEL HERBICIDE RESISTANCE GENES
<p>As the below named inventor, I hereby declare that:</p> <p>This declaration is directed to: ☐ The attached application, or ☒ United States application or PCT international application number <u>14/491,197</u> filed on <u>09/19/2014</u></p> <p>The above-identified application was made or authorized to be made by me.</p> <p>I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.</p>	
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<p>LEGAL NAME OF INVENTOR</p> <p>Inventor: <u>Gaofeng Lin</u> Date (Optional): <u>28 Apr 2018</u></p> <p>Signature: </p>	
<p>Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.</p>	

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt	
EFS ID:	32507880
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Adam Bobel/Sherri Cozart
Filer Authorized By:	Adam Bobel
Attorney Docket Number:	14764-245772
Receipt Date:	02-MAY-2018
Filing Date:	19-SEP-2014
Time Stamp:	10:43:47
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	Transmittal_Declarations_245772.pdf	50919 0c2b89b20475ee5f9458dbaf3c70a0a969ab91a6	no	2

Warnings:

Information:					
2	Oath or Declaration filed	Declarations_245772.pdf	731151	no	12
			b6cea552da8219ac92d38a5ac121ccde021e1854		
Warnings:					
Information:					
Total Files Size (in bytes):			782070		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Customer No.:</i>	107540	}	
		}	
<i>Group:</i>	1663	}	
		}	
<i>Confirmation No.:</i>	3336	}	
		}	
<i>Application No.:</i>	14/491,197	}	
		}	FILED ELECTRONICALLY:
<i>Invention:</i>	NOVEL HERBICIDE RESISTANCE GENES	}	<u>May 25, 2018</u>
		}	
<i>Inventors:</i>	Wright, Terry R., et al.	}	
		}	
<i>Filed:</i>	September 19, 2014	}	
		}	
<i>Attorney Docket:</i>	14764-245772	}	
		}	
<i>Examiner:</i>	KRUSE, DAVID H.	}	

RESPONSE TO OFFICE ACTION

Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed January 30, 2018, Applicant respectfully requests entry of the following amendments and consideration of the accompanying remarks.

The response was due on April 30, 2018 without extensions of time. Applicant hereby petitions for a one-month extension of time to extend the time for response to May 30, 2018. The Commissioner is hereby authorized to charge the one-month extension fee for a large

U.S. Application Serial No. 14/491,197

entity to the account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772. If any additional fees are required, the Commissioner is hereby authorized to charge those fees to the account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 8 of this paper.

Please type a plus sign (+) inside this box 

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i> Sheet 1 of 1	Complete if Known	
	Application Number:	14/491197
	Filing Date:	September 19, 2014
	First Named Inventor:	WRIGHT, Terry R.
	Group Art Unit:	1663
	Confirmation Number:	3336
	Examiner Name:	Kruse, David H.
	Attorney Docket Number:	14764-245772

U.S. PATENT DOCUMENTS						
Examiner Initials	Doc. No.	U.S. Patent Document		Name of Patentee or Applicant	Date of Publication	Filing Date If Appropriate
	B A	Application or Patent Number	Kind Code			
		4683195		Mullis et al.	07-28-1997	
	B B	4761373		Anderson et al.	08-02-1988	

FOREIGN PATENT DOCUMENTS								
Examiner Initials	Doc. No.	Foreign Patent Document			Name of Patentee or Applicant	Date of Publication	Translation	
		Office	Application or Patent Number	Kind Code			Yes	No* +
	B C	WO	2003/034813		U.S. Secretary of Agriculture	05-01-2003		

OTHER - NON PATENT LITERATURE DOCUMENTS				
Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.	Translation	
			Yes	No*+
	B D	Muller et al., "Purification and Characterization of Two Enantioselective α-Ketoglutarate-Dependent Dioxygenases, RdpA and SdpA, from Sphingomonas herbicidovorans MH," Appl. Environ. Microbiol., 72(7):4853-61 (2006).		
	B E	Schleinitz et al., "Delftia acidovorans plasmid pMC1, partial sequence," Appl. Environ. Microbiol. 70 (9), 5357-5365, Genbank accession no. AY327575 (2004).		
	B F	Westendorf et al., "The two enantiospecific dichlorprop/α-ketoglutarate-dioxygenases from Delftia acidovorans MC1 – protein and sequence data of RdpA and SdpA," Microbiol. Res.157: 317-322 (2002).		
	B G	Wright et al., "Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes," Natl. Acad. Sci., 107(47): 20240-5 (2010).		

DMS 12002559v1

EXAMINER		DATE CONSIDERED	
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* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).
 + An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:

WRIGHT, Terry R.

Application No. 14/491197

Filed: September 19, 2014

Confirmation No. 3336

Group Art Unit: 1663

Examiner: Kruse, David H.

For: NOVEL HERBICIDE RESISTANCE GENES

INFORMATION DISCLOSURE STATEMENT

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

Dear Sir:

Pursuant to 37 CFR 1.97 and 1.98, the references listed on the enclosed Form PTO-1449 and/or Substitute Form PTO-1449 ("Form 1449") are submitted for consideration by the Examiner in the examination of the above-identified patent application.

The full consideration of the references in their entirety by the Examiner is respectfully requested and encouraged. Also, it is respectfully requested that the references be entered into the record of the present application and that the Examiner place his or her initials in the appropriate area on the enclosed Form 1449, thereby indicating the Examiner's consideration of each of the references.

The submission of the references listed on the Form 1449 is for the purpose of providing a complete record and is not a concession that the references listed thereon are prior art to the invention claimed in the patent application. The right is expressly reserved to establish an invention date earlier than the above-identified filing date in order to remove any reference submitted herewith as prior art should it be deemed appropriate to do so.

Further, the submission of the references is not to be taken as a concession that any reference represents art that is relevant or analogous to the claimed invention. Accordingly, the right to argue that any reference is not properly within the scope of prior art relevant to an examination of the claims in the above-identified application is also expressly reserved.

The Information Disclosure Statement is being filed:

- ☐ **within** any one of the following time periods: (a) within three months of the filing date of a national application other than a continued prosecution application under 37 CFR 1.53(d); (b) within three months of the date of entry of the national stage as set forth in 37 CFR 1.491 of an international application; (c) before the mailing date of a first

First Named Inventor: WRIGHT, Terry R.
Application No. 14/491197

Office Action on the merits; or (d) before the mailing of a first Office Action after the filing of a request for continued examination under 37 CFR 1.114.

☒ **after** (a), (b), (c) or (d) above, but before the mailing date of a final action under 37 CFR 1.113, a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and includes *one* of:

☐ the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below).

or

☒ the fee as set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a final action under 37 CFR 1.113 or a Notice of Allowance under 37 CFR 1.311, or an action that otherwise closes prosecution in the application, and on or before payment of the issue fee, and includes the Statement under 37 CFR 1.97(e) (see "Statement under 37 CFR 1.97(e)" below), and the fee as set forth in 37 CFR 1.17(p) (see "Fees" below).

☐ **after** the mailing date of a Notice of Allowance under 37 CFR 1.311, and on or before payment of the issue fee, and **within** thirty days of receiving each item of information contained in the Information Disclosure Statement, and includes the Statement under 37 CFR 1.704(d) (see "Statement under 37 CFR 1.704(d)" below), and the fee as set forth in 37 CFR 1.17(p) (see "Fees" below).

NOTE: This is for original applications except applications for a design patent, filed on or after May 29, 2000, wherein a paper containing only an Information Disclosure Statement in compliance with 37 CFR 1.97 and 1.98 is being filed.

Copies of the References

☒ Copies of all foreign patents and non-patent literature listed on the enclosed Form 1449 are enclosed herewith.

☐ Attached to each reference not in the English language is a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3). An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of the relevance pursuant to 37 CFR 1.98(a)(3).

☐ A copy of the international search report is enclosed herewith.

☐ The references listed on the enclosed Form 1449 were previously identified in the parent application(s) of the present application, and copies of the references were furnished at that time. Accordingly, additional copies of the references are not submitted herewith, so as not to burden the file with duplicate copies of references. The Examiner is respectfully requested to carefully review the references in accordance with the requirements set out in the Manual of Patent Examining Procedure. In accordance with 37 CFR 1.98(d), the details of the parent application(s) relied upon for an earlier filing date under 35 USC 120 in which copies of the references were previously furnished are set out below:

First Named Inventor: WRIGHT, Terry R.
Application No. 14/491197

U.S. APPLICATIONS		Status (<i>check one</i>)		
U.S. APPLICATIONS	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
1.				
2.				
3.				

Fees

- ☐ No fee is owed by the applicant(s).
☒ Charge Deposit Account No. 10-0435 in the amount of \$240.00.

Authorization to Charge Additional Fees

- ☒ Please charge any additional required fees or credit any overpayments in connection with this communication to Deposit Account No. 10-0435.

Respectfully submitted,

Date: May 25, 2018

/Rebecca L. Ball/
Rebecca L. Ball, Reg. No. 46535
BARNES & THORNBURG LLP
Customer No. 23643

Purification and Characterization of Two Enantioselective α -Ketoglutarate-Dependent Dioxygenases, RdpA and SdpA, from *Sphingomonas herbicidovorans* MH

Tina A. Müller,^{1†} Thomas Fleischmann,¹ Jan Roelof van der Meer,^{1,2} and Hans-Peter E. Kohler^{1*}

Swiss Federal Institute of Aquatic Science and Technology (Eawag), Überlandstrasse 133, CH-8600 Dübendorf, Switzerland,¹ and
Département de Microbiologie Fondamentale, Bâtiment de Biologie, University of Lausanne, 1015 Lausanne, Switzerland²

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α -Ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA) and α -ketoglutarate-dependent (*S*)-dichlorprop dioxygenase (SdpA), which are involved in the degradation of phenoxyalkanoic acid herbicides in *Sphingomonas herbicidovorans* MH, were expressed and purified as His₆-tagged fusion proteins from *Escherichia coli* BL21(DE3)(pLysS). RdpA and SdpA belong to subgroup II of the α -ketoglutarate-dependent dioxygenases and share the specific motif HXD_{2,4}TX₁₃HX₁₀R. Amino acids His-111, Asp-113, and His-270 and amino acids His-102, Asp-104, and His-257 comprise the 2-His-1-carboxylate facial triads and were predicted to be involved in iron binding in RdpA and SdpA, respectively. RdpA exclusively transformed the (*R*) enantiomers of mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid] and dichlorprop [2-(2,4-dichlorophenoxy)propanoic acid], whereas SdpA was specific for the (*S*) enantiomers. The apparent K_m values were 99 μ M for (*R*)-mecoprop, 164 μ M for (*R*)-dichlorprop, and 3 μ M for α -ketoglutarate for RdpA and 132 μ M for (*S*)-mecoprop, 495 μ M for (*S*)-dichlorprop, and 20 μ M for α -ketoglutarate for SdpA. Both enzymes had high apparent K_m values for oxygen; these values were 159 μ M for SdpA and >230 μ M for RdpA, whose activity was linearly dependent on oxygen at the concentration range measured. Both enzymes had narrow cosubstrate specificity; only 2-oxoadipate was able to replace α -ketoglutarate, and the rates were substantially diminished. Ferrous iron was necessary for activity of the enzymes, and other divalent cations could not replace it. Although the results of growth experiments suggest that strain MH harbors a specific 2,4-dichlorophenoxyacetic acid-converting enzyme, *tfdA*-, *tfdA*-, or *cadAB*-like genes were not discovered in a screening analysis in which heterologous hybridization and PCR were used.

Phenoxyalkanoic acid herbicides are widely used to control broadleaf weeds in agriculture, lawns, and industry. These compounds are systemic postemergence herbicides that act as synthetic auxins (1, 2, 28). Well-known representatives are mecoprop [2-(*R,S*)-2-methyl-4-chlorophenoxypropanoic acid], dichlorprop [2-(*R,S*)-2,4-dichlorophenoxypropanoic acid], and 2,4-D (2,4-dichlorophenoxyacetic acid). Mecoprop and dichlorprop are chiral molecules that have one stereogenic center and therefore exist as two enantiomers. In the 1950s, it was shown that only the (*R*) enantiomers had herbicidal effects (29). However, both enantiomers were and still are applied as racemic mixtures, resulting in the introduction of large amounts of unwanted chemicals into the environment (10, 50, 51). *Sphingomonas herbicidovorans* MH, an *Alphaproteobacterium*, is able to grow on both enantiomers of mecoprop and dichlorprop, as well as on 2,4-D, as sole carbon and energy sources (21, 24, 54). In batch cultures, this organism degrades the two enantiomers of mecoprop and dichlorprop sequentially, and the (*S*) enantiomers are degraded first (25, 32).

In the first reaction, both mecoprop and dichlorprop are enantioselectively converted by *S. herbicidovorans* MH to

achiral phenols and pyruvate (Fig. 1). Nickel et al. (35) showed in experiments with cell extracts that two distinct enzymes are involved. These enzymes are highly specific for the corresponding enantiomers and belong to the family of α -ketoglutarate-dependent dioxygenases. The α -ketoglutarate-dependent dioxygenases are a group of enzymes which are classified on the basis of their biochemical characteristics. They are nonheme iron-dependent dioxygenases that require both oxygen and α -ketoglutarate as substrates. For many of these dioxygenases ascorbate has been used as a reducing agent. α -Ketoglutarate-dependent dioxygenases catalyze a wide range of oxidative processes, such as hydroxylations, epoxidations, desaturations, ring formations, and expansion reactions (7, 17, 27, 38, 44). Despite the diversity of their primary sequences, all of these dioxygenases have a 2-His-1-carboxylate facial triad at the catalytic center (19) and also common amino acid motifs, on the basis of which they are classified into three subgroups (20). Relevant for the work described here is subgroup II, and the representatives of this subgroup have the motif HX(D/E)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R. More prominent members of this subgroup are taurine dioxygenase (TauD) from *Escherichia coli*, which is involved in taurine utilization (11), and 2,4-D dioxygenase (TfdA) from *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4). TfdA is involved in 2,4-D degradation and catalyzes the oxidative cleavage of the ether bond in 2,4-D, yielding 2,4-dichlorophenol (15, 16, 18).

Recently, several genes coding for the enzymes involved in phenoxypropanoic acid herbicide degradation in *S. herbi-*

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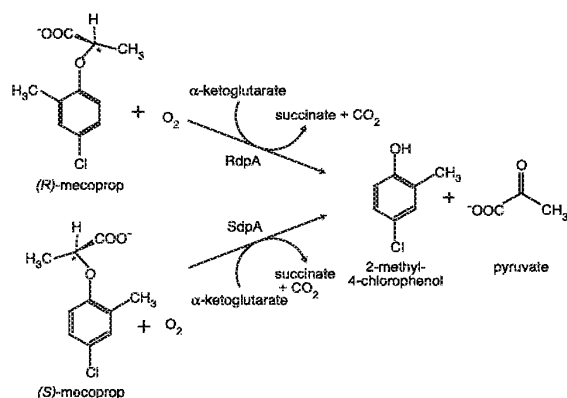


FIG. 1. Initial step in the degradation of chiral phenoxypropanoic acids in *S. herbicidovorans* MH catalyzed by the α -ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA) and the α -ketoglutarate-dependent (*S*)-dichlorprop dioxygenase (SdpA).

cidovorans MH (31) and *Delftia acidovorans* MC1 (43) were identified and isolated. The genes coding for two α -ketoglutarate-dependent dioxygenases designated RdpA and SdpA were suggested to be responsible for the initial steps in the degradation of (*R*)- and (*S*)-mecoprop, respectively. The deduced amino acid sequence of RdpA from *S. herbicidovorans* MH is 30% identical to that of TfdA and 100% identical to that of RdpA from *D. acidovorans* MC1. The deduced amino acid sequence of SdpA from *S. herbicidovorans* MH exhibits only 60% identity to that of SdpA from *D. acidovorans* MC1 and about 30% identity to that of RdpA (30, 31, 43, 48).

In this study, we expressed and purified RdpA and SdpA from *S. herbicidovorans* MH as His₆-tagged fusion proteins. By measuring enzyme activities with a novel coupled enzyme assay, we verified that RdpA and SdpA are α -ketoglutarate-dependent dioxygenases belonging to subgroup II. We also characterized the kinetic behavior of the enzymes with various substrates and cosubstrates. In addition, we determined the substrate and cosubstrate specificities and obtained clear evidence that the two enzymes have opposite enantioselectivities.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5 α was used as a host for cloning experiments, and *E. coli* BL21(DE3)(pLysS) was used as a host for protein expression studies with pET-15b-based constructs (Novagen, Darmstadt, Germany). The *E. coli* strains were grown at 30°C or 37°C in Luria-Bertani medium (42). Ampicillin and chloramphenicol were added at final concentrations of 50 μ g/ml and 25 μ g/ml, respectively. Solid media were prepared by addition of 1.5% (wt/vol) agar.

Standard molecular techniques. Cloning and digestion were done by using established procedures (4, 42). Restriction enzymes and other DNA-modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling miniprep method or the alkaline lysis method described by Sambrook et al. (42) or by using an E.Z.N.A. plasmid miniprep kit II (Qiagen Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Purification of DNA fragments from agarose gel was carried out by using a MinElute gel extraction kit (Qiagen AG, Basel, Switzerland) according to the protocol of the supplier.

Construction of N-terminal His₆-tagged recombinant enzyme expression plasmids. Expression plasmids pMec15 and pMec19 were constructed by reamplification by PCR of the *rdpA* and *sdpA* genes from pMec10 and pMec16, respec-

tively, (31) with the following primers: 5'-CGC TCA TAT GCA TGC TGC ACT-3' and 5'-AGC GGG GAT CCG CGT CGC C-3' for *rdpA* and 5'-CAG GAG GAT TCA TAT GTC A-3' and 5'-GCC AGC TGG ATC CGC CGA TGA-3' for *sdpA*. In this way, an NdeI restriction site at the translational start and a BamHI restriction site after the stop codon were introduced (underlined nucleotides). The PCR-amplified fragments were first cloned into pGEM-T Easy (Promega), generating pMec14 and pMec18, which was verified by DNA sequencing. The plasmids were then digested with NdeI and BamHI, and the *rdpA* or *sdpA* inserts were recovered, purified, and ligated into the same sites of vector pET-15b. After transformation, this yielded plasmids pMec15 and pMec19.

Expression and purification of recombinant RdpA and SdpA. *E. coli* BL21-(DE3)(pLysS) harboring pMec15 or pMec19 was grown in Erlenmeyer flasks containing Luria-Bertani medium supplemented with ampicillin and chloramphenicol at 30°C with constant shaking. To express the recombinant proteins, cultures were grown until an optical density at 546 nm of 0.4 to 0.6 was reached, after which isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mM. The culture was incubated for another 3 h at 30°C, and the cells were harvested by centrifugation at 7,500 $\times$ g for 30 min at 4°C. The pellet was stored at -20°C until it was used. For preparation of cell extracts, 1 g (wet weight) of cells was resuspended in 3 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole; pH 8.0), and the cells were broken by one passage through a French press at 1 MPa. The lysate was centrifuged for 45 min at 16,000 $\times$ g at 4°C, and aliquots were stored at -20°C.

His₆-tagged RdpA was purified by a one-step procedure. Up to 1 mg of protein was loaded on a 2-ml Ni-nitrilotriacetic acid (NTA) Superflow agarose column (QIAGEN) at a flow rate of 0.5 ml/min. Unbound protein was removed stepwise by washing with five bed volumes (~10 ml) of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole; pH 8.0). After the initial washing step, wash buffers containing 50 mM imidazole and then 100 mM imidazole were applied. RdpA was then eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole; pH 8.0) at a flow rate of 0.8 ml/min and collected in 1-ml fractions. Fractions were analyzed for α -ketoglutarate-dependent dioxygenase activity; active fractions were pooled and desalted with 20 mM Tris-HCl (pH 7.5) with PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland). The purified RdpA solution was stored at 4°C.

Recombinant SdpA was purified by a two-step procedure. Unless indicated otherwise, all steps were carried out at 4°C. Up to 1 mg protein was loaded on a 5-ml Superflow agarose column (QIAGEN) at a flow rate of 0.3 ml/min. Unbound protein was removed stepwise with 5 column volumes of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl) containing 20 mM imidazole (pH 8) and 5 column volumes of wash buffer containing 50 mM imidazole (pH adjusted to 8). SdpA was then eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 200 mM imidazole; pH adjusted to 8) at a flow rate of 0.6 ml/min. One milliliter fractions were collected and analyzed, and active fractions were pooled and desalted with 20 mM Tris-HCl (pH 7.5) using PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland) at room temperature. The SdpA solution was then loaded on a Bioscale Q2 column (Bio-Rad) equilibrated in the same buffer. The enzyme was eluted with a linear gradient from 20 mM Tris-HCl (pH 7.5) to 20 mM Tris-HCl (pH 7.5)-600 mM NaCl in 6 column volumes. SdpA was found to elute at approximately 220 mM NaCl. The fractions that exhibited activity were pooled and stored at 4°C.

Thrombin protease (Amersham Biosciences) was used to cleave the N-terminal His₆ tags of RdpA and SdpA. The N-terminal His₆ tag of approximately 10 μ g enzyme was completely cleaved by 0.5 U thrombin protease within 2 h at room temperature.

Enzyme activity assays. (i) **Coupled continuous assay.** For purification and kinetic measurements of phenoxypropanoic acids, a coupled continuous assay with L-lactate dehydrogenase from rabbit muscle (Sigma-Aldrich, Buchs, Switzerland) as a reporter enzyme was developed. RdpA and SdpA stoichiometrically convert (substituted) phenoxypropanoic acids to the corresponding phenols and pyruvate. Pyruvate is converted by L-lactate dehydrogenase to lactate with concomitant consumption of NADH. NADH consumption was monitored spectrophotometrically by determining the decrease in absorbance at 340 nm. An extinction coefficient of 6.3 mM⁻¹ cm⁻¹ was used to calculate activities. The standard 0.5-ml assay mixture contained 50 mM imidazole (pH 6.75), 1 mM substrate, 1 mM α -ketoglutarate, 100 μ M (NH₄)₂Fe(SO₄)₂, 1 mM ascorbic acid, 0.32 mM NADH, 1.2 U of L-lactate dehydrogenase, and 0.05 μ g of purified RdpA or SdpA. Activity assays were done at 30°C unless indicated otherwise and were started by addition of the substrate. NADH consumption was monitored for 30 s, and the linear portion of the curve was used to calculate the activity. The substrate-specific oxidation rates were corrected for nonspecific NADH oxidation in the absence of substrate (endogenous rate). Specific activities were expressed as turnover rate (min⁻¹).

TABLE 1. Purification of α -ketoglutarate-dependent (*R*)-dichlorprop dioxygenase and α -ketoglutarate-dependent (*S*)-dichlorprop dioxygenase

Fraction	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (min^{-1})	Recovery (%)	Purification (<i>n</i> -fold)
α-Ketoglutarate-dependent (<i>R</i>)-dichlorprop dioxygenase						
Cell extract	10	133.1	30.9	7	100	1
Ni-NTA Superflow	7	1.8	7	133	22.7	19
α-Ketoglutarate-dependent (<i>S</i>)-dichlorprop dioxygenase						
Cell extract	3.5	42.57	18.48	14	100	1
Ni-NTA Superflow	5	1.05	7.71	247	41.70	17.1
BioScale Q2	3	0.71	6.34	299	34.32	20.7

(ii) **Discontinuous assay.** Experiments with alternative substrates were performed using a discontinuous assay by measuring the formation of the product by high-performance liquid chromatography. The standard assay mixture contained 50 mM imidazole (pH 6.75), 1 mM substrate, 1 mM α -ketoglutarate, 100 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, and 1 mM ascorbic acid. The assay mixtures were incubated at 30°C for 1 min and were inactivated in a boiling water bath for 2 min. Negative controls without enzyme were treated in the same manner. The products were analyzed on a Nucleodex α -PM column (200 by 4 mm; Macherey-Nagel, Düren, Germany) as described previously (35). Specific activity was defined as the amount of phenol derivative (μmol) that was formed per min per mg of protein. Oxidation rates were corrected for nonspecific formation of the product in the absence of the enzyme.

Oxygen electrode measurements. Kinetic measurements with oxygen were obtained with a Rank Brothers oxygen electrode at 30°C. The standard 1-ml assay mixture contained 50 mM imidazole (pH 6.75), 2 mM substrate, 2 mM α -ketoglutarate, 100 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, and 1 mM ascorbic acid. The assays were started by addition of the substrate, and the O_2 concentration was monitored at 2-s intervals for 15 min. An assay without substrate showed that the amount of background O_2 consumed was small and could be neglected for calculation of activities. Specific activity was defined as the amount of oxygen (μmol) reduced per min per mg of protein in the presence of a substrate and expressed as turnover rate (min^{-1}).

Kinetic measurements. The kinetic parameters K_m and k_{cat} were calculated using IgorPro software (WaveMetrics Inc., Lake Oswego, OR). The method used for calculation was based on a weighted nonlinear regression analysis of the Michaelis-Menten model. To calculate K_m and k_{cat} of RdpA with respect to oxygen, we used the first derivative (i.e., the velocity, expressed in $\mu\text{mol min}^{-1}$) of the oxygen concentration monitored with time.

Determination of the protein concentration and SDS-PAGE. Protein concentrations were determined by the Bradford method (6) with the Bio-Rad reagent dye concentrate reagent by following the instructions of the manufacturer (Bio-Rad, Reinach, Switzerland). Bovine serum albumin fraction V (Sigma-Aldrich) was used as a reference protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out with 12% acrylamide slab gels by using established procedures (26).

Molecular weight determination. Molecular weights of purified enzymes were determined by using a Superdex 200 preparation grade HiLoad 16/60 size exclusion column (Amersham Biosciences). Approximately 0.25 mg of protein was loaded on the HiLoad column and eluted at a flow rate of 0.2 ml/min with 50 mM $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7) containing 150 mM NaCl. A calibration curve was produced with the gel filtration standard from Bio-Rad containing bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa). Blue dextran was used to determine the void volume.

Screening for additional α -ketoglutarate-dependent phenoxylalkanoic acid dioxygenases. To screen for *tfdA*- and *tfdAa*-like genes, we amplified DNA by PCR performed with the *tfdA*-derived primers *tfdAfor* (5'-TGG CAG AGC GAC AGI TCC TTT-3'), *tfdArev* (5'-CIG CGG TTG TCC CAG ATC IC-3'), *tfdAfor2* (5'-ACG GAG TTC TG[C/T] GA[C/T] AT-3'), and *tfdArev2* (5'-AAC GCA GCG [G/A/T]G[G/T] ATC CCA-3') (46) in all possible combinations and with chromosomal DNA of *S. herbicidovorans* MH. The oligonucleotides used for PCR were obtained from Microsynth GmbH (Balgach, Switzerland). As a positive control, total DNA of *C. necator* JMP134(pJP4) was used.

For Southern hybridization, total DNA of *S. herbicidovorans* MH fractionated on an agarose gel was blotted on a Hybond-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) with a vacuum blotter (VacuGene XL; Amer-

sham Biosciences) by the procedure described by Ravatn et al. (40). Total DNA was hybridized with a 0.7-kb EcoRI fragment from pCBA101 (0.7-kb EcoRI fragment with *tfdA* in pGEM7; van der Meer, unpublished) and with a 1.1-kb SalI fragment of pBHSI harboring part of *cadAB* (23). The fragments were radioactively labeled with [α -³²P]dATP using a Random Primed DNA labeling kit or with digoxigenin-dUTP using a DIG High Prime II DNA labeling and detection starter kit according to the protocol of the manufacturer (Roche Applied Science, Rotkreuz, Switzerland). Hybridization experiments were performed in SDS/bovine serum albumin hybridization buffer (0.5 M sodium phosphate [pH 7], 7% sodium dodecyl sulfate, 1% bovine serum albumin) at 55°C for 16 h. The membranes were washed twice with 5 $\times$ SSC containing 1 mM EDTA for 2 min at room temperature and twice with 2 $\times$ SSC containing 0.1% SDS for 30 min at 55°C (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). When digoxigenin-labeled probes were used, immunological detection was carried out by using the supplier's protocol (Roche Applied Science).

Sequencing. DNA sequencing was performed with double-stranded DNA templates by using a Thermo Sequenase primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (5). Sequencing reaction mixtures were analyzed with an automated DNA sequencer (model 4200 IR²; LI-COR Inc., Lincoln, NE). The primers used for sequencing were labeled at the 5' end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR Inc., Madison, WI) and were compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/BLAST; (45)).

Chemicals. Phenoxylalkanoic acids were purchased from Riedel-de Haën (Buchs, Switzerland) (PESTANAL; purity, 99%) or Sigma-Aldrich (purity, 97 to 99%). (*S*)-Mecoprop and (*S*)-dichlorprop were synthesized by C. Zipper (52). α -Ketoglutarate and other (di)carboxy acids were purchased from Sigma-Aldrich. All other chemicals were obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

RESULTS

Expression and purification of recombinant RdpA and SdpA. The *rdpA* and *sdpA* genes were expressed as N-terminal His₆-tagged fusion proteins in *E. coli* BL21(DE3)(pLysS) harboring pMec15 and pMec19, respectively. Expression resulted in high levels of recombinant RdpA and SdpA in the soluble fraction that accounted for approximately 16% and 10%, respectively, of the total protein as determined by measuring the band intensities of SDS-PAGE gels. A decrease in the incubation temperature during expression in *E. coli* did not increase the amount of the His₆-tagged enzymes in the cell extract. His₆-tagged RdpA was purified about 16-fold to apparent homogeneity by Ni-NTA affinity chromatography, whereas His₆-tagged SdpA was purified to apparent homogeneity by a two-step procedure (Table 1 and Fig. 2). The calculated molecular masses of the subunits of RdpA and SdpA were 34.9 kDa and 33.7 kDa, respectively, which was consistent with the sizes estimated by SDS-PAGE (approximately 36 kDa and 32 kDa, respectively).

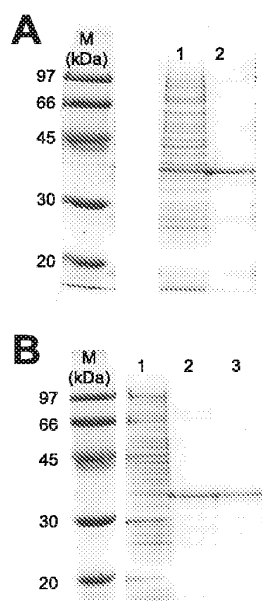


FIG. 2. Purification of RdpA (A) and SdpA (B) from *S. herbicidovorans* MH expressed as His₆-tagged fusion proteins. Protein samples were analyzed by denaturing gel electrophoresis on a 12% polyacrylamide gel and were stained with Coomassie brilliant blue. Lane M contained molecular mass markers. (A) Lane 1, extract of induced cells; lane 2, apparently pure RdpA (after Ni-NTA Superflow chromatography). (B) Lane 1, extract of induced cells; lane 2, partially purified SdpA (after Ni-NTA Superflow chromatography); lane 3, apparently pure SdpA (after ion-exchange chromatography).

The molecular weights of the native proteins were determined by size exclusion chromatography after the His₆ tag was cleaved by thrombin treatment. A molecular mass of 104 ± 7 kDa was obtained for native RdpA. This is consistent with a homotrimeric structure based on the calculated subunit molecular mass, 33.2 kDa. Native SdpA had a molecular mass of 32 kDa, a strong indication that the structure is monomeric.

The fusion proteins were treated with thrombin protease to cleave the His₆ tag, and the enzyme activities of the native and the His₆-tagged proteins were determined. In both cases, the His₆-tagged and native enzymes were equally active, showing that the His₆ tag did not affect activity. Therefore, all further experiments were carried out with the His₆-tagged recombinant proteins. Below, RdpA and SdpA refer to the His₆-tagged enzymes, unless indicated otherwise.

RdpA and SdpA are α -ketoglutarate-dependent dioxygenases that catalyze enantioselective reactions. RdpA and SdpA have been classified as α -ketoglutarate-dependent dioxygenases based on their amino acid sequences (31). This classification was verified by enzyme assays. RdpA and SdpA activities were completely absent in the absence of α -ketoglutarate, were strongly reduced in assays without ferrous iron, and were slightly reduced in the absence of ascorbate (Table 2).

The results of three different assays, a coupled continuous assay, a discontinuous assay, and an O₂ measurement assay, showed that there was stoichiometric product formation and

substrate consumption. The specific activity of RdpA ranged from 2 to 4 U/mg protein. Determinations of the specific activity of RdpA were reproducible within single purified RdpA batches but could vary between independently induced *E. coli* cultures by a factor of two.

RdpA converted only the (*R*) enantiomers of phenoxypropanoic acids, and no activity was observed with the (*S*) enantiomers. The enantioselectivity was exactly the opposite for SdpA, which converted only the (*S*) enantiomers. In contrast to RdpA, SdpA was slightly inhibited in the presence of the non-substrate enantiomer (data not shown).

Physicochemical characterization. RdpA was stable at 4°C for up to 8 weeks and at room temperature for up to 7 days, but it was inactivated by several freezing and thawing cycles. Therefore, RdpA was stored at 4°C. Optimum activity was observed at pH 6.5 (Fig. 3A). Temperature dependence was also studied, but no optimum could be determined (Fig. 3B). The experiments were carried out with two independently purified RdpA batches. To avoid possible aberrant results due to inactivation of L-lactate dehydrogenase in the coupled continuous assay, specific activities were determined once by the coupled continuous assay and then by the discontinuous assay. RdpA activity was stable over a relatively wide temperature range, 20 to 55°C. Slightly higher activity was observed at 10°C and 20°C, but only in the discontinuous assay. However, the general trends for the two types of enzyme assays were similar and revealed no Arrhenius-like behavior.

SdpA was stable at room temperature for several hours and in 3 days lost less than 10% of its activity when it was stored at 4°C. Storage on ice and freeze-thaw cycles reduced the activity to about 80% of the original activity. More consistent specific activities were obtained when SdpA was prewarmed at room temperature prior to enzymatic assays. The pH optimum was pH 6.5 (Fig. 3C). In contrast to the RdpA activity, the SdpA activity had an optimum temperature between 35° and 40°C (Fig. 3D).

Substrate, cosubstrate, and cofactor specificities. Substrate specificities were determined by the coupled continuous assay for phenoxypropanoic acids and by the discontinuous assay for all other substrates. We examined the influence of the alkanolic acid group attached by an ether bond to the phenoxy ring by determining the activities with phenoxyacetic, phenoxypropanoic, and phenoxybutyric acids as substrates. The effects of different substituents, such as hydroxy, methyl, and chloride

TABLE 2. Dependence of α -ketoglutarate-dependent (*R*)- and (*S*)-dichlorprop dioxygenase activities on substrate, cosubstrate, and cofactors

Assay mixture	RdpA		SdpA	
	Activity (%)	Sp act (min ⁻¹)	Activity (%)	Sp act (min ⁻¹)
Complete	100	118.8	100	236.2
Complete without:				
(<i>R</i>)-Mecoprop	0	0	ND ^a	ND
(<i>S</i>)-Mecoprop	ND	ND	0	0
α -Ketoglutarate	0	0	0	0
Fe(II)	17.6	21.0	8.2	19.1
Ascorbate	85.3	101.4	37.8	89.2

^a ND, not determined.

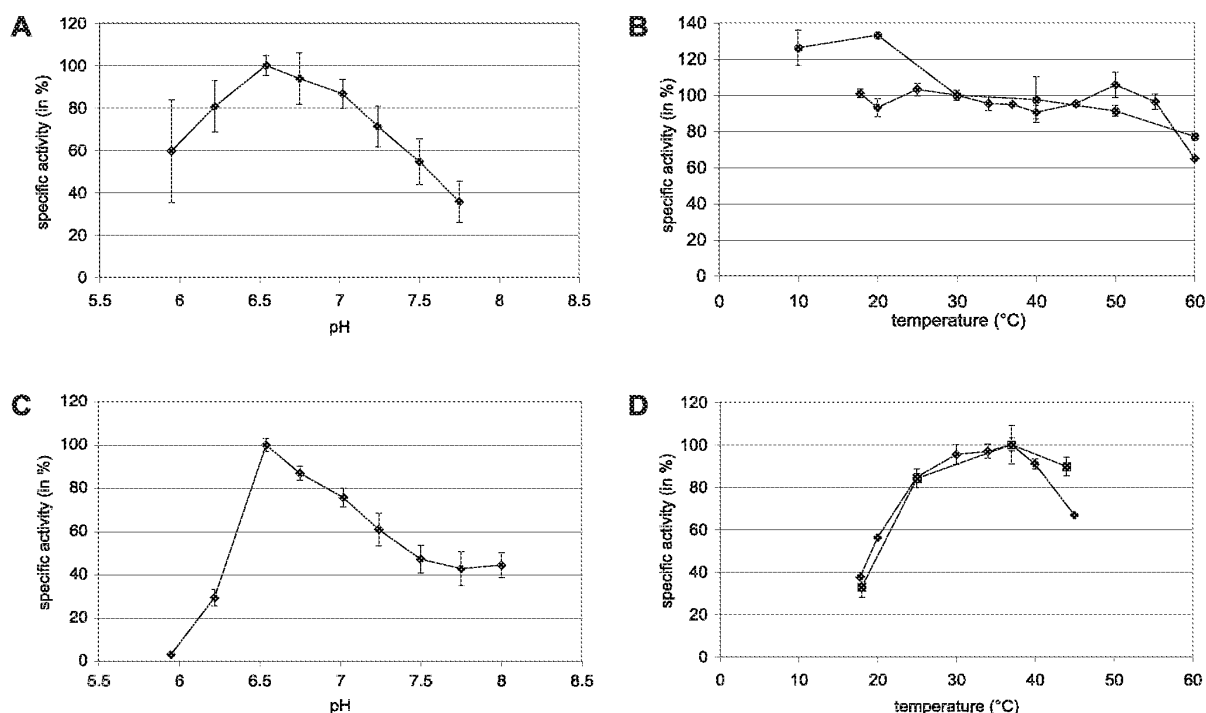


FIG. 3. Dependence of RdpA and SdpA on pH (A and C, respectively) and temperature (B and D, respectively). (A) The symbols indicate the means of six measurements. Enzyme activity was determined by the coupled continuous assay. (B) Symbols: $\blacklozenge$, data obtained with the discontinuous assay; $\bullet$, data obtained with the coupled continuous assay. The experiments were performed with two independently purified RdpA samples. (C) The symbols indicate the means of six measurements for two independent experiments. Enzyme activity was determined by the coupled continuous assay. (D) Symbols: $\blacksquare$, data obtained with the discontinuous assay; $\blacklozenge$, data obtained with the coupled continuous assay. The experiments were performed with two independently purified SdpA samples.

groups on the aromatic ring, were investigated as well. RdpA converted phenoxypropanoic acids and none of the phenoxyacetic or phenoxybutyric acids except 3-methyl-5-chlorophenoxyacetic acid (MCPA). 2-(*R,S*)-4-Chlorophenoxypropanoic acid was turned over at the highest rate, followed by dichlorprop and mecoprop. Interestingly, the hydroxylated phenoxypropanoic acids and the unsubstituted phenoxypropanoic acids were converted (Table 3). SdpA exhibited the highest turnover rates with substituted phenoxypropanoic acids, and mecoprop was transformed fastest (Table 3). In contrast to RdpA, SdpA was able to convert MCPA and 2,4-D at low but measurable rates.

We also tested whether several carboxylic and dicarboxylic acids could replace α -ketoglutarate as a cofactor. Since the reporter enzyme in the coupled continuous assay, *L*-lactate dehydrogenase, showed activity with some cosubstrates, in this case the RdpA and SdpA activities were determined by the discontinuous assay. RdpA and SdpA were quite specific for α -ketoglutarate, with specific activities of 132.8 min^{-1} and 226.1 min^{-1} , respectively, and exhibited only low levels of activity in the presence of 2-oxoadipate (1.9% and 5%, respectively). There was no activity with any of the other carboxylic acids tested (oxaloacetate, 2-oxobutyrate, and 2-oxovalerate).

In the absence of ferrous iron, the activities of the two enzymes were substantially reduced (Table 2). The activity was

independent of the iron salt, and other divalent cations could not substitute for Fe(II) (Table 4). Some of the divalent cations, including Co(II), Mg(II), Mn(II), and Ni(II), slightly inhibited enzyme activity.

Kinetic analysis. The apparent kinetic constants are shown in Table 5. For RdpA, Michaelis-Menten kinetics was observed for all substrates except oxygen; the reaction rate was linearly dependent on the oxygen concentration up to a concentration of about 1.0 mM. Therefore, apparent K_m and k_{cat} values could not be determined. However, it was possible to calculate a specificity constant (k_{cat}/K_m) for oxygen, which was $0.64 \mu\text{M}^{-1} \text{ min}^{-1}$. For the phenoxypropanoic acid substrates, RdpA exhibited the lowest K_m value and highest specificity for (*R*)-mecoprop, whereas the k_{cat} was highest for (*R,S*)-4-chlorophenoxypropanoic acid. The apparent K_m value for α -ketoglutarate was $2.8 \mu\text{M}$; this value is below the lowest α -ketoglutarate concentration for which activity could be measured, but the data fit a Michaelis-Menten curve well, and according to the goodness of fit of the nonlinear regression analysis the value was reliable. SdpA exhibited Michaelis-Menten kinetics with all substrates and cosubstrates tested. Mecoprop was a better substrate than dichlorprop; i.e., the apparent K_m value was lower and the catalytic efficiency was higher. SdpA had an apparent K_m of $19.6 \mu\text{M}$ for α -ketoglutarate and an apparent K_m of $159 \mu\text{M}$ for oxygen.

TABLE 3. Substrate specificities of α -ketoglutarate-dependent (R)- and (S)-dichlorprop dioxygenases^a

Substrate	RdpA		SdpA	
	Activity (%) (mean $\pm$ SD)	Sp act (min ⁻¹)	Activity (%) (mean $\pm$ SD)	Sp act (min ⁻¹)
(R,S)-Mecoprop	100	108.3/140.9 ^b	100	274.8/209.7 ^b
(R,S)-Dichlorprop	115.5 $\pm$ 3.6	125.8	65.3 $\pm$ 9.7	170.4
2-(R,S)-Phenoxypropanoic acid	39.7 $\pm$ 7.6	41.9	11.6 $\pm$ 2.6	31.5
2-(R,S)-3-Chlorophenoxypropanoic acid	14.8 $\pm$ 2.0	17.5	33.2 $\pm$ 2.5	96.6
2-(R,S)-4-Chlorophenoxypropanoic acid	152.1 $\pm$ 9.0	171.3	66.1 $\pm$ 3.2	198.6
2-(R,S)-2,4,5-Trichlorophenoxypropanoic acid	49.4 $\pm$ 4.3	55.9	71.5 $\pm$ 6.6	217.7
2-(R,S)-4-Hydroxyphenoxypropanoic acid	51.3 $\pm$ 7.3	55.9	19.6 $\pm$ 3.2	60.4
2,4-D ^{c,d}	<2	<3.5	5.6 $\pm$ 5.2	11.1
MCPA ^c	7.7 $\pm$ 4.3	10.5	20.3 $\pm$ 6.2	41.9
2,4-Dichlorophenoxybutyric acid ^{c,e}	<2	<3.5	<1	<3.4
4-Chloro-2-methylphenoxybutyric acid ^c	<2	<3.5	2.3 $\pm$ 1.2	4.7

^a The substrate range was determined with RdpA and SdpA purified from two independent batches, and the enzyme activity of each batch was measured three times with all substrates.

^b Activity determined by the coupled continuous assay/activity determined by the discontinuous assay.

^c Enzyme activity was determined by the discontinuous assay.

^d Only one SdpA batch showed slight activity with 2,4-D. The means might be overestimates.

^e In one experiment, very low activity was observed. However, the 2,4-dichlorophenol concentration was below the detection limit (1 μ M), and no increase in the amount of 2,4-dichlorophenol converted per unit of time was observed when the amount of RdpA in the assay was increased. Therefore, the activity was defined as <2%.

Screening for other 2,4-D-degrading genes. We screened the total DNA of *S. herbicidovorans* MH for genes encoding a TfdA-like dioxygenase and the aromatic ring-hydroxylating dioxygenase CadAB by performing hybridization and PCR experiments. Hybridization experiments with both *tfdA*- and *cadAB*-derived probes did not give positive results, and no *tfdA*-like gene could be amplified with the PCR primers derived from the conserved regions of the *tfdA* and *tfdA* α genes.

DISCUSSION

RdpA and SdpA are α -ketoglutarate-dependent dioxygenases. α -Ketoglutarate-dependent dioxygenases are grouped according to their cosubstrate and cofactor requirements. Enzyme activity measurements demonstrated that RdpA and SdpA belong to the α -ketoglutarate-dependent dioxygenase enzyme family, since their activities were strictly dependent on oxygen and α -ketoglutarate. Without ascorbate the activities were reduced to about 85% and 39%, respectively, of the highest activities. Ascorbate is not formally needed in the reaction, and it is generally thought to maintain Fe(II) in the reduced state. Two other α -ketoglutarate-dependent dioxygenases, prolyl 4-hydroxylase and lysyl hydroxylase, are almost completely inactivated within 1 min in the absence of ascorbate (9, 33, 34, 39). The inactivation is thought to involve a side

reaction in which α -ketoglutarate is decarboxylated. This reaction is coupled to the oxidation of Fe(II) to Fe(III). Ascorbate reduces the ferric iron, thereby reactivating the enzyme. TfdA, on the other hand, is not as sensitive to the absence of ascorbate and is inactivated only after 30 to 40 min (16). The reductions in RdpA and SdpA activities in the absence of ascorbate indicated that α -ketoglutarate decarboxylation side reactions took place, although the inactivation was not as fast as the inactivation of the peptide hydroxylases.

Alignment of RdpA and SdpA with other α -ketoglutarate-dependent dioxygenases revealed conservation of the 2-His-1-carboxylate facial triad in RdpA and SdpA, as well as several other conserved amino acid residues (Fig. 4). RdpA and SdpA have the metal binding motif of subgroup II of the α -ketoglutarate-dependent dioxygenase family, which is HXDX₂₄TX₁₃₁

TABLE 5. Kinetic parameters of α -ketoglutarate-dependent (R)-dichlorprop dioxygenase and α -ketoglutarate-dependent (S)-dichlorprop dioxygenase

Substrate	K_m (μ M)	k_{cat} (min ⁻¹)	k_{cat}/K_m (μ M ⁻¹ min ⁻¹)
α -Ketoglutarate-dependent			
(R)-dichlorprop dioxygenase			
(R)-Mecoprop (10–2,000 μ M)	98.8 $\pm$ 6.4	114.2	1.16
(R)-Dichlorprop (50–2,000 μ M)	163.7 $\pm$ 13.5	170.1	1.04
(R,S)-4-Chlorophenoxypropanoic acid (120–2,000 μ M)	262.4 $\pm$ 10.1	241.9	0.92
O ₂ (0.02–1.0 mM)	ND ^a	ND	0.64
α -Ketoglutarate (7–1,900 μ M)	2.8 $\pm$ 0.8	135.6	48.82
α -Ketoglutarate-dependent			
(S)-dichlorprop dioxygenase			
(S)-Mecoprop (50–2,000 μ M)	132 $\pm$ 17.7	303.9	2.3
(S)-Dichlorprop (120–2,000 μ M)	494.8 $\pm$ 43.9	284.6	0.58
O ₂ (0.02–0.2 mM)	159.32	116 ^b	0.75
α -Ketoglutarate (8–1,000 μ M)	19.6 $\pm$ 2.5	229.7	11.72

^a ND, not determined.

^b The kinetic values for O₂ were determined with material from two different SdpA purification batches, both of which were less active than the purification batches used for other kinetic experiments. However, the stoichiometry of the reaction was verified with material from a third purification batch (data not shown).

TABLE 4. Cofactor specificities of α -ketoglutarate-dependent (R)- and (S)-dichlorprop dioxygenases

Cofactor	RdpA		SdpA	
	Activity (%)	Sp act (min ⁻¹)	Activity (%)	Sp act (min ⁻¹)
Fe(NH ₄) ₂ (SO ₄) ₂	100	97.9	100	243.2
Fe(SO ₄) ₂	97.5	94.4	92.8	229.1
CaCl ₂	15.9	14.0	0	0
CoCl ₂	0	0	0	0
MgCl ₂	9.4	10.5	0	0
MnCl ₂	0	0	0	0
NiCl ₂	0	0	0	0

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RdpA_MH      --MHAALSPLSQRFERIAVQPLTGVSGAEI-----
SdpA_MH      -----MSPAFDIAPLDATGCAVV-----
SdpA_MC1     -----MQTTLQITPTGATGATV-----
TfdA_pJP4    -----MSVVANPLHPLFAAGV-----
TauD_E.coli  -----MSERLSITPLGPYIGAGI-----
AtsK_P.putida MSAALATAP--HALELDVHPVAGRIAGAEI-----
CASI1        -----MTSVDC--TAYGFELRALAARSPRTPRADLYAFDLAAHTAAASLPGA

RdpA_MH      -----TGVDKREPLDDST-----WNEILDAFHTY
SdpA_MH      -----TGVKSLAD--LDDAG-----WLDLQAALVEY
SdpA_MC1     -----TCVHSLAT--LDDAG-----FAALHAALQHQ
TfdA_pJP4    -----EDIDREALGSTE-----VREIERIMDEK
TauD_E.coli  -----SGADQTRPLSDNQ-----FEQLYHVALRH
AtsK_P.putida -----RGVKSPLDLAAT-----VEAIQAALVRH
CASI1        LATALDTFNAEGSEDGHLLRGLFYSADADLPTTFSSTPAPEDRSLLTME

RdpA_MH      QVYFEPGAA--ITNEQHIA--SRRFQGPVD-----PVPLLSK-----I
SdpA_MH      AELVFPDQH--LTREQQIA--FARRFGPLE-----FEMAALSN
SdpA_MC1     AELVFPDQH--LSDQQAIA--FARRFGATE-----RIGGGDTVAISN
TfdA_pJP4    SVYVFRGQP--LSQDQQAIA--FARRFGLEGFIKVNQRPSRFYAEADISN
TauD_E.coli  QVYFEPGAA--ITPQQQIA--FARRFGELH-----IHPVYFH-----A
AtsK_P.putida KYVFPFGQT--LHDDQQAIA--FARRFGEPV-----AHPTVVP-----V
CASI1        AMGCVGGR-----KLCGHTGYRELRSQTVYHDVTPSPGAH-----

RdpA_MH      EGYFEPQMIHR--EANES--GRV--IGDDP-----SSTFLDAPPAVVMRAIDV
SdpA_MH      VRPDGSLRVES--DNDOM--MKILKGNMGN--SSTFMPVQAQGAFAAEVV
SdpA_MC1     VKADGTVRQSPAEWDDM--MKVIVGNMGN--SSTFMPVQAQGAFAAEVV
TfdA_pJP4    VSLDGKVAQDAR--EYVGNF--ANQLK-----SSSQQQAARYSLSAEVV
TauD_E.coli  EGVDELIVL-----DTHND--NPF--DNDNR--DVTRETPFAGASLAAKEL
AtsK_P.putida DGTLYLLQL-----DGAQG--QRA-----NSG--DVTREVEAYPKASLSLSVA
CASI1        -----HLSSETSET--LL-----EYK--EMAKHRLQPNYVRLACGRA
* *

RdpA_MH      P--EHGGD--GFLSMYTHWET--SPTMQATIEGLNVVHSATRVGSLYQAQMR
SdpA_MH      P--SVGGG--GFADMRAYDA--DEDLKAHVETLQARHSLYNSQSLGHQTKA
SdpA_MC1     P--AVGGG--GFADMRAYDA--DEATKALVHQRSARHSLYNSQSLGHVQQA
TfdA_pJP4    P--PSGGG--EFCDMRAYDA--PRDLQSELEGLRAEHYALNSRFLGSD--
TauD_E.coli  P--STGGG--LWTSGLIAYEA--SVFPLLSGLRAEHDFRKSFPETKYRKE
AtsK_P.putida P--ASGGG--VWANTARYQY--PEPLRELADKLVAHVSNEYDYSLSKFDLPD
CASI1        DHERTAA--LVA SVRK--LPI--DERTARLLDRMPCCVDVAFRGVDVDPGA
▲

RdpA_MH      RFSNTSVKVMVDVAGDRETVHP--VVTTPGSS--RKGSLYNQVYQCR--EG
SdpA_MH      ADGEYSYGYGLHDG----PVPLRFPVKIHPET--RKSLILGR--HAHAK--PG
SdpA_MC1     GSA--YIGYGMDDT----ATPLRFPVKIHPET--RPSLLIGR--HAHAK--PG
TfdA_pJP4    --VDYSEAQGNAM----PPVNMWVVRTEAGS--RKFLFIGA--HASHY--EG
TauD_E.coli  EEFQRWRRAVAKN----EPLLHPVVRTEAGS--KQALFVNEGETTTR--VD
AtsK_P.putida AKLERHRKRVFTSTV--YETEHFVVRTEAGS--ERALQGH--FVKRE--KG
CASI1        --LAQVRLD--DADDFPGY--DRELAPE

RdpA_MH      MTDASKEPI--QFLYEHATRFDFTCRVRWKKDVL--N--LCTMRAVP--
SdpA_MH      LEPASERL--QQLIDFACQPPRIYHHDWAPGSAV--N--RCLLDQATP--
SdpA_MC1     MDAASERL--EGLVDWACQAPRVHARQWAGGV--N--RCLLDQATP--
TfdA_pJP4    LPVAGRMIGAELEHATQREFVYRRRWVGV--N--RCVLRAGRR--
TauD_E.coli  VSEKSEAL--SPLFAHITKPEFQVRRWQPNLIA--N--RVTRQYANA--
AtsK_P.putida YSLASQHL--AVLQGHVTRLENTFVRWRWAGV--N--RATOR--YAVD--
CASI1        PADKQAA--SKALDEVTEAVYL-----EPGGL--N--FRTTARTPSP
*

RdpA_MH      DYAGKEP--ITTTVGVGRPAR-----
SdpA_MH      WDMTQK--TMMRSRIAGDPASETALAH-----
SdpA_MC1     WDFKLP--TMMRSRIAGDPASETALAH-----
TfdA_pJP4    YDISAPR--LRRATTLLDDAVV-----
TauD_E.coli  DYLQPR--LRRATTLLDDKPFYRAG-----
AtsK_P.putida DYGTQPR--LRRATTLLAGEVPGVDQQLSRTTRKG-----
CASI1        RWGDK--LRRATTLLRYINTDR-----NQGLSGGERAGDVVAFPTFG
▲

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FIG. 4. Alignment of amino acid sequences of RdpA and SdpA from strain MH and other group II α -ketoglutarate-dependent dioxygenases. The alignment was performed with the T-Coffee software (<http://figs-server.cnrs-mrs.fr/Tcoffec/tcoffec.cgi/index.cgi>). TfdA_pJP4, 2,4-D dioxygenase from *C. necator* JMP134(pJP4) (accession no. A4421983); RdpA_MH, (R)-dichlorprop dioxygenase from *S. herbicidovorans* MH (accession no. CAF32811); SdpA_MH, (S)-dichlorprop dioxygenase from *S. herbicidovorans* MH (accession no. CAF32813); SdpA_MC1, (S)-dichlorprop dioxygenase from *D. acidovorans* MC1 (accession no. AAM90963); TauD_E. coli, taurine dioxygenase from *E. coli* (accession no. AAC73471); AtsK_P. putida, alkylsulfate dioxygenase from *P. putida* (accession no. AAD31784); CAS1, clavamate synthase from *Streptomyces clavuligeres* (accession no. A44241). Highly conserved residues (dark gray boxes) and conserved residues (light gray boxes) are highlighted. Asterisks indicate the conserved or predicted Fe(II)-binding residues, and triangles indicate the α -ketoglutarate binding residues (12, 36).

HX₁₀R. On the basis of the crystal structure of TauD, a structural model of TfdA, and mutagenesis studies (12, 20, 36), we concluded that His-111-Asp-113-His-270 and His-102-Asp-104-His-257 comprise the facial triads of RdpA and SdpA, respectively, which are responsible for iron binding. Residues Thr-138 and Arg-281 of RdpA and residues Thr-129 and Arg-268 of SdpA might act as α -ketoglutarate binding sites.

A comparison of kinetic data for RdpA from *S. herbicidovorans* MH with kinetic data for RdpA from *D. acidovorans* MC1 (48) revealed certain differences. Although the amino acid sequences of the enzymes are identical, the reported K_m values of the phenoxypyranoic acid derivatives for RdpA from *D. acidovorans* MC1 are less than the K_m values that we report here by a factor of about 3. The differences might be explained by the fact that different methods were used to obtain the kinetic data. In our hands, the method based on 2-aminoantipyridine (13–15) that was used for determination of the kinetic parameters of RdpA from *D. acidovorans* MC1 (48) never gave reproducible results for kinetic measurements at low substrate concentrations because of uncontrolled formation of background color. Nonspecific background activity might lead to overestimation of enzyme velocity at low substrate concentrations, which in turn might lead to underestimation of K_m values. Our problem with the aminoantipyridine method was the main reason for developing the continuous coupled enzyme assay described in Materials and Methods. With this assay, we were able to monitor the enzyme reaction continuously and to determine true initial velocities.

To our knowledge, the RdpA proteins from *S. herbicidovorans* MH and *D. acidovorans* MC1 are the only α -ketoglutarate-dependent dioxygenases with a homotrimeric structure (47). Most enzymes of this class are monomers or homodimers, such as SdpA and TfdA; tetramers, such as the alkylsulfatase from *Pseudomonas putida*, are also known (11, 16, 47).

RdpA from *S. herbicidovorans* was surprisingly insensitive to an increase in temperature (Fig. 3B). No Arrhenius-like activity profile was observed, and RdpA was found to be stable at temperatures up to 55°C. This finding correlates with the findings for an α -ketoglutarate-dependent 2,4-D dioxygenase from *Burkholderia cepacia* 2a, which also showed an unexpectedly small increase in activity with increasing temperature and at 10°C still exhibited 77% of the activity observed at 25°C (37). In contrast, TfdA from *C. necator* JMP134(pJP4) exhibits more typical temperature-dependent activity; the activity increases with higher temperatures up to 30°C, and the enzyme is completely inactivated at 40°C within 5 min (16).

RdpA and SdpA catalyze enantioselective reactions. RdpA converted only the (R) enantiomers of phenoxypyranoic acid herbicides, and SdpA converted only the (S) enantiomers. This confirmed the results of previous experiments with *S. herbicidovorans* cell extracts in which the existence of two distinct α -ketoglutarate-dependent dioxygenases in strain MH was proposed (35). We firmly concluded that *S. herbicidovorans* MH harbors two distinct enzymes with opposite enantioselectivities. Enantioselective conversion is more widespread in α -ketoglutarate-dependent dioxygenases. Also, RdpA from *D. acidovorans* MC1 did not significantly convert (S) enantiomers of phenoxypyranoic acids (48). Some α -ketoglutarate-dependent 2,4-D dioxygenases are also capable of enantioselective conversion of mecoprop and/or dichlorprop. For example, TfdA from *C. necator* JMP134(pJP4) and TfdA from *Burkhold-*

eria cepacia RASC cleave only the ether bond of (*S*)-dichloroprop, whereas TfdA from *Alcaligenes denitrificans* converts only the (*R*) enantiomer (41). This difference is surprising, given the high degree of sequence conservation among these three TfdA dioxygenases. None of the TfdA dioxygenases, however, is able to convert both enantiomers of phenoxypropanoic acids at significant catalytic rates. To oxidize both enantiomers, two distinct dioxygenases are required in strains such as *S. herbicidovorans* MH or *D. acidovorans* MC1 (31, 35, 47, 48). Kinetic investigations with site-directed variants of the enzymes demonstrated that not just one but many residues in RdpA and SdpA play a structural role in directing enantio-specificity (T. A. Müller, M. I. Zavodsky, M. Feig, L. A. Kuhn, and R. P. Hausinger, submitted for publication).

Substrate and cosubstrate specificities. RdpA exhibited narrow substrate specificity with respect to the alkyl moiety in the alkanolic acid side chain; only phenoxypropanoic acids were converted, and 2,4-D and phenoxybutyric acids were not converted. This is in contrast to SdpA, which showed measurable activity with 2,4-D. It is also in contrast to RdpA from *D. acidovorans* MC1, whose activity with 2,4-D was reported to be 14% of its activity with (*R*)-dichloroprop (48). This is yet another difference between the two enzymes for which there is not a satisfactory explanation at the moment. Neither RdpA nor TfdA from *C. necator* converted phenoxybutyric acids (16, 48). These results indicate that the active sites of RdpA and of TfdA are specific with regard to the alkanolic acid substituent and are indifferent with regard to the substituents of the aromatic ring. Additionally, RdpA was not inhibited by the (*S*) enantiomers of phenoxypropanoic acids, which indicates that there is no binding of these enantiomers at the active site.

α -Ketoglutarate-dependent dioxygenases have different cosubstrate specificities. AtsK, an alkylsulfatase from *P. putida* S-313, and TfdA, for instance, exhibit broad cosubstrate ranges, and many oxo acids substitute for α -ketoglutarate (16, 22). TauD and the inorganic α -ketoglutarate-dependent hypophosphite dioxygenase HtxA exhibited stricter specificities, and other than α -ketoglutarate, they used only 2-oxoadipate as a cosubstrate, albeit with reduced activities in the range from 4 to 23% (11, 16, 49). RdpA and SdpA showed the same cosubstrate specificities as these two enzymes, but the remaining activities with 2-oxoadipate were only 2% and 5%, respectively. The adaptation of RdpA and SdpA for α -ketoglutarate is apparent from the low apparent K_m values for α -ketoglutarate (3 to 20 μ M).

A linear relationship between the O_2 concentration (0 to 1.0 mM) and the reaction rate was observed; hence, only the value for the apparent second-order rate constant k_{cat}/K_m that can be calculated from this relationship is shown in Table 5. Although the oxygen dependence of SdpA followed Michaelis-Menten kinetics, the apparent K_m value (0.16 mM O_2) was also quite high. Compared to other α -ketoglutarate-dependent dioxygenases, RdpA and SdpA have unusually high apparent K_m values for oxygen. Apparent K_m values of 40 to 50 μ M O_2 were observed for lysyl hydroxylase and an α -ketoglutarate-dependent 4-hydroxylase from *Catharanthus roseus* which is involved in vindoline biosynthesis (8, 39). Flavonol dioxygenase exhibits even a lower apparent K_m value, 5.26 μ M (3). The activities of RdpA and SdpA are therefore highly dependent on the oxygen concentration in laboratory assays.

Existence of another dioxygenase involved in phenoxyalkanoic acid degradation in strain MH. RdpA did not convert 2,4-D, and SdpA only converted this compound at low rates. However, *S. herbicidovorans* MH does grow on 2,4-D, and it grows at a higher rate than it grows on mecoprop and dichloroprop (53). Therefore, another phenoxyalkanoic acid-converting enzyme, which is specific for 2,4-D, must be present in *S. herbicidovorans* MH. Our results of hybridization and PCR experiments suggest that *S. herbicidovorans* MH did not harbor a gene sufficiently similar to the *tfdA*, *tfdA α* , or *cadAB* genes to allow detection. Therefore, the nature of this additional enzyme in *S. herbicidovorans* MH remains unclear.

In conclusion, we describe the characteristics of two α -ketoglutarate-dependent dioxygenases, RdpA and SdpA, from *S. herbicidovorans* MH that catalyze enantioselective ether cleavage of phenoxypropanoic acids to form achiral phenols. These two dioxygenases are a good example of an enzyme pair in which each enzyme converts only one enantiomer of a chiral substrate.

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The two enantiospecific dichlorprop/ α -ketoglutarate-dioxygenases from *Delftia acidovorans* MC1 – protein and sequence data of RdpA and SdpA

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Abstract

Two α -ketoglutarate-dependent dioxygenases carrying enantiospecific activity for the etherolytic cleavage of racemic phenoxypropionate herbicides [(*RS*)-2-(2,4-dichlorophenoxy)propionate and (*RS*)-2-(4-chloro-2-methylphenoxy)propionate] from *Delftia acidovorans* MC1 were characterized with respect to protein and sequence data. The (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA) appeared as a monomeric enzyme with a molecular weight of 32 kDa in the presence of SDS. N-terminal sequences revealed relationship to α -ketoglutarate-dependent taurine dioxygenase (TauD) and to 2,4-dichlorophenoxyacetate/ α -ketoglutarate-dioxygenase (TfdA). The (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) referred to 36 kDa in the presence of SDS and to 108 kDa under native conditions. Internal sequences of fragments obtained after digestion made evident relationship to TfdA and TauD. Two-dimensional electrophoretic separation resulted in the resolution of up to 3 individual spots with almost identical molecular weights but different isoelectric points with both RdpA and SdpA. The structural differences of these isoenzyme forms are not yet clear.

Key words: chiral herbicides – (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA) – (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) – partial amino acid sequences – *Delftia acidovorans* MC1

Introduction

A few strains are known which are capable of completely degrading the chiral herbicides (*RS*)-2-(2,4-dichlorophenoxy)propionate (2,4-DP; dichlorprop) and (*RS*)-2-(4-chloro-2-methylphenoxy)propionate (MCPP; mecoprop) in addition to phenoxyacetate derivatives (2,4-dichlorophenoxyacetate; 2,4-D) (Zipper *et al.* 1996; Ehrig *et al.* 1997; Müller *et al.* 1999). This contrasts with the experience gained with most of the phenoxy herbicide-degrading strains, which have been shown, based on the activity of 2,4-D/ α -ketoglutarate-dioxygenase (TfdA) (Fukumori and Hausinger 1993a, b; but cf. Saari *et al.* 1999), to be highly specific for the productive degradation of phenoxyacetates (Hägglom 1992; Hoffmann *et al.* 1996). The enzymes of one of the above strains, *Delftia acidovorans* MC1, have been purified and characterized (Müller and Babel 1999; Westendorf *et al.* 2003). Both enzymes were found to be α -ketoglutarate-dependent dioxygenases (Müller *et al.* 2001). This matches the reaction mechanism described for the cleavage of 2,4-D by the respective enzyme, i.e. TfdA, from the canonical strain *Ralstonia eutropha* JMP134 (Fukumori and Hausinger 1993a, b). Kinetic investigations revealed that one of the enzymes of strain MC1 is highly specific for the cleavage of the *R*-configuration of 2,4-DP and MCPP, i.e. (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase, consecutively designed RdpA. The other enzyme, i.e. (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase and designed SdpA, prefers the *S*-configuration but also accepts phenoxyacetate derivatives as substrate (Westendorf *et al.* 2003). Sequence data of *tfdA* are known

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(Streber *et al.* 1987; Suwa *et al.* 1996; Mäe *et al.* 1993; Ka *et al.* 1994; Vallaeys *et al.* 1996; Hoffmann *et al.* 2001) revealing high similarity in the gene of the various strains of β - and γ -*Proteobacteria* (but form a separate cluster, *tfdA* α , with α -*Proteobacteria*; Itoh *et al.* 2002). The application of primers derived from conserved regions of *tfdA* (Vallaeys *et al.* 1996), however, failed to result in amplification products in PCR with genomic DNA from strain MC1 (Müller *et al.* 2001). No data are available on a molecular level with respect to the phenoxypionate-degrading strains. The present investigation is designed to elucidate the protein characteristics of the two different dioxygenases of strain MC1. Special attention is directed to sequence data whose elucidation should enable the assignment of the two enzymes to known proteins by databank comparison. Moreover, these data should favor targeting the genetic basis.

Materials and methods

Enzyme purification. The (*R*)-phenoxypionate/ α -ketoglutarate dioxygenase (RdpA) and the (*S*)-phenoxypionate/ α -ketoglutarate dioxygenase (SdpA) were purified as described by Westendorf *et al.* (2003). RdpA and SdpA were enriched by this procedure by a factor of 20 and 30, respectively, resulting in 71% and >98% purity.

Electrophoretic and chromatographic separations. For one-dimensional electrophoresis, a PowerEase 500 system was used (Novex, San Diego, USA). SDS PAGE of the enzyme preparation was carried out on 12% Tris glycine pre cast acrylamide gels (Novex). The samples contained 1–2 μ g of protein. The running buffer was composed of 2.9 g/l Tris base, 14.4 g/l glycine and 1.0 g/l SDS. The sampling buffer contained 2 ml glycerol, 4 ml 10% SDS (w/v), 0.5 ml 1% bromophenol blue and 2.5 ml 0.5 M Tris/HCl, pH 6.8 in 10 ml distilled water. The gels were treated at 125 V. Mark 12™ Wide Range Standard (Novex) was applied as a reference.

Peptides obtained from the digested enzymes were separated by peptide electrophoresis with Tris/Tricin buffer as described by Schägger and von Jagow (1987) in Mini Protean II Cell (Bio-Rad, Hercules, CA, USA). The following concentrations of acrylamide were used: separation gel (16% T and 3% C), spacer gel (10% T and 3% C) and stacking gel (4% T and 3% C). Electrophoresis was run with a constant current of 10 mA/gel for 0.5 h and a constant current of 20 mA/gel for 4 h. Ultra low range proteins (Sigma) served as a standard.

Two dimensional electrophoresis was performed as described by Benndorf and Babel (2002).

Gels were stained by following a slightly modified procedure described by Blum *et al.* (1987), or by using Coomassie brilliant blue.

Gel exclusion chromatography was performed by HPLC at 23°C using a Bio-Sil SEC-250 column (300 mm $\times$ 7.8 mm, Biorad) and a mobile phase containing 5 mM Na₂HPO₄, 5 mM NaH₂PO₄, 150 mM NaCl (pH 6.8). The flow rate was 1 ml/min. The molecular weight was determined by using a gel filtration standard (Biorad).

Protein digestion. Before enzymatic and chemical in-gel digestion, bands containing protein separated by PAGE were cut out and the gel slices washed three times with 50% methanol and 5% acetic acid in water for at least 3 h. The gel slices were reduced and alkylated by adding and removing the following solutions: 200 μ l acetonitrile (5 min), 30 μ l 1,4-dithioerythrole in 100 mM ammonium hydrogencarbonate (30 min), 30 μ l 100 mM iodoacetamide in 100 mM ammonium hydrogencarbonate (30 min), 200 μ l acetonitrile (5 min), 200 μ l 100 mM ammonium hydrogencarbonate (10 min) and 200 μ l acetonitrile (5 min). After drying the gel slices by vacuum centrifugation, enzymatic digestion was performed by adding 30 μ l LysC buffer (25 mM Tris/HCl pH 8.5) containing 1.5 μ l LysC (Lys-C, enzyme preparation from *Lysobacter enzymogenes*, Merck, Germany), 1 mM EDTA and 0.05% SDS, and incubating the sample overnight. Chemical digestion was performed by adding 30 μ l BNPS-scatole solution (500 μ g BNPS in 500 μ l 75% acetic acid; Crimmins *et al.*, 1990) and incubating the sample for 1 h at 47°C by shaking. To recover the peptides, the following solution were added and the respective supernatants collected: twice 30 μ l of 50 mM ammonium hydrogencarbonate for 10 min and twice 30 μ l extraction buffer (10 ml acetonitrile, 5 ml dist. water, 1.176 ml 85% formic acid) for 10 min. The unified supernatants were centrifuged under vacuum until a dry pellet was obtained. The latter was solubilized in sample buffer for electrophoretic separation.

Amino acid sequencing. Peptides separated by PAGE were blotted onto PVDF membranes (Jin and Cerletti 1992). Gels were washed for 5 min in distilled water and for 15 min in 10 mM CAPS buffer pH 11. Blotting was performed for 2 h at 3 mA/cm² with 10 mM CAPS buffer pH 11 (Trans Blot, Semi-Dry Transfer Cell, Biorad, USA). After staining with Coomassie brilliant blue, the bands were cut and applied to N-terminal sequencing (PROCISE® 491 cLC Protein Sequencer or PROCISE® 473A Protein Sequencer, Applied Biosystems; Foster City, CA, USA).

Results

One-dimensional SDS PAGE of the enzyme preparation resulted in distinct protein bands corresponding to molecular weights of about 32 kDa for SdpA and to 36 kDa for RdpA. Gel exclusion chromatography of the preparations of RdpA under native conditions resulted in a protein fraction corresponding to about 108 kDa which showed cleavage activity for (*R*)-2,4-DP. In the presence of SDS, the 108 kDa band disappeared in favor of a band with an apparent molecular weight of 36 kDa. This pattern indicates a trimeric structure of the R-specific enzyme. By contrast, with SdpA no fraction could be detected which corresponded to a protein larger than the monomeric form in gel exclusion chromatography. The protein appeared at a retention time corresponding to a molecular weight of about 23.7 kDa under native conditions. The enzyme separated under these conditions showed specific activity corresponding to that found before. Applying this fraction to SDS PAGE or performing gel exclusion chromatography in the presence of SDS resulted in separation patterns which correlated this protein to a mass of about 32 kDa.

Table 1. Isoelectric points (pI) and molecular weight (MW) of the isoforms of RdpA (R) and SdpA (S)

Spot	pI	MW [kDa]
R1	5.9	36.3
R2	5.86	35.9
R3	5.8	35.7
S1	6.33	32.8
S2	6.16	32.5

Data were obtained from electropherograms by software analysis (Phoretix 2D, 5.01, NonLinear Dynamics Ltd., G.B.)

Table 2. N-terminal sequences of the isoforms of RdpA and SdpA and internal sequences from selected peptide fragments of the two proteins

Position		Sequence	Accession No
N-terminal	R-Spot1	MHAALSPLSQRFERI	P 83310
	R-Spot2	MHAALSPLSQRFERI	
	R-Spot3	MHAALSPLSQRFEIIVQPLTGGV	
	S-Spot1	MQTTLQITPTGATLG	P 83309
	S-Spot2	MQTTLQITPTGATLGATVTGVHLAT	
Internal	R-Frac1E	SIEGYPEVQMIRREANESGRVIGDDXHTXII	P 83310
	R-Frac5C	ETLSPTMQATIEGLN	
	R-Frac8C	NEILDAFHTYQVIYFPGQAITNEQHIAFSR	P 83309
	S-Frac1E	LGHVQQAGSAYI	
	S-Frac2E	VIVGNTAG	

R, S refers to RdpA and SdpA; Spot designates the respective proteins in the 2D gels (Fig. 1 for SdpA; RdpA not shown); Frac refers to the protein fractions after enzymatic (E) and chemical (C) digestion

The purified enzymes were treated by 2D-electrophoresis which resulted in the resolution of the respective enzymes into 2–3 individual protein spots. This is convincingly shown for SdpA in Fig. 1. Apparently, the isoforms were characterized by an almost identical molecular weight but differed in their charges (Table 1). A similar pattern was observed for RdpA (Table 1). Although the latter preparation contained significant impurities which could not be eliminated by the purification protocol applied, three spots were clearly identified to correspond to RdpA by admixture of crude extracts of pyruvate grown cells (not shown). The individual protein spots with respect to RdpA and SdpA preparations were used to determine N-terminal amino acid sequences. The results are shown in Table 2. The sequences were clearly identical with respect to the isoforms of RdpA and SdpA except position 14 in spot R3 which may be attributed to analytical uncertainties in this late cycle of sequencing.

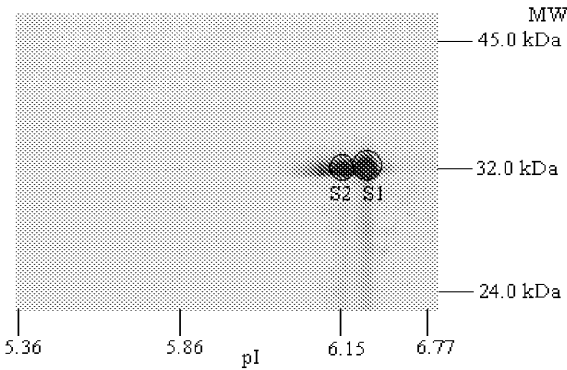


Fig. 1. Part of the 2D-gel of SdpA. Isoelectric focusing from right to left, SDS PAGE from up to down.

P96312_BURCE MSINSEYHLFLVAEYEEADQGALSPTEV
TfdA_BURSR MSINSEYHLFLVGVQVDNLAQGALSPAEV
TfdA_ALCEU MSVVANPLHFLFAAGVEDIDREALGSTEV
TauD_ECOLI SERLSITPLGFIYGAQISGADTRPLSDNQ
SdpA_MC1 MQTTLQITPTGATLGATVTVHAT
Consensus

P96312_BURCE, 2,4-D-dioxygenase [*Burkholderia cepacia*]; TFDA_Bursr, *Burkholderia* sp. RASC; TfdA_Alceu, α -ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase [*Ralstonia eutropha*]; TauD_Ecoli, taurine dioxygenase [*Escherichia coli* O157:H7];

Fig. 2. Alignment of the N-terminus of SdpA (SdpA_MC1) with TfdA and TauD.

RdpA_MC1 MHAALSPLSQRFEELAVQPTTGGV N L D FHTYQ IY P E - A ENE H A S
AF170704_7 LKQFLFPG - - HPE L I V S N I V - - - - - E N G Q P I G L G D A G K F - - S L S Y K E L P S L G M H Q E L E - E - L F A D M H K
F83356 MSVSLPAPARADVPLQIRADAAGFEVLGLDGLPAAEDFRFHHHDEHLVDE - R P A A S R R E Q I H V
TauD_Ecoli MSERLSITPGYIGQISGADTRPSDNQFE LYH V R T Q V F D - A E P Q R A L Q R E E H I H P
YY06_MYCTU MTLITVKKGSRIQIDGVREGGDPAAVN R A L L A K K V F D - H Q D D A E L A G L L T P I G H P
TfdA_Alceu MSVVANPLHFLFAAGVEDIDREALGSTEVRLMDKESLV - - P L S Q D - A - N P E G G F
AAB47567 MSINSEYHPLFVVEYEADLQGA S P T E V R L E H Q M D K K A L V - - P L D Q D - A - N P E G G F
Consensus

RdpA_MC1 LAPEGSD - - - - - P H L E I S A D K - - - - - D S R N V A G - - - - - H C - - - - - G T A D L K P S L G M H Y V T R T E I G S - - - - - M F S N M H L
AF170704_7 LKQFLFPG - - HPE L I V S N I V - - - - - E N G Q P I G L G D A G K F - - S L S Y K E L P S L G M H Q E L E - E - L F A D M H K
F83356 VYPHA-EG - - - - - V D E I V L D T H N - - - - - D N P F D - - - - - N D N - - - - - V T F I E T P P A G A L - - K E L S - - - - - L W T S G I A
TauD_Ecoli A A I A L A D D - - - - - A P I T P I N S E F - - - - - G K A - - - - - N P - - - - - V T F A A N Y P A A V - - V S L S - - - - - S L W A N T A A
YY06_MYCTU I K V N Q R P S R F K Y A E L A D I S N V S L D G K V A Q R D A R E V V G N F A N - Q I - - - - - S S F Q Q P A A R Y M S V V V P - - - - - E F C D M R A
TfdA_Alceu I K V N Q R P S R F K Y A E L A D I S N V S V D G K V A D R E A R E S V G N F A N N Q L - - - - - S S F Q Q A A R Y M S S V L P - - - - - E F W D I H A T
AAB47567 I K V N Q R P S R F K Y A E L A D I S N V S V D G K V A D R E A R E S V G N F A N N Q L - - - - - S S F Q Q A A R Y M S S V L P - - - - - E F W D I H A T
Consensus

RdpA_MC1 M P T Q A T I N N
AF170704_7 M P A K E L L P T V - - - - - N G L L A W E G A T P P P E - - Y D V P V N V - - - - - A R E D T R K L - - - - - I N G I Y V S H - - - - - E Q S K G
F83356 W S P E A R K A I L R T A S Y T A R Y S E P R F E G N W R P T L S A A Q - L A E V R E V V H I - - - - - E S R K A - - - - - V S E G F T T R - - - - - V G P A D
TauD_Ecoli A S V P F R Q L L S R F E D F R K S F P E Y K Y R K T E E E H Q R W R E A V A K N P L L H V - - - - - E V S K Q A - - - - - V N E G F T T R - - - - - V D V S E K
YY06_MYCTU G A E P E P K C L T N W L T N R Y D V T T K P L T A A Q R A F R Q V F E K P D F R - T E R - - - - - V E V R E T E R T L A G D - F V R S F V G D S H
TfdA_Alceu A S P R D Q S E L R E R - - - - - Y A L N S R F L L G D T D Y S E A Q R N A - M P P V N W L - - - - - E T A G S R K F - - - - - I G A - H A S H - - - - - E G P V A
AAB47567 N L G G R D D P R E L R E R - - - - - Y L Q N S R F I L G D T D Y S E S Q R N A - M P P V S W L - - - - - E T A G S R K F - - - - - I G A - H A G H - - - - - E G R P V A
Consensus

RdpA_MC1 S I E G Y V Q M I R E A N E S G R E G X H X I I X
AF170704_7 R A I D M V K Q I N T A L L S C V R F P N T L F - - - - - C V O H I W D Y F P H S Y A O V A I A H R E T A
F83356 A Q L A E Y A H S V R H I - Y H R Q A H L F - - - - - S L I L G G C P A H L R K L Y T I Q H A F F
TauD_Ecoli E A L G F F A H I K F Q - V W Q P N I A S - - - - - V O Y N A D Y L P Q R I M H A I L R K F F Y R A G
YY06_MYCTU R V F E V Q R R I M N T - I W N A P G V A S - - - - - A O R I D D Y D D Q H L M H V L M R Y V D V Y G Q A S R V I S G A P M E I A G
TfdA_Alceu G R M L A E L E H A Q R K F V - Y H R N V G G L M - - - - - G V L R G R R Y D I S A R E L P A T L D A V V
AAB47567 G R M L A E L E H A Q R K F V - Y H R K V G G L M - - - - - G V L R G R G Y D I T A R E L P A T V D G G V V
Consensus

Fig. 3. Alignment of the N-terminal and internal amino acid sequences of RdpA (RdpA_MC1) with different proteins (dioxygenases).

N-terminal sequence data were applied for databank comparison using NCBI BLAST, Matrix Blossom 80. The data from SdpA revealed similarity to other α -ketoglutarate-dependent dioxygenases, i.e. taurine dioxygenase (TauD) and 2,4-D/ α -ketoglutarate-dioxygenase (TfdA) (Fig. 2). This was supported further by data derived from internal sequences (see Discussion) the alignment of which is not shown in detail. With respect to RdpA, however, the N-terminal sequence did not exhibit significant homology with any known protein. Elucidation was expected from determining internal sequences. Three fragments obtained by electrophoretic separation after chemical and enzymatic proteolysis gave unequivocal sequence data for their individual N-termini (Table 2), in other cases the results were obviously disrupted by the presence of different fragments in the respective fractions. The sequence data

from the positive results were again applied to databank comparison, which now resulted in significant correlation to known proteins. Accordingly, homology of the individual fragments to sequences of TfdA and TauD became evident (Fig. 3). The results of alignment also highlight that the N-terminal sequence of RdpA deviates significantly from the other cluster of proteins, which explains the failure to obtain any positive correlation in the initial attempts.

Discussion

The results make evident a monomeric structure with the (*S*)-phenoxypropionate/ α -ketoglutarate dioxygenase (SdpA) and a trimeric structure with the (*R*)-phenoxypropionate/ α -ketoglutarate dioxygenase (RdpA) from

D. acidovorans MC1. The molecular weights of the monomers, 32 and 36 kDa respectively, are in the range found for analog enzymes from other sources (Fukumori and Hausinger 1993b; Nickel *et al.* 1997). With TfdA from *R. eutropha* JMP134 a dimeric structure was deduced (Fukumori and Hausinger 1993b). Surprisingly, with the two enzymes of strain MC1, isomeric enzyme forms were detected being separated from each other by only slight differences in their isoelectric points. The structural reasons are not yet apparent. The existence of isoenzymes is a likely explanation of the complex, multiphasic characteristics observed in estimating the kinetic properties with preparations of both RdpA and SdpA (Westendorf *et al.* 2003).

The two dioxygenases from *D. acidovorans* MC1 were shown to be related to other α -ketoglutarate-dependent dioxygenases. According to the N-terminal and two internal sequences, SdpA showed closest homology to taurine dioxygenase (TauD) from *E. coli* (Eichhorn *et al.* 1997) and *Pseudomonas aeruginosa* (36%/43% identical/similar to *E. coli* enzyme) and to TfdA from strains JMP134 and RASC (18%/20% identical/similar to JMP134) based on 44 amino acids aligned. With respect to RdpA, homology with other α -ketoglutarate-dependent dioxygenases was detected from sequence data of various internal fragments, according to which highest homology was attributed to TfdA (32%/37% identical/similar; strain JMP134), followed by TauD (21%/30% identical/similar; *E. coli*) on the basis of 101 amino acids aligned. The present sequence data are considered an appropriate basis to target the genetic background and elucidate the whole structure of these enzymes.

Acknowledgements

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Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes

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Engineered glyphosate resistance is the most widely adopted genetically modified trait in agriculture, gaining widespread acceptance by providing a simple robust weed control system. However, extensive and sustained use of glyphosate as a sole weed control mechanism has led to field selection for glyphosate-resistant weeds and has induced significant population shifts to weeds with inherent tolerance to glyphosate. Additional weed control mechanisms that can complement glyphosate-resistant crops are, therefore, urgently needed. 2,4-dichlorophenoxyacetic acid (2,4-D) is an effective low-cost, broad-spectrum herbicide that controls many of the weeds developing resistance to glyphosate. We investigated the substrate preferences of bacterial aryloxyalkanoate dioxygenase enzymes (AADs) that can effectively degrade 2,4-D and have found that some members of this class can act on other widely used herbicides in addition to their activity on 2,4-D. AAD-1 cleaves the aryloxyphenoxypropionate family of grass-active herbicides, and AAD-12 acts on pyridyloxyacetate auxin herbicides such as triclopyr and fluroxypyr. Maize plants transformed with an AAD-1 gene showed robust crop resistance to aryloxyphenoxypropionate herbicides over four generations and were also not injured by 2,4-D applications at any growth stage. Arabidopsis plants expressing AAD-12 were resistant to 2,4-D as well as triclopyr and fluroxypyr, and transgenic soybean plants expressing AAD-12 maintained field resistance to 2,4-D over five generations. These results show that single AAD transgenes can provide simultaneous resistance to a broad repertoire of agronomically important classes of herbicides, including 2,4-D, with utility in both monocot and dicot crops. These transgenes can help preserve the productivity and environmental benefits of herbicide-resistant crops.

herbicide resistance | weed management | genetically modified crops | AOPP herbicides

Since their introduction in the mid-1990s, genetically modified (GM) crops have delivered many benefits to agricultural growers by providing increased productivity, reduced pesticide use, and greater flexibility and efficiency in farm management. The area devoted to GM crop plantings has steadily increased at a rate of ~20%/y globally for over a decade, with 134 million ha of GM crops planted worldwide in 2009. Continued growth is expected in the near future because of rapid adoption of this technology in developing countries (1). Over 90% of global GM crop plantings contain a trait conferring resistance to the broad-spectrum herbicide glyphosate (1). These include soybean, cotton, maize, and canola. Because glyphosate resistance provides a simple and convenient solution for control of a wide spectrum of broadleaf and grass weeds, farmers have rapidly adopted glyphosate-resistant (GR) crops. However, in many instances, traditional best agronomic practices for avoiding natural development of GR weeds in the field have been abandoned or reduced. These practices include crop rotation, herbicide mode of action rotation, tank mixing of multiple herbicide modes of action, and incorporation of mechanical weed control with chemical and cultural methods.

Glyphosate is commonly used in noncrop areas for total vegetation control. With the introduction of GR crops, glyphosate has been applied to some crop fields one to three times per year for more than 15 consecutive years. This increased application frequency, in combination with higher use rates and the increasing hectareage being treated, has placed heavy selection pressure on weed species to acquire naturally occurring resistance mechanisms to glyphosate (2–5). To date, 19 weeds, including both grass and broadleaf species, have developed natural resistance to glyphosate (6), most of which have been reported in the past 8 y, which is coincident with increasing use of GR crops (7, 8). Moreover, weeds that had previously not been an agronomic problem before the wide deployment of GR crops, such as *Ipomoea*, *Amaranthus*, *Chenopodium*, *Taraxacum*, and *Commelina* species (9–11), are now becoming more prevalent and difficult to control. With this trend, growers are rapidly losing the advantages of GR crops.

One of the most effective approaches for weed resistance management is the use of herbicides with differing modes of action, either in rotation as part of a weed management program or in combination as mixtures (12, 13). One strategy to complement and sustain the powerful weed control technology conferred by the GR trait is to deploy an additional trait that can confer resistance to herbicides with alternative modes of action. The plant hormone mimic 2,4-dichlorophenoxyacetic acid (2,4-D) was the first synthetic herbicide to be commercially developed and has been used agronomically and in noncrop applications for broad-spectrum broadleaf weed control for over 60 y (14). In addition to low cost, 2,4-D has environmentally friendly properties such as short environmental persistence and low toxicity to humans and wildlife (15, 16). Although 2,4-D remains one of the most widely used herbicides globally, its complex, plant-specific mode of action has not led to widespread natural resistance development (17), and only isolated cases of resistant weed species have been reported (6). Engineered 2,4-D resistance could offer a potentially robust, cost-effective herbicide-resistant trait that could complement glyphosate resistance.

In this work, we show that members of a class of bacterial enzymes [aryloxyalkanoate dioxygenases (AADs)], which effi-

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ciently cleave 2,4-D in vitro into nonherbicidal dichlorophenol and glyoxylate, can provide robust field resistance to 2,4-D when expressed in maize and soybean crops. In addition, we have discovered that two unique members of this class of enzymes have surprisingly broad substrate ranges for xenobiotic herbicides. AAD-1 has the additional ability to inactivate potent grass-active herbicides that act through a different mode of action (inhibition of lipid biosynthesis). AAD-12 can cleave pyridyloxyacetate auxins that are structurally diverse members of the synthetic auxin herbicide family related to 2,4-D and provide extended spectra of weed control. These attributes indicate the utility of these types of enzymes for herbicide-resistant crop applications. They may offer growers options to overcome the significant developing issues of GR weeds as well as provide the ability to use herbicides that differ in both chemical structure and mode of action through a single transgene.

Results and Discussion

Identification of 2,4-D Degrading Enzymes. Previous studies have established that the enzyme TfdA is responsible for the first enzymatic step in the 2,4-D mineralization pathway of the soil bacterium *Ralstonia eutropha* (18). TfdA catalyzes the oxygenolytic cleavage of 2,4-D to nonherbicidal dichlorophenol and glyoxylate through an Fe(II)/ α -keto acid-dependent dioxygenase reaction (Fig. 1A) (19). The enzyme was also shown to confer 2,4-D resistance when expressed in transgenic cotton plants (20). Sequences for a large number of bacterial genes that encode homologs of TfdA have now been deposited in genetic databases. However, most of these have not been biochemically characterized or more significantly, tested for attributes that could confer optimal 2,4-D resistance in transgenic crops. We identified sequences of candidate genes for potential 2,4-D-resistant crop utility by using a bioinformatic approach to identify homologs of TfdA in the National Center for Biotechnology Information (NCBI) genetic sequence database. Fe(II)/ α -ketoglutarate-dependent dioxygenases typically contain a triad of conserved histidine residues comprising the Fe(II)-coordinating active site (21), and therefore, although overall similarity to TfdA may be relatively low, sequences with this histidine triad motif can be attributed to the Fe(II)/ α -ketoglutarate-dependent dioxygenase family. Genes encoding candidate enzymes were synthesized and expressed in *Escherichia coli*, and the biochemical and physiological properties of the recombinant enzymes were assessed. This range-finding study enabled us to focus on two lead homologs, RdpA from *Sphingobium herbicidivorans* and SdpA

from *Delftia acidovorans*, that have 28% and 31% amino acid sequence identity to TfdA, respectively.

To establish a uniform and unbiased nomenclature for the enzymes that we have characterized, we refer to these enzymes generically as AADs and further refer to RdpA and SdpA as AAD-1 and AAD-12, respectively. The chemical structures of herbicidal compounds included in the generic aryloxyalkanoate chemical family descriptor are shown in Fig. 1B. AAD-1 has been previously shown to cleave the herbicidally active *R*-enantiomer of dichlorprop and was also reported to have a low level of activity on 2,4-D (22). Screening an expanded set of structurally related herbicides, we unexpectedly found that AAD-1 has a unique ability to enantioselectively cleave members of the aryloxyphenoxypionate (AOPP) class of potent grass-selective herbicides, including *R*-cyhalofop and *R*-quizalofop (Table 1). These herbicides act by an entirely different mechanism than that of the synthetic auxins and specifically inhibit the monomeric acetyl-CoA carboxylases of grasses (23). The efficiency of in vitro AOPP cleavage approaches that of the optimal substrate, *R*-dichlorprop, and is significantly higher than the activity of AAD-1 on 2,4-D (Table 1). This was surprising given the extended and bulky aryloxy substituents on the AOPPs. These data show that AAD-1 can cleave two distinct classes of herbicides covering broadleaf and grass-selective modes of action.

In contrast to AAD-1, AAD-12 has been previously reported to selectively cleave the *S*-enantiomer of dichlorprop (24). We found in our biochemical studies that AAD-12 can also cleave the *S*-enantiomers of the AOPP herbicides (Table 1). Because these are the herbicidally inactive enantiomers of these compounds, this AAD-12 activity is not of utility for providing crop resistance to these chiral herbicides. However, AAD-12 has significantly greater in vitro activity on 2,4-D (an achiral substrate) than AAD-1 (Table 1) (25). Testing a series of pyridyloxyacetate compounds as substrates, we found that AAD-12 was capable of degrading the synthetic auxin herbicides triclopyr and fluroxypyr at rates of 4% and 16%, respectively, of 2,4-D (at 1 mM substrate). AADs have not previously been shown to work on substrates containing a pyridine ring. This activity gives AAD-12 potential utility for providing resistance to a wider repertoire of synthetic auxins beyond 2,4-D and thus enables expanded broadleaf weed control.

Validation of Plant Herbicide Resistance Provided by AADs. Our initial investigations into the broad in vitro substrate specificities of the AAD-1 and AAD-12 enzymes were intriguing. Our next goal was to evaluate if expression of these enzymes in transgenic plants could provide protection against the damaging effects of 2,4-D and other herbicides. *Arabidopsis thaliana* was first used as

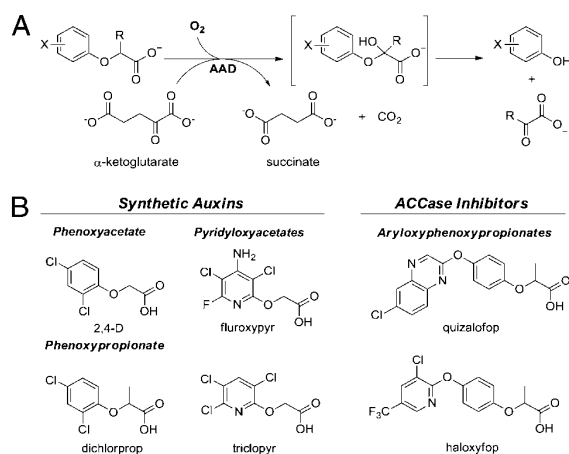


Fig. 1. (A) General reaction catalyzed by AAD enzyme. (B) Aryloxyalkanoate herbicidal compounds that are substrates for AADs.

Table 1. In vitro kinetic constants of AAD-1 and AAD-12 for various herbicide substrates

Enzyme	Substrate	K _m (μ M)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ ·s ⁻¹ × 10 ³)
AAD-1	2,4-D	683 ± 76	0.31 ± 0.01	0.45
	MCPA	527 ± 38	0.11 ± 0.002	0.21
	<i>R</i> -dichlorprop	93 ± 16	5.22 ± 0.36	56.3
	<i>R</i> -cyhalofop	96 ± 7	3.12 ± 0.06	32.5
	<i>R</i> -quizalofop	144 ± 9	3.34 ± 0.09	23.3
AAD-12	2,4-D	185 ± 25	3.46 ± 0.14	18.6
	MCPA	283 ± 67	4.18 ± 0.60	14.7
	<i>S</i> -dichlorprop	118 ± 35	3.57 ± 0.46	30.2
	<i>S</i> -cyhalofop	279 ± 22	1.52 ± 0.08	5.43

Assays were performed using 0.1- to 1.5- μ M enzyme. MCPA, 2-methyl-4-chlorophenoxyacetic acid.

a rapid and facile model system for validation of in planta activity of the AADs. An expression cassette containing either *aad-1* or *aad-12* genes (synthesized with plant-preferred codon usage) under control of a constitutive promoter was introduced into *Arabidopsis* plants through *Agrobacterium tumefaciens*-mediated gene delivery using phosphinothricin acetyltransferase (PAT) gene as a selectable marker (Figs. S1 and S2). T₁ generation plants were selected with glufosinate herbicide, and transformants were analyzed by Southern blot and PCR for presence of the genes and by Western blot and ELISA for expression of the appropriate proteins.

Application of 2,4-D onto untransformed *Arabidopsis* plants severely injured them, even at doses as low as 50 g acid equivalent (ae)/ha. In contrast, transgenic *Arabidopsis* plants expressing either AAD-1 and AAD-12 exhibited little or no visible signs of 2,4-D damage, even when treated with up to 3.2 kg ae/ha of the herbicide (Fig. 2 and Fig. S3). This application rate is about 5- to 10-fold higher than typical field use rates of 2,4-D and therefore, indicated that the introduced AAD enzymes could function well in plants.

In addition, plants expressing AAD-12 survived applications of triclopyr and fluroxypyr at rates of up to 2.24 kg ae/ha. Some temporary epinastic symptoms were visible on rates exceeding 0.28 g ae/ha fluroxypyr on AAD-12-expressing plants; however, the treated plants recovered, whereas control plants were severely injured or died.

Crop Transformation with AADs. For broad utility, a transgene must provide robust herbicide resistance in major crops where management of weeds is critical to maximize production. Maize, soybean, cotton, rice, and canola were chosen as crop targets for transformation with AAD-1 or AAD-12 expression cassettes similar to those used in *Arabidopsis*. Here, we describe the results obtained for maize and soybean as relevant representative monocot and dicot crops. To obtain transgenic events with adequate expression of AAD-1 and AAD-12 in these crops, various vectors with different regulatory elements were designed.

For soybean transformation, a binary vector pDAB4468 was constructed with a transfer DNA (T-DNA) insert carrying an *aad-12* gene cassette under the control of an *A. thaliana* ubiquitin 10 promoter (AtUbi10) and a *pat* gene cassette driven by the Cassava Vein Mosaic Virus promoter (CsVMV) as the selectable marker for transformation (Fig. S4). Multiple transgenic soybean events were produced through *Agrobacterium*-mediated transformation of the public elite variety Maverick. Events with one intact copy of T-DNA insert were selected for further evaluation and characterization. T₁ seed was collected from self-pollinated T₀ putative transformants, T₁ plants were screened for resistance to glufosinate, and seeds from multiple subsequent generations of transformants (up to T₅) were collected. The heritability and stability of the gene in soybean were investigated over multiple generations. Progeny testing and molecular analysis revealed that the inheritance of the *aad-12* gene was stable across all of the tested generations and followed typical single-locus Mendelian segregation for selected soybean events (Table 2). When

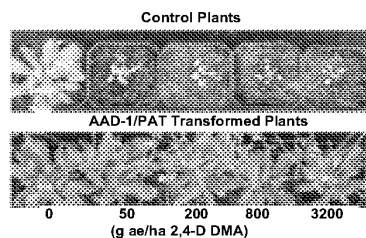


Fig. 2. *Aad-1* transformed *Arabidopsis* response to 2,4-D in the greenhouse.

sprayed with varying levels of 2,4-D, most of the transgenic soybean events showed 2,4-D resistance exceeding typical agricultural use rates, and the selected events were resistant to rates of ≥ 4.48 kg ae/ha under greenhouse and field conditions. The gene and herbicide resistance were heritable across multiple generations from T₀ through T₅ (Table S1 and Fig. S5). Selected events showed excellent resistance to 2,4-D at 4.48 kg ae/ha when applied at the V3 stage in field trials (eight times the recommended level of agricultural applications) (Fig. 3). Initial field studies with multiple selected soybean events at several locations in the United States showed agronomic performance equivalent to that of the nontransformed control variety, including date to flowering, lodging, phenotype, and yield. Transgenic plots were treated with 2,4-D applications comprising a seasonal total of 3.36 kg ae/ha (or 3 lb ae/acre) at both preemergence (postplant) and approximately V4 or R2 growth stages.

For maize transformation, plasmid pDAS1740 was constructed containing an *aad-1* gene under the control of the maize ubiquitin promoter (ZmUbi1) (Fig. S6). The recipient maize line Hi-II was transformed using direct insertion of the DNA fragment from plasmid pDAS1740 through silicon carbide fiber-mediated transformation (26). Putative transgenic maize plants were screened with *R*-haloxyfop, and multiple transgenic maize events were produced. Events with one single intact copy of *aad-1* plant transcription unit (PTU) were selected for further evaluation and characterization. Seeds from multiple generations, including self-pollinated generations (up to T₄), converted inbred generations (BC₁, BC₂, and BC₃S₁), and hybrid test crosses, were collected. Progeny testing and molecular analysis revealed that the inheritance of the *aad-1* gene was stable across all of the generations evaluated and followed typical single-locus Mendelian segregation (Table 2).

The transgenic maize plants were resistant to applications of the grass-active AOPP herbicides (cyhalofop or quizalofop) at rates of 0.28–0.56 kg ae/ha under greenhouse and field conditions. Maize is normally highly susceptible to these herbicides. Although 2,4-D is used as a broadleaf herbicide with little or no activity on grasses, it can produce significant malformations of maize plants when applied at late seedling stages. This leads to restrictions on its use in maize to plants that are before the six-leaf stage or under 18 cm. Transgenic maize events showed resistance to 2,4-D application rates up to 4.48 kg ae/ha, with no 2,4-D-induced brace root malformations being observed. The gene and herbicide resistance was heritable over multiple generations of selfed lines (T₀ through T₄), converted inbred lines (BC₁, BC₂, and BC₃S₁), and test hybrid crosses (Table S2 and Fig. S7). Selected events showed excellent resistance to quizalofop at rates of up to 0.28 kg ae/ha when applied at the V6 stage in field trials. This is four to eight

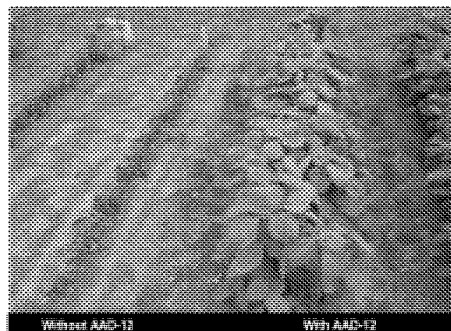


Fig. 3. Transgenic and nontransgenic soybean plants response to 2,4-D in the field.

Table 2. Inheritance of the AAD-1 maize and AAD-12 soybean

	AAD-1 maize event A T ₂ seeds	AAD-1 maize event B T ₂ seed	AAD-12 soybean event C F ₂ seed	AAD-12 soybean event D F ₂ seed	WT maize	WT soybean
Resistant	61	55	102	78	0	0
Sensitive	27	18	45	22	30	30
Totals	88	73	147	100	30	30
R (%)	69.3	75.3	69.4	78.0	0	0
S (%)	30.7	24.7	30.6	22.0	100	100
χ^2	0.2184	0.9461	2.4694	0.7866		
P value	>0.05	>0.05	>0.05	>0.05		
Ratio	3:1	3:1	3:1	3:1		

T₁ maize events were self-pollinated to produce T₂ seed; T₂ seeds were progeny-tested. Maize was sprayed with quizalofop and verified with either ELISA or Southern blot analysis. T₄ soybean events were crossed with elite soybean variety to produce F₁ seeds that were planted to produce F₂ seeds. Soybeans were sprayed with 2,4-D and verified with either ELISA or Southern blot analysis. WT, wild-type.

times the recommended field rate for control of volunteer maize, showing that the *aad-1* gene can serve as an excellent selectable marker in field situations (Fig. 4). Initial field studies with multiple selected maize events across multiple locations in the United States showed equivalent agronomic performance to conventional controls, including date to silking, date to pollination, date to maturity, lodging, phenotype, and yield. Transgenic plots were treated with 2,4-D applications, with a seasonal total of 3.36 kg ae/ha at preemergence (postplant) and approximately V4 and V8 growth stages and/or a quizalofop application at a rate of 0.092 kg ae/ha at approximately V6 growth stage. These data show that a single *aad-1* gene confers robust field resistance to two different herbicides with distinct modes of action. This is in contrast to other transgenic mechanisms of field-level herbicide resistance, such as glyphosate and glufosinate resistance, that confer resistance to a single herbicide chemistry.

Potential Impact of AAD-Derived Herbicide-Resistant Traits. 2,4-D is a synthetic auxin herbicide that kills the target weed by mimicking the natural plant growth hormone auxin [indole-3-acetic acid (IAA)] and binds to recently discovered IAA receptors in plants (17). It has been used effectively for over six decades to efficiently control a wide spectrum of broadleaf weeds (27). When applied to dicotyledonous plants at effective doses, 2,4-D causes uncontrolled and disorganized plant growth that leads to death (17). It can also have undesirable morphological effects on seedling grass species. Despite its widespread use, very few 2,4-D-resistant weed species have been identified (6). Of these few species, none have showed significant spread from initial sites of discovery, and none have significant economic importance (28). The lack of widespread development of 2,4-D-resistant weeds may be because of the genetic redundancy in auxin/2,4-D recep-

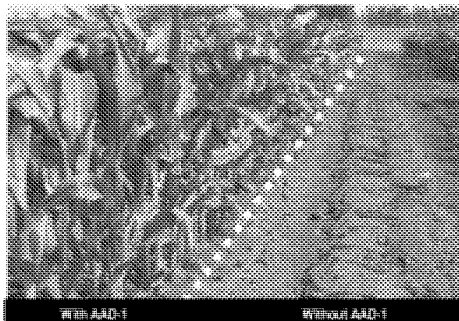


Fig. 4. Transgenic and nontransgenic maize plants response to quizalofop in the field.

tors, the essentiality of auxin perception for plant development, and/or the pleiotropic nature of the downstream auxin effects (29–31). These observations suggest that the frequency of 2,4-D-resistant weed appearance may be low.

Robust crop 2,4-D resistance can assist growers by increasing the flexibility of weed control practices at low cost. Moreover, it can help overcome or slow the development of GR weeds. A significant advantage provided by higher rates of 2,4-D is the potential for residual control of broadleaf weeds. Glyphosate is a nonresidual herbicide and does not provide control of later-germinating weeds. Resistance provided by AADs can also provide the ability to tank mix a broad-spectrum broadleaf weed control herbicide with commonly used residual weed control herbicides. These herbicides are typically applied before or at planting but often are less effective on emerged, established weeds that may exist in the field before planting. By extending the utility of aryloxyacetate auxin herbicides such as 2,4-D and fluroxypyr to include at-plant, preemergence, or preplant applications, the flexibility of residual weed control programs can be increased.

In grass crops such as maize, the AAD-1 gene confers robust resistance to grass-active AOPP herbicides, such as quizalofop and cyhalofop, that are otherwise highly injurious to these crops. AAD-1 simultaneously provides resistance to 2,4-D. The enhanced resistance of maize and other monocot crops to 2,4-D conferred by AAD-1 will enable use of these auxinic herbicides without the potential for known auxin-induced effects, such as crop leaning, inhibited leaf unfurling (rat-tailing), stalk brittleness, or deformed brace roots, that lead to the current growth-stage restrictions on 2,4-D application to grass crops.

In summary, the broad xenobiotic substrate selectivity of the AAD enzymes that we have characterized provides diverse mechanisms to incorporate additional established highly effective herbicide modes of action with the convenience of herbicide-resistant crops in an integrated weed-shift management strategy. When stacked with other herbicide resistance traits, for example, with the widely used GR traits, AAD-derived herbicide resistance will provide farmers with a practical tool to control current GR weeds, such as horseweed, as well as multiple pigweed and ragweed species and newly developing GR weeds. It also may slow weed-resistance development by diversifying herbicide selection pressures with additional modes of action and differing chemical structures. AAD-based herbicide-resistant traits will provide a tool to protect and sustain the value of current herbicides and herbicide-resistant crops.

Materials and Methods

Expression and Purification of AAD-1 and AAD-12 Enzymes. *Aad-1* and *aad-12* genes were cloned using standard PCR amplification and molecular cloning techniques. The primers were designed according to published DNA sequences to amplify the mature protein coding region (24, 25). The fragment encoding the full-length gene was then cloned in frame into a pET-based vector (pET280)

and transformed into *E. coli* BL21 (DE3) for protein expression. Typical induction conditions for the AAD-1 and AAD-12 proteins were with 75 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) in Luria Broth with appropriate antibiotics and cells grown overnight at 25 °C for optimal soluble protein expression. Cell paste was harvested by centrifugation, and cell lysate was prepared either by using sonication or microfluidization methods. The protein was purified from the soluble fraction using standard ion exchange and hydrophobic interaction chromatography separations followed by size exclusion chromatography. Typically, after three steps of column separation, the target protein had a purity of >99%. The homogeneity and enzymatic activity of the protein were further validated by HPLC and activity assays. Protein concentration was determined by total amino acid hydrolysis.

In Vitro Enzyme Assay. AAD enzymatic activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (32) in a 96-well microplate format. Assays were performed in a total volume of 0.15 mL 20 mM Mops (pH 6.75) containing 200 μ M NH_4FeSO_4 , 200 μ M sodium ascorbate, 1 mM α -ketoglutarate, and the appropriate substrate and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme, or α -ketoglutarate at time 0. After 15 min incubation at 25 °C, the reaction was terminated by addition of 30 μ L of a 1:1:1 mix of 50 mM Na EDTA, pH 10 buffer, and 0.2% 4-aminocoumarin; then, 10 μ L 0.8% potassium ferricyanide were added. After 15 min, the absorbance at 510 nm was recorded in a spectrophotometer. Blanks contained all reagents except for enzyme. Assays with pyridoxalacetate substrates were performed in the same way, except that release of the pyridinol was directly observed by the increase of absorbance at 325 nm or by a succinyl-CoA synthetase-coupled assay (33).

Gene Redesigns for Plant Expression. Both of the *aad-1* and *aad-12* genes (*RdpA* and *SdpA*) were identified from the NCBI database (<http://www.ncbi.nlm.nih.gov>) with accession numbers of AF516752 and AY327575, respectively. To improve production of the recombinant protein in plants, both AAD DNA sequences were codon-optimized for plant expression. The resulting synthetic gene sequences encoded identical protein sequences, except for the addition of an alanine residue in the second position resulting from the creation of an NcoI site (CCATGG) across the ATG start codon to enable subsequent cloning operations. The plant-optimized *aad-1* and *aad-12* genes were synthesized at Picoscript and confirmed by sequencing.

Constructs for testing in Arabidopsis. The *aad-1* gene was cloned into plasmid pDAB726 as an NcoI-SacI fragment. The resulting construct was designated pDAB720, containing AtUbi10/*aad-1*/NtOsm3'UTR/AtuORF1 3'UTR. The fragment containing the described cassette in pDAB720 was then cloned into the corresponding NotI site of the binary vector pDAB3038. The resulting binary vector, pDAB721 (Fig. 2), containing the following cassette (AtUbi10 promoter/*aad-1*/NtOsm3'UTR/AtuORF1 3'UTR/CsVMV promoter/*pat*/AtuORF25/26 3'UTR) was confirmed through restriction enzyme digestion and DNA sequencing.

The *aad-12* gene was cloned into plasmid pDAB726 as an NcoI-SacI fragment. The resulting construct was designated pDAB723, containing AtUbi10 promoter/*aad-12*/NtOsm3'UTR/AtuORF1 3'UTR. The fragment containing the described cassette in pDAB723 was then cloned into the NotI site of the binary vector pDAB3038. The resulting binary vector, pDAB724 (Fig. S1), containing the following cassette (AtUbi10 promoter/*aad-12*/NtOsm3'UTR/AtuORF1 3'UTR/CsVMV promoter/*pat*/AtuORF25/26 3'UTR) was confirmed through restriction enzyme digestion and DNA sequencing.

AAD constructs for soybean and maize. *Aad-12*-containing construct pDAB4468 for soybean transformation was similar to construct pDAB724 with some modification. The T-DNA insert encompassing the *aad-12* and *pat* expression cassettes consisted of RB7 MAR/AtUbi10 promoter/*aad-12*/AtuORF23 3'UTR/CsVMV promoter/*pat*/AtuORF1 3'UTR (Fig. S3). The resulting binary vector, pDAB4468, was confirmed by restriction enzyme digestion and DNA sequencing.

Aad-1-containing construct pDAS1740 was derived from pDAB721 by simply eliminating the PAT expression cassette. *Aad-1*-containing construct pDAS1740 was digested by restriction enzyme FseI flanking the *aad-1* expression cassette. The released fragment containing RB7 MAR/ZmUbi1 promoter/*aad-1*/ZmPer5 3'UTR/RB7 MAR was purified and used for silicon carbide fiber-mediated transformation of maize (Fig. S5).

Plant Transformation. Agrobacterium-mediated transformation of Arabidopsis and soybean. The genetically engineered *aad* gene cassettes in the binary vectors pDAB721 and pDAB724 were introduced into electrocompetent *A. tumefaciens* strain Z7075 using a protocol from Weigel and Glazebrook (34). The presence of the gene insert in selected *Agrobacterium* colonies was verified with PCR analysis using vector-specific primers. *Arabidopsis* was transformed using the floral dip method (35).

The disarmed *A. tumefaciens* strain EHA101 carrying the binary vector pDAB4468 was used for soybean transformation. Transformation of soybean varieties was carried out by following the cotyledonary node *Agrobacterium*-mediated transformation system (36). Briefly, Maverick soybean seeds were germinated on basal media, and cotyledonary nodes were isolated and infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin, and vancomycin for removal of *Agrobacterium*. Glufosinate selection was used to inhibit the growth of nontransformed shoots. Selected shoots were transferred to rooting medium for root development and then, transferred to soil mix for acclimatization of plantlets. Terminal leaflets of selected plantlets were leaf-painted with glufosinate to screen for putative transformants. Surviving plantlets were deemed to be putative transformants. The screened plants were sampled and analyzed by molecular approaches to confirm the insertion of selectable marker gene and/or the gene of interest.

Silicon carbide fiber-mediated transformation of maize. DNA fragment containing the genetically engineered *aad-1* gene cassette was released from plasmid pDAS1740 by FseI digestion and introduced into recipient maize line Hi-II through silicon carbide fiber (Whiskers)-mediated transformation (26). Immature embryos of maize were aseptically removed from the developing caryopsis, callused on semisolid media, initiated in liquid suspension cultures, cryopreserved, thawed, and reestablished as embryogenic suspensions. The reestablished suspensions were agitated with isolated pDAS1740/FspI DNA fragment and silicon carbide fibers to introduce the DNA into the cells. After 3 d of growth on nonselective, semisolid media, the cells were transferred to a medium containing the herbicide (*R*)-haloxyfop to select for cells expressing the *aad-1* gene. The callus that survived on the herbicide-containing medium proliferated and produced embryogenic tissue that was presumably genetically transformed. Callus samples were taken for molecular analysis to verify the presence of the transgene and the absence of the ampicillin resistance gene from the vector backbone, although they were not included in the DNA fragment used for transformation. The embryogenic tissue was then manipulated to regenerate whole transgenic plants and transferred to a greenhouse. The plants were sprayed with the AOPP herbicide quizalofop to confirm herbicide resistance. Surviving plants were crossed with proprietary inbred maize lines to obtain T₁ seed from the initially transformed T₀ plants.

Characterization of Herbicide Resistance in Transformed Plants. Freshly harvested transformed *Arabidopsis* T₁ seeds were planted, and the T₁ plants at cotyledon [5 d after planting (DAP)] and two to four leaf-growth stages (10 DAP) were sprayed with glufosinate at a rate of 0.28 kg active ingredient (ai)/ha per application. Survivors (plants actively growing) were identified 5–7 d after the final spraying and transplanted individually into 3-in pots filled with potting media. Surviving T₁ plants from glufosinate spray were confirmed for expression of the PAT protein using a PAT ELISA (EnviroLogix, Inc.). Plants were then treated with various rates of 2,4-D at 0.0125 kg ae/ha and elevated rates of 0.05, 0.2, 0.8, or 3.2 kg ae/ha. Similarly, AAD-1 maize plants were selected either in the greenhouse or field with quizalofop (0.14–0.56 kg ai/ha) and subsequently challenged with 2,4-D (0.56–4.48 kg ae/ha). AAD-12 transgenic soybean plants were selected with herbicide glufosinate (0.411 kg ai/ha) and subsequently challenged with 2,4-D (0.56–4.48 kg ae/ha) in the field.

Heritability of Genes in Transgenic Plants. Herbicide resistance. T₁ and T₂ *Arabidopsis* populations (from self-pollination of T₁ plants) were screened with glufosinate. Approximately 75% of the T₂ individuals survived selective rates of glufosinate. Survivors were sprayed with varying rates of 2,4-D (0.05–3.2 kg ae/ha) as previously noted. Plant survival and level of injury were rated at 7 and 14 d after treatment (DAT). The level of injury observed among T₂ siblings was consistent with that observed in the individuals in T₁ generation. For soybean events, up to generation T₃ and the F₂ generation after crossing with elite varieties were used to study transgene heritability. For maize events, self-pollinated inbred generations (up to T₄) and back-cross generations (BC₁, BC₂, and BC₃S₁) were studied for the AAD-1 gene heritability. The data gathered indicated that the resistance is transferable across all of the tested generations (Table S1 and Fig. S6).

Southern blot analysis. For DNA analysis, total genomic DNA was isolated from individual plant tissues using the cetyltrimethylammonium bromide (CTAB) method. A total of 9 μ g leaf genomic DNA or 10 μ g genomic callus DNA was subjected to an overnight digestion using selected restriction enzymes. DNA was then separated by gel electrophoresis and transferred to a nylon membrane as described by Memelink et al. (37). The hybridization probes were generated using a PCR-based incorporation of a digoxigenin (DIG)-labeled nucleotide, (DIG-11)-dUTP, to DNA fragments generated by primers

specific to the gene elements and other regions from target plasmid. The PCR synthesis of the probes was performed using PCR DIG Probe Synthesis Kit (Roche Diagnostics). Labeled probes were hybridized to the target DNA on the nylon membranes using the DIG Easy Hyb Solution (Roche Diagnostics). DIG-labeled DNA molecular weight marker II was used to determine the hybridizing fragment size on the Southern blots.

DIG-labeled probes bound to the nylon membranes were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System (Roche Diagnostics). Blots were exposed to chemiluminescent film for one or more time points to detect the hybridizing fragments and visualize the molecular weight standards.

Western blot analysis. For Western blot analysis, ~50–100 mg leaf tissue were extracted with 500 μ L plant extraction buffer (PBS containing 0.05% Tween 20 and 0.5% BSA) using the Geno/Grinder (Model 2000–115; Certiprep). Extracts were centrifuged, and the supernatant containing the soluble proteins was used for Western blots or ELISA. Plant extracts were incubated with Laemmli sample buffer at 95 °C for 10 min and electrophoretically separated in 8–16% Tris-Glycine Precast gel. Proteins were then electrotransferred onto nitrocellulose membrane using a standard protocol. After blocking in 4% skim milk in PBS, protein was detected by antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate (ECL Western Analysis Reagent; Amersham).

ELISA Analysis of Plant Tissues. AAD-1 ELISA. An AAD-1-specific monoclonal antibody (2 μ g/mL) was immobilized on microtiter wells to capture AAD-1 protein in plant extracts (described above). Various concentrations of plant extracts were incubated in the wells. To prepare a standard curve, different concentrations of purified AAD-1 proteins (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 ng/mL) were also put into the microtiter wells on the same assay plate in duplicates. The plate was incubated for 1 h and washed four times with PBS with 0.05% Tween 20 (PBST). Biotinylated anti-AAD-1 monoclonal antibody (1 μ g/mL) solution in PBST was added and incubated for 1 h to detect the bound AAD-1 protein. After another wash step, neutravidin-alkaline phosphatase (AP) conjugate (Pierce Biotech) was added to each well and incubated for another 1 h. pNPP (p-nitrophenyl phosphate) solution (AP substrate; 100 μ L) was added, and after the 30- to 45-min incubation, the absorbance at 405 nm was measured using a microtiter plate reader. The concentration of proteins in plant extracts was extrapolated from a standard curve developed on the same plate.

AAD-12 ELISA. Purified rabbit antibody (1 μ g/mL) was immobilized on microtiter wells to capture AAD-12 protein in plant extracts as described above for AAD-1. HRP-conjugated anti-AAD-12 monoclonal antibody (1 μ g/mL) solution in PBST was added and incubated for 1 h to detect the bound AAD-12 protein. After another wash step, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (100 μ L) was added, and after the 15-min incubation, 100 μ L stop solution (1 N HCl) was added to stop the reaction. The absorbance at 450 nm was measured using a microtiter plate reader.

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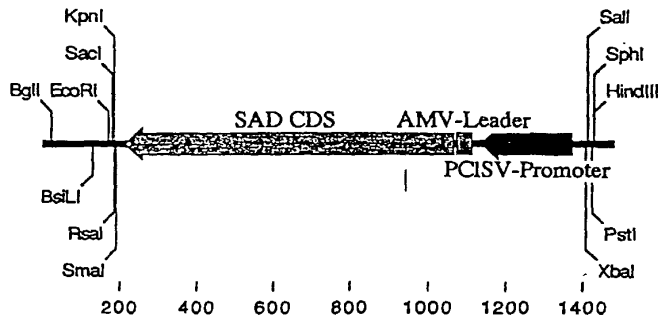
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: SYNTHETIC HERBICIDE RESISTANCE GENE



(57) Abstract: The invention provides a DNA molecule, a DNA construct, a transgenic plant and a transgenic plant part comprising a synthetic DNA sequence. The synthetic DNA sequence encodes an enzyme that degrades 2,4-dichlorophenoxyacetic acid (2,4-D) to dichlorophenol. The synthetic DNA sequence comprises a natural microbial sequence in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by a plant. The invention also provides a method of controlling weeds in a field containing transgenic plants according to the invention by applying an auxin herbicide, such as 2,4-D, to the field. The

invention further provides methods of selecting plants and plant cells that have been transformed with a DNA construct according to the invention using an auxin herbicide.

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SYNTHETIC HERBICIDE RESISTANCE GENE

FIELD OF THE INVENTION

The present invention relates to a synthetic herbicide-resistance gene, its use to prepare herbicide-resistant transgenic plants and its use as a selection marker.

BACKGROUND OF THE INVENTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is a herbicide used to control broadleaf weeds. 2,4-D is degraded by *Alcaligenes eutrophus* and other microorganisms. The gene which encodes the first enzyme in the *A. eutrophus* 2,4-D degradation pathway is *tfdA*. This gene encodes a dioxygenase which catalyzes the conversion of 2,4-D to 2,4-dichlorophenol (DCP). DCP is much less toxic to plants than 2,4-D, and transgenic tobacco plants, cotton plants, and hardwood trees containing the *tfdA* gene have been reported to have increased tolerance to 2,4-D. Streber et al., *Bio/Technology*, **7**, 811-816 (1989); Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989); Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992); Llewellyn and Last, in *Herbicide-Resistant Crops*, Chapter 10, pages 159-174 (Duke, ed., CRC Press (1996)); Last and Llewellyn, *Weed Science*, **47**, 401-404 (1999); U.S. Patents Nos. 6,153,401, 6,100,446, and 5,608,147; and PCT applications WO 98/38294 and WO 95/18862. However, transgenic plants resistant to levels of 2,4-D that might be encountered in agricultural situations have not been obtained. See Last and Llewellyn, *Weed Science*, **47**, 401-404 (1999). These authors suggest that codon optimization of the *tfdA* gene "might enhance tolerance levels." *Id.* at 404. The *tfdA* gene has also been used as a selection marker to identify transformed plants and plant cells. U.S. Patent No. 5,608,147; PCT application WO 95/18862.

SUMMARY OF THE INVENTION

The invention provides a DNA molecule comprising a synthetic DNA sequence. The synthetic DNA sequence encodes an enzyme that

degrades 2,4-dichlorophenoxyacetic acid to dichlorophenol. The synthetic DNA sequence comprises a natural microbial sequence that encodes the enzyme in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by a plant.

The invention also provides a DNA construct comprising the synthetic DNA sequence just described. In this construct, the synthetic DNA sequence is operatively linked to plant gene expression control sequences.

The invention further provides a transgenic plant or part of a plant. The transgenic plant or plant part comprises the synthetic DNA sequence operatively linked to plant gene expression control sequences.

The invention also provides a method of controlling weeds in a field containing transgenic plants according to the invention. The method comprises applying an amount of an auxin herbicide to the field effective to control the weeds in the field. The transgenic plants are tolerant to the auxin herbicide as a result of comprising and expressing the synthetic DNA sequence. Indeed, for the first time, transgenic plants have been produced which are tolerant to levels of auxin herbicides substantially greater than those normally used in agriculture for controlling weeds.

The invention further provides methods of selecting transformed plants and plant cells. The method of selecting transformed plant cells comprises providing a population of plant cells. At least some of the plant cells in the population are transformed with the DNA construct of the invention. Then, the resulting population of plant cells is grown in a culture medium containing an auxin herbicide at a concentration selected so that transformed plant cells proliferate and untransformed plant cells do not proliferate.

The method of selecting transformed plants comprises providing a population of plants suspected of comprising a transgenic plant according to the invention. Then, an auxin herbicide is applied to the population of plants, the amount of herbicide being selected so that transformed plants will grow and growth of untransformed plants will be inhibited.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Diagram of pProPClSV-SAD.

Figure 2: Diagram of pPZP211-PNPT-311g7.

Figure 3: Diagram of pPZP211-PNPT-512g7.

Figure 4: Diagram of pPZP211-PNPT-311-SAD.

Figure 5: Diagram of pPZP211-PNPT-512-SAD.

In these figures, SAD = 2,4-D-degrading synthetic gene adapted for dicots; CDS = coding sequence; AMV-Leader = 5' untranslated leader sequence from the 35S transcript of alfalfa mosaic virus; PClSV-Promoter = peanut chlorotic streak virus promoter; T-Left = T-DNA left border from *Agrobacterium tumefaciens* nopaline Ti plasmid pTiT37; 35SPolyA = 3' polyadenylation (polyA) termination signal sequence from the cauliflower mosaic virus (CaMV) 35S transcript; NPTII = neomycin phosphotransferase II; g7PolyA = 3' polyA termination signal from gene 7 within the T-Left border of an *A. tumefaciens* octopine plasmid; MCS = multiple cloning site; T-Right = T-DNA right border from *A. tumefaciens* Ti plasmid pTiT37.

DETAILED DESCRIPTION OF THE PRESENTLY-PREFERRED EMBODIMENTS OF THE INVENTION

The invention provides a synthetic DNA sequence. "Synthetic" is used herein to mean that the DNA sequence is not a naturally-occurring sequence.

The synthetic DNA sequence of the invention encodes an enzyme that degrades 2,4-dichlorophenoxyacetic acid (2,4-D) to dichlorophenol (DCP). The synthetic DNA sequence comprises a natural microbial

sequence that encodes the enzyme, in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by a plant.

A "natural microbial sequence" is the coding sequence of a naturally-occurring microbial gene that encodes an enzyme that can degrade 2,4-D to DCP. Thus, the "natural microbial sequence" may be the coding sequence of a cDNA or genomic clone isolated from a microorganism, may be a chemically-synthesized DNA molecule having the same coding sequence as that of such a clone, or may be a combination of such sequences.

Multi-enzyme pathways for 2,4-D degradation have been demonstrated in several genera of bacteria. See, e.g., Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989), and references cited therein. Strains of *Alcaligenes eutrophus* have been the most extensively studied of these bacteria. The first enzyme in the *A. eutrophus* degradation pathway converts 2,4-D to DCP. This enzyme, which is often referred to as a monooxygenase, but which is now known to be a dioxygenase (see Fukumori et al., *J. Bacteriol.*, **175**, 2083-2086 (1993)), is encoded by the *tfdA* gene. Thus, the natural microbial sequence may be the coding sequence of a cDNA or genomic clone encoding a *tfdA* dioxygenase. Such clones and their isolation are described in Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992), Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989), Streber et al., *J. Bacteriology*, **169**, 2950-2955 (1987), Perkins and Lurquin, *J. Bacteriology*, **170**, 5669-5672 (1988), and U.S. Patents Nos. 6,100,446 and 6,153,401. See also Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989).

It is known that many bacteria are capable of degrading 2,4-D, including strains of *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, *Flavobacterium*, *Pseudomona* and

strains of Actinomycetes (e.g., *Nocardia* spp. and *Streptomyces viridochromogenes*) (see, e.g., Llewellyn and Last, in *Herbicide-Resistant Crops*, Chapter 10 (Stephen O. Duke ed., CRC Press Inc. (1996)), Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992), Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989), and Streber et al., *J. Bacteriology*, **169**, 2950-2955 (1987), Loos, in *Degradation Of Herbicides*, pages 1-49 (Kearney and Kaufman, eds., Marcel Dekker, Inc., New York 1969), and references cited in these references), and additional strains of bacteria that degrade 2,4-D can be isolated by methods well known in the art (e.g., by isolation from soils where 2,4-D is used by the enrichment culture technique) (see, e.g., Loos, in *Degradation Of Herbicides*, pages 1-49 (Kearney and Kaufman, eds., Marcel Dekker, Inc., New York 1969)). Additional cDNA and genomic clones encoding an enzyme which converts 2,4-D to DCP can be obtained from these other bacteria in a similar manner as for the *tfdA* clones. See, e.g., Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992); Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989); Streber et al., *J. Bacteriology*, **169**, 2950-2955 (1987); Perkins and Lurquin, *J. Bacteriology*, **170**, 5669-5672 (1988); U.S. Patents Nos. 6,100,446 and 6,153,401. See also Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989). In addition, or alternatively, isolated clones, portions of them, or sequences from them, can be used as probes to identify and isolate additional clones. See, e.g., Perkins and Lurquin, *J. Bacteriology*, **170**, 5669-5672 (1988); Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992); U.S. Patents Nos. 6,100,446 and 6,153,401. See also Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989). The natural microbial sequence may be the coding sequence of one of these cDNA or genomic clones.

It is also known that yeasts and fungi are capable of degrading 2,4-D (see, e.g., Llewellyn and Last, in *Herbicide-Resistant Crops*, Chapter 10 (Stephen O. Duke ed., CRC Press Inc. (1996)); Han and New, *Soil Biol. Biochem.*, **26**, 1689-1695 (1994); Donnelly et al., *Applied And Environmental Microbiology*, **59**, 2642-2647 (1993); Loos, in *Degradation Of Herbicides*, pages 1-49 (Kearney and Kaufman, eds., Marcel Dekker, Inc., New York 1969), and references cited in these references), and additional strains of yeast and fungi that degrade 2,4-D can be obtained by methods well known in the art (e.g., by isolation from soils where 2,4-D is used by the enrichment culture technique) (see, e.g., Loos, in *Degradation Of Herbicides*, pages 1-49 (Kearney and Kaufman, eds., Marcel Dekker, Inc., New York 1969); Han and New, *Soil Biol. Biochem.*, **26**, 1689-1695 (1994)). Additional cDNA and genomic clones encoding an enzyme which converts 2,4-D to DCP can be obtained from yeast and fungi by methods well known in the art (see references cited above in the discussion of obtaining clones from bacteria), and the natural microbial sequence may be the coding sequence of one of these cDNA or genomic clones.

In addition, as noted above, the natural microbial sequence may be fully or partially chemically synthesized. To do so, a cDNA or genomic clone, obtained as described in the previous paragraphs, is sequenced by methods well known in the art. See, e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989). A synthetic DNA sequence comprising the coding sequence of the cDNA or genomic clone can be fully or partially chemically synthesized using methods well known in the art. See, e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989). For instance, DNA sequences may be synthesized by phosphoamidite chemistry in an automated DNA synthesizer. Also, the sequence of the *tfdA* gene from A.

eutrophus JMP134 is publically available (see Streber et al., *J. Bacteriology*, **169**, 2950-2955 (1987), U.S. Patents Nos. 6,100,446 and 6,153,401, and GenBank (accession number M16730)), and a synthetic DNA sequence comprising the coding sequence of the *A. eutrophus tfdA* gene can also be fully or partially chemically synthesized.

The preferred natural microbial sequence is a natural bacterial sequence. A "natural bacterial sequence" is the coding sequence of a naturally-occurring bacterial gene that encodes an enzyme that can degrade 2,4-D to DCP. Thus, the "natural bacterial sequence" may be the coding sequence of a cDNA or genomic clone isolated from a bacterium, may be a chemically-synthesized DNA molecule having the same coding sequence as that of such a clone, or may be a combination of such sequences. Most preferably the natural bacterial sequence is the coding sequence of a cDNA or genomic clone isolated from a strain of *A. eutrophus*, a chemically-synthesized DNA molecule having the same coding sequence as that of such a clone, or a combination of such sequences.

As noted above, at least a plurality of the codons of the natural microbial sequence will be replaced by codons more preferred by a plant (also referred to herein as "plant-preferred codons"). A "codon more preferred by a plant" or a "plant-preferred codon" is a codon which is used more frequently by a plant to encode a particular amino acid than is the microbial codon encoding that amino acid. Preferably, the plant-preferred codon is the codon used most frequently by the plant to encode the amino acid. The plant codon usage may be that of plants in general, a class of plants (e.g., dicotyledonous plants), a specific type of plant (e.g., cotton or soybeans), etc. The codon usage or preferences of a plant or plants can be deduced by methods known in the art. See, e.g., *Maximizing Gene Expression*, pages 225-85 (Reznikoff & Gold, eds., 1986), Perlak et al., *Proc. Natl. Acad. Sci. USA*, **88**,

3324-3328 (1991), PCT WO 97/31115, PCT WO 97/11086, EP 646643, EP 553494, and U.S. Patents Nos. 5,689,052, 5,567,862, 5,567,600, 5,552,299 and 5,017,692. For instance, the codons used by the plant or plants to encode all of the different amino acids in a selection of proteins expressed by the plant or plants, preferably those proteins which are highly expressed, are tabulated. This can be done manually or using software designed for this purpose (see PCT application WO 97/11086).

The use of codons more preferred by the plant in which the synthetic DNA sequence will be expressed will improve expression as compared to use of the natural microbial sequence. The published reports indicate that codon usage affects gene expression in plants at the level of mRNA stability and translational efficiency. See, e.g., Perlak et al., *Proc. Natl. Acad. Sci. USA*, **88**, 3324-3328 (1991); Adang et al., *Plant Molec. Biol.*, **21**:1131-1145 (1993); Sutton et al., *Transgenic Res.*, **1**:228-236 (1992). Not all of the codons of the natural microbial sequence need to be changed to plant-preferred codons in order to obtain improved expression. However, preferably at least the codons least preferred by the plant are changed to plant-preferred codons. "Codons least preferred by the plant" are those codons in the natural microbial sequence that are used least by the plant or plants in question to encode a particular amino acid. Preferably greater than about 50%, most preferably at least about 80%, of the microbial codons are changed to plant-preferred codons.

Plant-preferred codons can be introduced into the natural microbial sequence by methods well known in the art. For instance, site-directed mutagenesis can be used. See Perlak et al., *Proc. Natl. Acad. Sci. USA*, **88**, 3324-3328 (1991). See also Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989). However, the plant-

preferred codons are preferably introduced into the natural microbial sequence by chemically synthesizing the entire DNA sequence encoding the 2,4-D degrading enzyme. In particular, chemical synthesis is highly preferred when a large number of microbial codons are replaced by plant-preferred codons. In addition, chemical synthesis has a number of advantages. For instance, using chemical synthesis allows other changes to the sequence of the DNA molecule or its encoded protein to be made to, e.g., optimize expression (e.g., eliminate mRNA secondary structures that interfere with transcription or translation, eliminate undesired potential polyadenylation sequences, and alter the A+T and G+C content), add unique restriction sites at convenient points, delete protease cleavage sites, etc.

The synthetic DNA sequence having plant-preferred codons substituted for at least a plurality of microbial codons will encode the same amino acid sequence as the natural microbial sequence if these substitutions are the only differences in the sequence of the synthetic DNA sequence as compared to the natural microbial sequence. However, the synthetic DNA sequence may comprise additional changes as compared to the natural microbial sequence. For instance, the synthetic DNA sequence may encode an enzyme which degrades 2,4-D to DCP, but which has an altered amino acid sequence as compared to the enzyme encoded by the (unmutated) natural microbial sequence as a result of one or more substitutions, additions or deletions in the natural microbial sequence. Methods of making such substitutions, additions and deletions are well known in the art and are described above.

Assays for determining whether 2,4-D has been degraded to DCP are well known in the art. See, e.g., Streber et al., *J. Bacteriol.*, **169**, 2950-2955 (1987); Perkins et al., *J. Bacteriol.*, **170**, 5669-5672 (1988); Streber et al., *Bio/Technology*, **7**, 811-816 (1989); Lyon et al., *Plant Molec. Biol.*, **13**, 533-540 (1989); Bayley et al., *Theor. Appl. Genet.*, **83**, 645-649 (1992); Fukumori et al., *J.*

Bacteriol., **175**, 2083 (1993); Lyon et al., *Transgenic Res*, **2**, 162-169 (1993); Llewellyn and Last, in *Herbicide-Resistant Crops*, Chapter 10, pages 159-174 (Duke, ed., CRC Press (1996)); Last and Llewellyn, *Weed Science*, **47**, 401-404 (1999). Also, tolerance to 2,4-D and other auxin herbicides may be used to demonstrate this conversion. See below and references just cited.

The invention also provides DNA constructs comprising the synthetic DNA sequence operatively linked to plant gene expression control sequences. "DNA constructs" are defined herein to be constructed (non-naturally occurring) DNA molecules useful for introducing DNA into host cells, and the term includes chimeric genes, expression cassettes, and vectors.

As used herein "operatively linked" refers to the linking of DNA sequences (including the order of the sequences, the orientation of the sequences, and the relative spacing of the various sequences) in such a manner that the encoded protein is expressed. Methods of operatively linking expression control sequences to coding sequences are well known in the art. See, e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982), Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989).

"Expression control sequences" are DNA sequences involved in any way in the control of transcription or translation. Suitable expression control sequences and methods of making and using them are well known in the art.

The expression control sequences must include a promoter. The promoter may be any DNA sequence which shows transcriptional activity in the chosen plant cells, plant parts, or plants. The promoter may be inducible or constitutive. It may be naturally-occurring, may be composed of portions of various naturally-occurring promoters, or may be partially or totally synthetic.

Guidance for the design of promoters is provided by studies of promoter structure, such as that of Harley and Reynolds, *Nucleic Acids Res.*, **15**, 2343-61 (1987). Also, the location of the promoter relative to the transcription start may be optimized. See, e.g., Roberts, et al., *Proc. Natl Acad. Sci. USA*, **76**, 760-4 (1979). Many suitable promoters for use in plants are well known in the art.

For instance, suitable constitutive promoters for use in plants include: the promoters from plant viruses, such as the peanut chlorotic streak caulimovirus (PClSV) promoter (U.S. Patent No. 5,850,019), the 35S promoter from cauliflower mosaic virus (CaMV) (Odell et al., *Nature* **313**:810-812 (1985)), promoters of *Chlorella* virus methyltransferase genes (U.S. Patent No. 5,563,328), and the full-length transcript promoter from figwort mosaic virus (FMV) (U.S. Patent No. 5,378,619); the promoters from such genes as rice actin (McElroy et al., *Plant Cell* **2**:163-171 (1990)), ubiquitin (Christensen et al., *Plant Mol. Biol.* **12**:619-632 (1989) and Christensen et al., *Plant Mol. Biol.* **18**:675-689 (1992)), pEMU (Last et al., *Theor. Appl. Genet.* **81**:581-588 (1991)), MAS (Velten et al., *EMBO J.* **3**:2723-2730 (1984)), maize H3 histone (Lepetit et al., *Mol. Gen. Genet.* **231**:276-285 (1992) and Atanassova et al., *Plant Journal* **2**(3):291-300 (1992)), *Brassica napus* ALS3 (PCT application WO 97/41228); and promoters of various *Agrobacterium* genes (see U.S. Patents Nos. 4,771,002, 5,102,796, 5,182,200, 5,428,147).

Suitable inducible promoters for use in plants include: the promoter from the ACE1 system which responds to copper (Mett et al. *PNAS* **90**:4567-4571 (1993)); the promoter of the maize In2 gene which responds to benzenesulfonamide herbicide safeners (Hershey et al., *Mol. Gen. Genetics* **227**:229-237 (1991) and Gatz et al., *Mol. Gen. Genetics* **243**:32-38 (1994)), and the promoter of the Tet repressor from Tn10 (Gatz et al., *Mol. Gen. Genet.* **227**:229-237

(1991). A particularly preferred inducible promoter for use in plants is one that responds to an inducing agent to which plants do not normally respond. An exemplary inducible promoter of this type is the inducible promoter from a steroid hormone gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone (Schena et al., *Proc. Natl. Acad. Sci. USA* **88**:10421 (1991)) or the recent application of a chimeric transcription activator, XVE, for use in an estrogen receptor-based inducible plant expression system activated by estradiol (Zuo et al., *The Plant Journal*, **24**:265-273 (2000)). Other inducible promoters for use in plants are described in EP 332104, PCT WO 93/21334 and PCT WO 97/06269.

Finally, promoters composed of portions of other promoters and partially or totally synthetic promoters can be used. See, e.g., Ni et al., *Plant J.*, **7**:661-676 (1995) and PCT WO 95/14098 describing such promoters for use in plants.

The promoter may include, or be modified to include, one or more enhancer elements. Preferably, the promoter will include a plurality of enhancer elements. Promoters containing enhancer elements provide for higher levels of transcription as compared to promoters that do not include them. Suitable enhancer elements for use in plants include the PClSV enhancer element (U.S. Patent No. 5,850,019), the CaMV 35S enhancer element (U.S. Patents Nos. 5,106,739 and 5,164,316) and the FMV enhancer element (Maiti et al., *Transgenic Res.*, **6**, 143-156 (1997)). See also PCT WO 96/23898 and *Enhancers And Eukaryotic Expression* (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1983).

For efficient expression, the coding sequences are preferably also operatively linked to a 3' untranslated sequence. The 3' untranslated sequence will include a transcription termination sequence and a polyadenylation sequence. The 3' untranslated region can be obtained from the flanking regions of genes from

Agrobacterium, plant viruses, plants or other eukaryotes. Suitable 3' untranslated sequences for use in plants include those of the cauliflower mosaic virus 35S gene, the phaseolin seed storage protein gene, the pea ribulose biphosphate carboxylase small subunit E9 gene, the soybean 7S storage protein genes, the octopine synthase gene, and the nopaline synthase gene.

A 5' untranslated sequence is also employed. The 5' untranslated sequence is the portion of an mRNA which extends from the 5' CAP site to the translation initiation codon. This region of the mRNA is necessary for translation initiation in plants and plays a role in the regulation of gene expression. Suitable 5' untranslated regions for use in plants include those of alfalfa mosaic virus, cucumber mosaic virus coat protein gene, and tobacco mosaic virus.

As noted above, the DNA construct may be a vector. The vector may contain one or more replication systems which allow it to replicate in host cells. Self-replicating vectors include plasmids, cosmids and viral vectors. Alternatively, the vector may be an integrating vector which allows the integration into the host cell's chromosome of the synthetic DNA sequence encoding the 2,4-D-degrading enzyme. The vector desirably also has unique restriction sites for the insertion of DNA sequences. If a vector does not have unique restriction sites, it may be modified to introduce or eliminate restriction sites to make it more suitable for further manipulations.

The DNA constructs of the invention can be used to transform any type of plant cells (see below). A genetic marker must be used for selecting transformed plant cells ("a selection marker"). Selection markers typically allow transformed cells to be recovered by negative selection (*i.e.*, inhibiting growth of cells that do not contain the selection marker) or by screening for a product encoded by the selection marker.

The most commonly used selectable marker gene for plant transformation is the neomycin phosphotransferase II (*nptII*) gene, isolated from Tn5, which, when placed under the control of plant expression control signals, confers resistance to kanamycin. Fraley et al., *Proc. Natl. Acad. Sci. USA*, **80**:4803 (1983). Another commonly used selectable marker gene is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin. Vanden Elzen et al., *Plant Mol. Biol.*, **5**:299 (1985).

Additional selectable marker genes of bacterial origin that confer resistance to antibiotics include gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase, and the bleomycin resistance determinant. Hayford et al., *Plant Physiol.* **86**:1216 (1988), Jones et al., *Mol. Gen. Genet.* **210**:86 (1987), Svab et al., *Plant Mol. Biol.* **14**:197 (1990), Hille et al., *Plant Mol. Biol.* **7**:171 (1986). Other selectable marker genes confer resistance to herbicides such as glyphosate, glufosinate or bromoxynil. Comai et al., *Nature* **317**:741-744 (1985), Stalker et al., *Science* **242**:419-423 (1988), Hinchee et al., *Bio/Technology* **6**:915-922 (1988), Stalker et al., *J. Biol. Chem.* **263**:6310-6314 (1988), and Gordon-Kamm et al., *Plant Cell* **2**:603-618 (1990).

Other selectable marker genes for plant transformation are not of bacterial origin. These genes include, for example, mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase, and plant acetolactate synthase. Eichholtz et al., *Somatic Cell Mol. Genet.* **13**:67 (1987), Shah et al., *Science* **233**:478 (1986), Charest et al., *Plant Cell Rep.* **8**:643 (1990), EP 154,204.

Commonly used genes for screening presumptively transformed cells include β -glucuronidase (GUS), β -galactosidase, luciferase, and chloramphenicol acetyltransferase. Jefferson, R.A., *Plant Mol.*

Biol. Rep. **5**:387 (1987)., Teeri et al., *EMBO J.* **8**:343 (1989), Koncz et al., *Proc. Natl. Acad. Sci. USA* **84**:131 (1987), De Block et al., *EMBO J.* **3**:1681 (1984), green fluorescent protein (GFP) (Chalfie et al., *Science* **263**:802 (1994), Haseloff et al., *TIG* **11**:328-329 (1995) and PCT application WO 97/41228). Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig et al., *Science* **247**:449 (1990).

According to another aspect of the present invention, tolerance to an auxin herbicide can be used as a selection marker for plants and plant cells. "Auxin herbicide" is used herein to refer to phenoxy auxins (phenoxy herbicides), which include 2,4-D, 4-chlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxybutyric acid, 4-(2-methyl-4-chlorophenoxy)butyric acid, 2-(4-chlorophenoxy)propionic acid, 2-(2,4-dichlorophenoxy)propionic acid, 2-(2,4,5-trichlorophenoxy)propionic acid, and salts (including amine salts) and esters of these acids. Auxin herbicides are available commercially. See *Crop Protection Reference* (Chemical & Pharmaceutical Press, Inc., New York, NY, 11th ed., 1995). The preferred auxin herbicides are 2,4-D and its salts (including amine salts) and esters. "Tolerance" means that transformed plant cells are able to grow (survive, proliferate and regenerate into plants) when placed in culture medium containing a level of an auxin herbicide that prevents untransformed cells from doing so. "Tolerance" also means that transformed plants are able to grow after application of an amount of an auxin herbicide that inhibits the growth of untransformed plants.

Methods of selecting transformed plant cells are well known in the art. Briefly, at least some of the plant cells in a population of plant cells (e.g., an explant or an embryonic suspension culture) are transformed with a DNA construct comprising the

synthetic DNA sequence of the invention. The resulting population of plant cells is placed in culture medium containing an auxin herbicide at a concentration selected so that transformed plant cells will grow, whereas untransformed plant cells will not. Suitable concentrations of an auxin herbicide can be determined empirically as is known in the art. At least in the case of 2,4-D, this amount may further need to be an amount which inhibits adventitious shoot formation from untransformed plant cells and allows adventitious shoot formation from transformed plant cells, since this is apparently the case with the natural-occurring bacterial *tfdA* gene. See U.S. Patent No. 5,608,147 and PCT application WO 95/18862. In general, 2,4-D should be present in an amount ranging from about 0.001 mg/l to about 5 mg/l culture medium, preferably from about 0.01 mg/l to 0.2 mg/l culture medium.

Methods of selecting transformed plants are also known in the art. Briefly, an auxin herbicide is applied to a population of plants which may comprise one or more transgenic plants comprising a DNA construct of the invention providing for 2,4-D degradation. The amount of the auxin herbicide is selected so that transformed plants will grow, and the growth of untransformed plants will be inhibited. The level of inhibition must be sufficient so that transformed and untransformed plants can be readily distinguished (*i.e.*, inhibition must be statistically significant). Such amounts can be determined empirically as is known in the art. See also *Crop Protection Reference* (Chemical & Pharmaceutical Press, Inc., New York, NY, 11th ed., 1995).

Selection based on tolerance to an auxin herbicide can be used in the production of plants tolerant to 2,4-D and other auxin herbicides, in which case the use of another selection marker may not be necessary. Absence of a separate selection marker is advantageous since it minimizes the number of foreign genes expressed.

Selection based on tolerance to an auxin herbicide can also be used in the production of transgenic plants that express other genes of interest. Many such genes are known and include genes coding for proteins of commercial value and genes that confer improved agronomic traits on plants (see, e.g., PCT WO 97/41228, the complete disclosure of which is incorporated herein by reference).

The DNA constructs of the invention can be used to transform a variety of plant cells. The synthetic DNA sequence coding for the 2,4-D-degrading enzyme and the selection marker, if a separate selection marker is used, may be on the same or different DNA constructs. Preferably, they are arranged on a single DNA construct as a transcription unit so that all of the coding sequences are expressed together. Also, the gene(s) of interest and the synthetic DNA sequence coding for the 2,4-D-degrading enzyme, when tolerance to an auxin herbicide is being used as a selection marker, may be on the same or different DNA constructs. Such constructs are prepared in the same manner as described above.

Suitable host cells include plant cells of any kind (see below). Preferably, the plant cell is one that does not normally degrade auxin herbicides. However, the present invention can also be used to increase the level of degradation of auxin herbicides in plants that normally degrade such herbicides.

Thus, the "transgenic" plants, plant parts, and plant cells of the invention include plants, plant parts and plant cells that do not normally degrade auxin herbicides, but which have been transformed according to the invention so that they are able to degrade these herbicides, and progeny of such transformed plants, plant parts and plant cells. The "transgenic" plants, plant parts and plant cells of the invention also include plants, plant parts and plant

cells that normally degrade auxin herbicides, but which have been transformed according to the invention so that they are able to degrade more of these herbicides or to degrade them more efficiently, and progeny of such transformed plants, plant parts and plant cells.

"Plant" should be understood as referring to a unicellular organism or a multicellular differentiated organism capable of photosynthesis, including algae, angiosperms (monocots and dicots), gymnosperms, bryophytes, ferns and fern allies. "Plant parts" are parts of multicellular differentiated plants and include seeds, pollen, embryos, flowers, fruits, shoots, leaves, roots, stems, explants, etc. "Plant cell" should be understood as referring to the structural and physiological unit of multicellular plants. Thus, the term "plant cell" refers to any cell that is a plant or is part of, or derived from, a plant. Some examples of cells encompassed by the present invention include differentiated cells that are part of a living plant, differentiated cells in culture, undifferentiated cells in culture, and the cells of undifferentiated tissue such as callus or tumors.

Methods of transforming plant cells are well known in the art. For instance, numerous methods for plant transformation have been developed, including biological and physical transformation protocols. See, for example, Miki et al., "Procedures for Introducing Foreign DNA into Plants" in *Methods in Plant Molecular Biology and Biotechnology*, Glick, B.R. and Thompson, J.E. Eds. (CRC Press, Inc., Boca Raton, 1993) pp. 67-88. In addition, vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are available. See, for example, Gruber et al., "Vectors for Plant Transformation" in *Methods in Plant Molecular Biology and Biotechnology*, Glick, B.R.

and Thompson, J.E. Eds. (CRC Press, Inc., Boca Raton, 1993) pp. 89-119.

The most widely utilized mechanism for introducing an expression vector into plants is based on the natural transformation systems of *Agrobacterium*. *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria which genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of the plant. See, for example, Kado, C.I., *Crit. Rev. Plant. Sci.* **10**:1 (1991). Descriptions of *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are provided by numerous references. See, for example, Horsch et al., *Science* **227**:1229 (1985), Hoekema et al., *Nature* **303**:179 (1983), de Framond et al., *Bio/Technology* **1**:262 (1983), Jordan et al., *Plant Cell Reports* **7**:281-284 (1988), Leple et al., *Plant Cell Reports* **11**:137-141 (1992), Stomp et al., *Plant Physiol.* **92**:1226-1232 (1990), Knauf et al., *Plasmid* **8**:45-54 (1982)), Gruber et al., supra, Miki et al., supra, Moloney et al., *Plant Cell Reports* **8**:238 (1989), PCT applications WO84/02913, WO84/02919 and WO84/02920, EP 116,718, and U.S. Patents Nos. 4,940,838, 5,464,763, and 5,929,300.

A generally applicable method of plant transformation is microprojectile-mediated transformation wherein DNA is carried on the surface of microprojectiles. The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds sufficient to penetrate plant cell walls and membranes. Sanford et al., *Part. Sci. Technol.* **5**:27 (1987), Sanford, J.C., *Trends Biotech.* **6**:299 (1988), Sanford, J.C., *Physiol. Plant* **79**:206 (1990), Klein et al., *Biotechnology* **10**:268 (1992), Klein et al., *Nature* **327**:70-73 (1987).

Another method for physical delivery of DNA to plants is sonication of target cells. Zhang et al., *Bio/Technology* **9**:996 (1991). Alternatively, liposome or spheroplast fusion have been used to introduce expression vectors into plants. Deshayes et al., *EMBO J.*, **4**:2731 (1985), Christou et al., *Proc Natl. Acad. Sci. USA* **84**:3962 (1987). Direct uptake of DNA into protoplasts using CaCl_2 precipitation, polyvinyl alcohol or poly-L-ornithine have also been reported. Hain et al., *Mol. Gen. Genet.* **199**:161 (1985) and Draper et al., *Plant Cell Physiol.* **23**:451 (1982). Electroporation of protoplasts and whole cells and tissues have also been described. Donn et al., In Abstracts of VIIth International Congress on Plant Cell and Tissue Culture IAPTC, A2-38, p. 53 (1990); D'Halluin et al., *Plant Cell* **4**:1495-1505 (1992), Spencer et al., *Plant Mol. Biol.* **24**:51-61 (1994), and Fromm et al., *Proc. Natl. Acad. Sci. USA* **82**:5824 (1985). Other techniques include microinjection (Crossway, *Mol. Gen. Genetics*, **202**:179-185 (1985)), polyethylene glycol transformation (Krens et al., *Nature* **296**:72-74 (1982)), fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies (Fraley et al., *Proc. Natl. Acad. Sci. USA* **79**:1859-1863 (1982)), and techniques set forth in U.S. Pat. No. 5,231,019).

After selection, transformed plant cells are regenerated into transgenic plants. Plant regeneration techniques are well known in the art and include those set forth in the *Handbook of Plant Cell Culture*, Volumes 1-3, Evans et al., eds. Macmillan Publishing Co., New York, N.Y. (1983, 1984, 1984, respectively); Predieri and Malavasi, *Plant Cell, Tissue, and Organ Culture* **17**:133-142 (1989); James, D. J., et al., *J. Plant Physiol.* **132**:148-154 (1988); Fasolo, F., et al., *Plant Cell, Tissue, and Organ Culture* **16**:75-87 (1989); Valobra and James, *Plant Cell, Tissue, and Organ Culture* **21**:51-54 (1990); Srivastava, P.S., et al., *Plant Science* **42**:209-214 (1985); Rowland and Ogden, *Hort. Science* **27**:1127-1129 (1992);

Park and Son, *Plant Cell, Tissue, and Organ Culture* 15:95-105 (1988); Noh and Minocha, *Plant Cell Reports* 5:464-467 (1986); Brand and Lineberger, *Plant Science* 57:173-179 (1988); Bozhkov, P.V. et al., *Plant Cell Reports* 11:386-389 (1992); Kvaalen and von Arnold, *Plant Cell, Tissue, and Organ Culture* 27:49-57 (1991); Tremblay and Tremblay, *Plant Cell Tissue, and Organ Culture* 27:95-103 (1991); Gupta and Pullman, U.S. Pat. No. 5,036,007; Michler and Bauer, *Plant Science* 77:111-118 (1991); Wetzstein, H.Y., et al., *Plant Science* 64:193-201 (1989); McGranahan, G.H., et al., *Bio/Technology* 6:800-804 (1988); Gingas, V.M., *Hort. Science* 26:1217-1218 (1991); Chalupa, V., *Plant Cell Reports* 9:398-401 (1990); Gingas and Lineberger, *Plant Cell, Tissue, and Organ Culture* 17:191-203 (1989); Bureno, M.A., et al., *Phys. Plant.* 85:30-34 (1992); and Roberts, D.R., et al., *Can. J. Bot.* 68:1086-1090 (1990).

Transgenic plants of any type may be produced according to the invention. Such plants include, for example, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Ceranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Sencia*, *Salpiglossis*, *Cucumis*, *Browalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, *Malus*, *Apium*, *Datura* and woody dicotyledonous forest tree species. In particular, broadleaf plants (including beans, soybeans, cotton, peas, potatoes, sunflowers, tomatoes, tobacco, fruit trees, ornamental plants and trees) that are currently known to be injured by auxin herbicides can be transformed so that they become tolerant to these herbicides. Other plants (such as corn, sorghum, small grains, sugarcane, asparagus, and grass) which are

currently considered tolerant to auxin herbicides can be transformed to increase their tolerance to these herbicides.

In yet another embodiment, the invention provides a method of controlling weeds in a field where transgenic plants are growing. The method comprises applying an effective amount of an auxin herbicide to the field to control the weeds. Methods of applying auxin herbicides and the amounts of them effective to control various types of weeds are known. See *Crop Protection Reference* (Chemical & Pharmaceutical Press, Inc., New York, NY, 11th ed., 1995). For the first time, as a result of the present invention, transgenic plants have been produced which are tolerant to levels of auxin herbicides substantially greater than those normally used in agriculture for controlling weeds.

EXAMPLES

EXAMPLE 1: Generation Of Synthetic Plant-Optimized Sequence Encoding A 2,4-D Dioxygenase

The DNA sequence of a 2,4-D dioxygenase (also often referred to as a monooxygenase; see above) gene isolated from *Alcaligenes eutrophus* was obtained from the sequence database GenBank (accession number M16730). From this DNA sequence, the amino acid sequence of the protein coded for by the single open-reading frame (ORF) was determined [SEQ ID NO:1]. A codon usage table reflecting dicotyledonous ORFs was derived from a composite selection of random cDNA sequences from cotton, Arabidopsis and tobacco extracted from the GenBank database. A codon usage table reflecting monocotyledonous ORFs was derived from a random selection of cDNA sequences from maize, also extracted from the GenBank database. These are Tables 1 and 2 below. Using these plant-specific codon usage tables, the derived primary amino acid sequence of the bacterial 2,4-D dioxygenase was converted into DNA coding sequences that reflected the codon preferences of dicotyledonous and monocotyledonous plants [SEQ ID NOS:2 and 3, respectively].

The synthetic plant-optimized 2,4-D dioxygenase ORFs [SEQ ID NOS:2 and 3], both dicot and monocot, were then used to design 2,4-D dioxygenase genes capable of efficient expression in transgenic plants. These synthetic genes were designated as SAD (Synthetic gene Adapted for Dicots) and SAM (Synthetic gene Adapted for Monocots) for the dicot and monocot versions, respectively. In order to generate a translatable transcript once the gene had been constructed and inserted into a plant genome, a 5' untranslated leader sequence representing the 5' untranslated leader sequence from the 35S transcript of alfalfa mosaic virus (AMV; Gallie et al., Nucleic Acids Res., 15:8693-8711 (1987)) was incorporated into the design of the synthetic genes. In addition, a signature sequence, encoding Cys Ala Gly, was added to the 3' end of the synthetic coding regions for each version of the synthetic gene. Finally, for ease of cloning, the designed sequences included a HindIII-specific overhang at the 5' end and a SalI-specific overhang at the 3' end. The complete designed sequences for the synthetic portions of the SAD and SAM genes are SEQ ID NOS:4 and 5.

To construct the designed synthetic portions of the SAD and SAM genes, each sequence was dissected into overlapping oligonucleotides, twelve oligonucleotides for each of the two strands resulting in a total of twenty-four oligonucleotides for each DNA sequence. A complete list of the oligonucleotides used to construct the synthetic portions of the SAD and SAM genes is given in Tables 3A, 3B, 4A, and 4B below. The oligonucleotides were synthesized using standard phosphoramidite chemistry by Integrated DNA Technologies, Inc., Coralville, Iowa. The synthetic DNA molecules were assembled using a procedure based upon the protocol described by Sutton et al. 1995 published on the World Wide Web (www.epicentre.com) using Ampliligase™ thermostable ligase (Epicentre Technologies Inc., Madison, WI). Oligonucleotides were first phosphorylated using T4 polynucleotide

kinase (Invitrogen Life Technologies, Carlsbad, CA) as mixtures of upper and lower strand oligonucleotides for each synthetic DNA construct. Each mixture contained 10 pmoles of each oligonucleotide, 70 mM Tris/HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.1 mM ATP, and 10 units of T4 polynucleotide kinase, for a total volume of 25 µl. Phosphorylation was achieved by incubation of the mixtures at 37°C for 30 minutes, followed by a denaturing incubation at 70°C for 10 minutes. To anneal and ligate the oligonucleotides, each reaction mixture was retreated at 70°C for 10 minutes in a thermocycler and subsequently cooled to 65°C over a 10-minute period. To each mixture, 65 µl of water, 10 µl of 10X Ampliligase buffer (Epicentre Technologies), and 2 µl of Ampliligase (5 units/µl) were added sequentially, and the temperature was reduced to 40°C over a three hour period.

At this stage, in order to improve the efficiency of cloning, the complete synthetic DNA sequences for SAD and SAM were recovered from their respective annealing/ligation reactions by polymerase chain reaction (PCR) in an MJ Research Inc. (Waltham, MA) Model PTC-100 Thermocycler using Amplitaq Gold™ DNA polymerase under conditions supplied by the manufacturer, Perkin Elmer Life Sciences (Boston, MA). The PCR primers used for the recovery of each sequence were AGATCCTTTTATTTTAATTTCTTTC [SEQ ID NO:6], a 28mer representing the 5' end of the AMV leader sequence and used for both the SAD and SAM recovery PCR reactions, and CTCCAGCACACTAAACAACAGCGTC [SEQ ID NO:7], a 25mer specific for the 3' end of the SAD sequence, and CTCCAGCACACTACACCACC [SEQ ID NO:8], a 20mer specific for the 3' end of the SAM sequence. PCR fragments corresponding to the appropriate size of 918 bp were gel purified as described in Ausubel et al., *Current Protocols In Molecular Biology* (Green/Wiley Interscience, New York, 1989) and cloned between two XcmI restriction sites in pUCR19, a modified pUC19 vector designed for rapid cloning of PCR fragments using T overhangs generated by XcmI digestion (described in O'Mahony and

Oliver, *Plant Molecular Biology*, 39:809-821 (1999)) to generate the plasmids pUCRs_{syn}SAD and pUCRs_{syn}SAM. Once cloned into these vectors, the inserts were sequenced to verify the sequence integrity of the designed synthetic portions of the SAD and SAM genes. DNA sequencing was performed by use of a dRhodamine Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence reactions were analyzed using a Perkin Elmer/ABI Prism 310 automated sequencer.

TABLE 1: Dicot Codon Usage

Amino Acid	Codon	% Usage	Amino Acid	Codon	% Usage
Alanine	GCU	45.10	Lysine	AAG	55.14
	GCA	25.41		AAA	44.86
	GCC	17.62	Methionine	AUG	100.00
	GCG	11.87	Phenylalanine	UUC	51.60
Arginine	AGA	32.71		UUU	48.40
	AGG	23.18	Proline	CCU	37.73
	CGU	18.53		CCA	34.72
	CGA	10.55		CCG	14.94
	CGG	7.70		CCC	12.61
	CGC	7.33	Serine	UCU	27.64
Asparagine	AAC	51.76		UCA	19.32
	AAU	48.24		AGU	15.84
Aspartic Acid	GAU	65.56		AGC	13.98
	GAC	34.44		UCC	13.50
Cysteine	UGU	56.16		UCG	9.72
	UGC	43.84	Threonine	ACU	36.71
Glutamic Acid	GAG	51.01		ACA	28.49
	GAA	48.99		ACC	21.89
Glutamine	CAA	54.26		ACG	12.91
	CAG	45.74	Tryptophan	UGG	100.00
Glycine	GGA	36.34	Tyrosine	UAC	52.04
	GGU	35.49		UAU	47.96
	GGC	14.14	Valine	GUU	40.71
	GGG	14.03		GUG	26.01
Histidine	CAU	57.39		GUC	20.56
	CAC	42.61		GUA	12.71
Isoleucine	AUU	43.44	Stop	UAA	41.50
	AUC	36.28		UGA	40.73
	AUA	20.28		UAG	17.77
Leucine	UUG	24.73			
	CUC	19.86			
	CUU	19.86			
	UUA	12.99			
	CAG	11.69			
	CUA	10.87			

TABLE 2: Monocot Codon Usage

Amino Acid	Codon	% Usage	Amino Acid	Codon	% Usage
Alanine	GCC	36.21	Lysine	AAG	79.42
	GCG	24.24		AAA	20.58
	GCU	23.10	Methionine	AUG	100.00
	GCA	16.45			
Arginine	AGG	27.35		UUU	29.32
	CGC	27.09	Proline	CCA	27.77
	CGG	15.18		CCG	27.06
	AGA	12.94		CCC	23.97
	CGU	11.41		CCU	21.20
	CGA	6.03	Serine	AGC	24.00
Asparagine	AAC	68.33		UCC	23.41
	AAU	31.67		UCU	15.47
Aspartic Acid	GAC	62.72		UCG	13.97
	GAU	37.28		UCA	13.59
Cysteine	UGC	73.56		AGU	9.57
	UGU	26.44	Threonine	ACC	40.33
Glutamic Acid	GAG	73.29		ACU	21.27
	GAA	26.71		ACG	20.39
Glutamine	CAG	58.31		ACA	18.00
	CAA	41.69	Tryptophan	UGG	100.00
Glycine	GGC	41.40	Tyrosine	UAC	72.14
	GGG	20.28		UAU	27.86
	GGU	20.25	Valine	GUG	37.35
	GGA	18.07		GUC	32.71
Histidine	CAC	63.83		GUU	22.11
	CAU	36.17		GUA	7.83
Isoleucine	AUC	57.82	Stop	UGA	44.57
	AUU	28.52		UAG	28.24
	AUA	136.5		UAA	27.18
Leucine	CUC	27.01			
	CUU	27.01			
	CUG	23.80			
	UUG	11.90			
	CUA	6.23			
	UUA	4.05			

TABLE 3A
Dicot Oligonucleotides: Sense Strand

AGCTAGATCCTTTTTATTTTAAATTTCTTTCAAATACTTCCAG	[SEQ ID NO:13]
ATCCATGTCTGTTGTTGCTAACCCTTTGCATCCTTTGTTTCGCTGCTGGAG TTGAGGATATTGATCTCAGAGAAGCATTGG	[SEQ ID NO:14]
GTTCTACTGAGGTGAGAGAAATTGAGAGACTCATGGACGAAAAGTCAGTT CTCGTTTTTCAGAGGTCAACCACTCTCACAG	[SEQ ID NO:15]
GATCAACAGATTGCTTTTGCTAGGAATTTTGACCTTTGGAGGGTGGATT CATCAAAGTGAACCAGAGACCATCTAGGTT	[SEQ ID NO:16]
CAAATATGCTGAACTCGCTGATATCTCTAATGTTTCATTGGATGGTAAGG TGGCACAAGAGACGCTAGAGAAGTTGTGG	[SEQ ID NO:17]
GAAATTTTGCAAATCAATTGTGGCATTCTGATTCTTCATTCCAACAGCCA GCAGCTAGATATTCTATGTTGTCAGCTGTT	[SEQ ID NO:18]
GTTGTGCCTCCTTCTGGAGGTGATACAGAATTTTGTGATATGAGGGCAGC TTACGATGCTCTCCCAAGGGATTTGCAGTC	[SEQ ID NO:19]
TGAACTCGAGGGATTGAGAGCTGAACATTACGCTTTGAACTCAAGATTTTC TCTTGGGAGATACTGATTACTCAGAGGCAC	[SEQ ID NO:20]
AGAGAAACGCTATGCCTCCTGTTAACTGGCCTCTCGTTAGGACTCATGCT GGTTCTGGTAGAAAGTTCTTGTATTGGA	[SEQ ID NO:21]
GCACATGCTTCACATGTTGAGGGTCTCCCTGTTGCTGAGGGAAGAATGTT GCTCGCTGAATTGCTCGAACATGCTACTCA	[SEQ ID NO:22]
AAGAGAGTTTGTATTATAGACACAGATGGAATGTTGGTGACTTGGTTATGT GGGATAATAGATGTGTGTTGCATAGAGGTA	[SEQ ID NO:23]
GGAGATATGATATTTCTGCTAGAAGGGAACCTCAGAAGGGCTACTACTTTG GATGACGCTGTTGTTTAGTGTGCTGGAG	[SEQ ID NO:24]

TABLE 3B
Dicot Oligonucleotides: Nonsense Strand

GAACAAAGGATGCAAAGGGTTAGCAACAACAGACATGGATCTGGAAGTAT TTGAAAGAAAATTAAAAATAAAAAGGATCT	[SEQ ID NO:25]
TCGTCCATGAGTCTCTCAATTTCTCTCACCTCAGTAGAACCCAATGCTTC TCTGAGATCAATATCCTCAACTCCAGCAGC	[SEQ ID NO:26]
CCAAAGGTCCAAAATTCCTAGCAAAAGCAATCTGTTGATCCTGTGAGAGT GGTTGACCTCTGAAAACGAGAACTGACTTT	[SEQ ID NO:27]
CAATGAAACATTAGAGATATCAGCGAGTTCAGCATATTTGAACCTAGATG GTCTCTGGTTCACTTTGATGAATCCACCCT	[SEQ ID NO:28]
AATGAAGAATCAGAATGCCACAATTGATTTGCAAAATTTCCCACAACCTTC TCTAGCGTCTCTTTGTGCCACCTTACCATC	[SEQ ID NO:29]
TATCACAAAATTCGTATCACCTCCAGAAGGAGGCACAACAACAGCTGAC AACATAGAATATCTAGCTGCTGGCTGTTGG	[SEQ ID NO:30]
GTTCAAAGCGTAATGTTTCAGCTCTCAATCCCTCGAGTTCAGACTGCAAAT CCCTTGGGAGAGCATCGTAAGCTGCCCTCA	[SEQ ID NO:31]
CTAACGAGAGGCCAGTTAACAGGAGGCATAGCGTTTCTCTGTGCCTCTGA GTAATCAGTATCTCCCAAGAGAAATCTTGA	[SEQ ID NO:32]
CCTCAGCAACAGGGAGACCCTCAACATGTGAAGCATGTGCTCCAATAAAC AAGAACTTTCTACCAGAACCAGCATGAGTC	[SEQ ID NO:33]
GTCACCAACATTCCATCTGTGTCTATAAACAAACTCTCTTTGAGTAGCAT GTTTCGAGCAATTCAGCGAGCAACATTCTTC	[SEQ ID NO:34]
GCCCTTCTGAGTTCCCTTCTAGCAGAAATATCATATCTCCTACCTCTATG CAACACACATCTATTATCCACATAACCAA	[SEQ ID NO:35]
TCGACTCCAGCACACTAAACAACAGCGTCATCCAAAGTAGTA	[SEQ ID NO:36]

TABLE 4A
Monocot oligonucleotides: Sense strand

AGCTAGATCCTTTTTATTTTAAATTTTCTTTCAAATACTTCCAG	[SEQ ID NO:37]
ATCCATGTCCGTGGTGGCCAACCCACTCCACCCGCTCTTCGCGGCCGGCG TGGAGGATATCGACCTCAGGGAGGCGCTGG	[SEQ ID NO:38]
GCAGCACCGAAGTGCGCGAAATCGAGAGGCTCATGGACGAGAAGAGCGTC CTCGTCTTCCGCGGCCAACCACCTCTCACAG	[SEQ ID NO:39]
GATCAACAGATTGCTTTTGCTAGGAATTTTGACCTTTGGAGGGTGGATT CATCAAGGTGAACCAGCGCCCGTCCAGGTT	[SEQ ID NO:40]
CAAGTACGCTGAACTGGCCGACATCAGCAACGTGTCCCTCGATGGGAAGG TGGCCCAGAGGGACGCTAGGGAAGTTGTGG	[SEQ ID NO:41]
GCAACTTCGCCAACCAACTGTGGCACTCCGATAGCTCTTTCCAACAGCCA GCAGCCAGGTACTCCATGCTGAGCGCCGTC	[SEQ ID NO:42]
GTCGTGCCACCATCCGGCGGTGACACCGAGTTCTGCGATATGCGCGCCGC GTACGACGCCCTCCCGAGGGATCTGCAGAG	[SEQ ID NO:43]
CGAGCTGGAGGGCCTCCGCGCGGAGCACTACGCCCTCAACAGCAGGTTCC TCCTGGGGGACACTGACTACTCCGAGGCC	[SEQ ID NO:44]
AGAGGAACGCGATGCCACCACTGAACTGGCCCCTCGTCCGCACCCACGCT GGCAGCGGCCGCAAGTTCCTGTTTCATCGGG	[SEQ ID NO:45]
GCCCATGCCTCCCATGTGGAGGGTCTCCCTGTGCGGAGGGCCGCATGCT CCTGGCCGAGCTCCTGGAGCACGCCACCCA	[SEQ ID NO:46]
ACGCGAGTTCGTCTACCGCCACAGGTGGAATGTGCGCGACCTCGTCATGT GGGATAACCGCTGCGTGCTGCACCGCGCA	[SEQ ID NO:47]
GGCGCTACGATATCAGCGCGCGCAGGGAACCTCAGGCGCGCCACCACCTC GACGACGCGGTGGTGTAGTGTGCTGGAG	[SEQ ID NO:48]

TABLE 4B
Monocot oligonucleotides: Nonsense strand

GAAGAGCGGGTGGAGTGGGTGGCCACCACGGACATGGATCTGGAAGTAT TTGAAAGAAAATTAAAAATAAAAAGGATCT	[SEQ ID NO:49]
TCGTCCATGAGCCTCTCGATTTTCGCGCACTTCGGTGCTGCCCAGCGCCTC CCTGAGGTCGATATCCTCCACGCCGCGCCGC	[SEQ ID NO:50]
CCTAAGGTCCAAAATTCTAGCAAAAGCAATCTGTTGATCCTGTGAGAGT GGTTGGCCGCGGAAGACGAGGACGCTCTTC	[SEQ ID NO:51]
GAGGGACACGTTGCTGATGTGCGCCAGTTCAGCGTACTTGAACCTGGACG GGCGCTGGTTACCTTGATGAATCCACCCT	[SEQ ID NO:52]
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EXAMPLE 2: Construction Of A Plant-Expressible SAD Gene.

For the generation of a complete and plant-competent SAD gene, the synthetic portions of the SAD gene contained in pUCRs_{syn}SAD were removed by first releasing the 5' end of the synthetic sequence by digestion with XbaI and filling in the overhang with DNA polymerase I (Klenow large fragment) followed by digestion with KpnI. This fragment was ligated into the plasmid pProPClSV, a pUC19 plasmid containing an enhanced Peanut Chlorotic Streak Virus (PClSV) promoter derived from pKLP36 (described by Maiti and Shepherd, *Biochem. Biophys. Res. Com.*, **244**:440-444 (1998)) by cutting first with NcoI, treating with DNA polymerase I (Klenow large fragment) to fill in the generated overhang, and

subsequently cutting with KpnI. This generated the plasmid pProPClSV-SAD within which the synthetic portion of the SAD gene, including the 5' AMV leader and 3' region coding for the Cys Ala Gly signature, is directly linked to the 3' end of the PClSV promoter (Figure 1). This plasmid served as the source for the PClSV-SAD construction for insertion into the binary vectors for final gene construction prior to introduction into *Agrobacterium* for plant transformation.

Two binary vectors were chosen for final SAD gene construction, pPZP211-PNPT-311g7 (Figure 2) and pPZP211-PNPT-512g7 (Figure 3). These two vectors are based on the pPZP family of vectors described by Hajdukiewicz et al., *Plant Molec. Biol.*, **25**:989-994 (1994) and are pPZP211 derivatives in which the neomycin phosphotransferase II (NPTII) gene for kanamycin resistance is driven by the PClSV promoter and a g7 polyA termination sequence is placed adjacent to a multicloning site (MCS, Figures 2 and 3). The only difference between these two vectors is the position of the MCS relative to the g7 polyA termination sequence. The g7 polyA termination sequence is the 3' polyA termination signal from gene 7 within the octopine T-Left region of an octopine *Agrobacterium tumefaciens* Ti plasmid and was isolated as an EcoRI-SalI fragment from pAP2034 (Velten and Schell, *Nucleic Acids*, **13**:6981-6998 (1985)).

The complete SAD gene was constructed by removal of the PClSV-SAD sequence from pProPClSV-SAD as a HindIII-SmaI fragment and insertion into both pPZP211-PNPT-311g7 and pPZP211-PNPT-512g7 that were first cut with BamHI, treated with DNA polymerase I (Klenow large fragment) to fill in the overhanging sequence, and subsequently digested with HindIII. These reactions generated the two vectors, pPZP211-PNPT-311-SAD (Figure 4) and pPZP211-PNPT-512-SAD (Figure 5), that contained the full plant expressible SAD gene in one of two orientations with respect to the PClSV-NPTII-35SpolyA construct. This design for insertion of the SAD gene

into plant genomes was implemented because of uncertainty as to the effect of having two PCISV promoter sequences in the same plasmid on both transformation and effective transmission of the expressed trait. By putting the SAD gene in the vectors such that the PCISV promoters were inserted as both direct and inverted repeats, the possibility of a negative outcome could be avoided.

After construction, the SAD genes in each vector were sequenced as described above to ensure fidelity. This sequencing revealed that, in the construction of pProPCISV-SAD, an out-of-frame ATG codon was introduced into the 5' untranslated leader sequence. The presence of this ATG codon could alter the translatability of the transcript that would be synthesized from the SAD gene and so was deleted by PCR mutagenesis to restore the normal AMV leader sequence. Following repair, the sequence was rechecked for fidelity. The original SAD gene containing the out-of-frame ATG was labelled SAD1 (since some transformation experiments had begun using this construct). The repaired SAD gene is referred to as SAD2 and is the only version of the gene used for integration of the SAD construct into the cotton genome.

EXAMPLE 3: Introduction Of SAD2 Into Cotton

The two binary vectors containing the SAD2 gene, pPZP211-PNPT-311-SAD2 and pPZP211-PNPT-512-SAD2, were individually introduced into the EHA 105 strain of *Agrobacterium tumefaciens* (Hood et al., *Transgenic Research*, 2:208-218 (1993)) by direct transformation as described by Walker-Peach and Velten, in *Plant Molecular Biology Manual*, section B1:1-19 (Gelvin, Shilperoort and Verma, eds., Kluwer Academic Publishers, Dordrecht, The Netherlands, 1994)). The constructs were subsequently introduced by *Agrobacterium* transfection into cotyledon explants from the cotton variety Coker 312 (Coker Seed Inc.). This was achieved by isolating sterile cotyledon tissue (derived from seedlings grown in culture from surface-sterilized seed as described by Trolinder and Gooden, *Plant Cell Reports*, 6:231-234 (1987)), generating

explants (by use of a sterile hole punch), and submerging the explants in a 48-hour-old culture of EHA 105, containing the appropriate construct, grown at 28°C. The explants were then transferred onto 2MST medium (MS medium + 0.2 mg/L 2,4-D and 0.1 mg/L kinetin) subsequent to removal of excess EHA 105. The infected cotyledon tissues were incubated on the 2MST medium for 2 days at 28°C prior to transfer to T1+KCL medium (MS medium + 0.1 mg/L 2,4-D and 0.1 mg/L kinetin + 50 mg/L kanamycin sulphate and 250 mg/L Cefotaxime). Once healthy callus tissue was formed, it was placed on fresh T1+KCL (with 0.05 mg/L 2,4-D) for a second round of selection. After six weeks, somatic embryos were generated from the surviving callus, and mature transgenic cotton plants were produced as described by Trolinder and Goodin, *Plant Cell Reports*, 6:231-234 (1987).

A total of 111 kanamycin-resistant cotton seedlings were generated (44 were generated in the pPZP211-PNPT-311-SAD2 transformations, and 67 in the pPZP211-PNPT-512-SAD2 transformations). Each plant was analyzed for the presence of the SAD synthetic coding sequence and the NPTII coding sequence by PCR to ensure the integrity of the inserted DNA. The PCR was performed as described above. The primers used for this analysis were GGAGTTGAGGATATTGATCTCAGAGAAGCATTG [SEQ ID NO:9] and GCGATCTGCTGATCCTGACTC [SEQ ID NO:10] for the SAD coding region and CGTCAAGAAGGCGATAGAAGG [SEQ ID NO:11] and GCTATGACTGGGCACAACAGAC [SEQ ID NO:12] for the NPTII coding region. Of the 44 pPZP211-PNPT-311-SAD seedlings that survived kanamycin treatment, 2 were shown to be negative by the PCR testing. Of the 67 pPZP211-PNPT-512-SAD seedlings that survived kanamycin treatment, 14 were negative in the PCR tests.

The remaining 95 plants were grown in pots in the greenhouse until at least the squaring stage (approximately 18" tall) and were then sprayed with 2,4-D amine (United Agri Products, Greeley, CO) at 1 lb/acre acid equivalents. This is twice the normal field rate

for 2,4-D applications. Of the 95 plants, 22 survived this treatment with little or no herbicide damage being evident. Eleven of these plants contained the pPZP211-PNPT-311-SAD2 construct, and 11 contained the pPZP211-PNPT-512-SAD2 construct. Each plant was grown to maturity in the greenhouse.

Of the 22 transgenic plants, only 7 produced seed. The remaining plants were apparently infertile. Presumably this infertility was an effect of the regeneration procedure, which is common for cotton. Of the seven fertile plants, 3 contained the pPZP211-PNPT-311-SAD2 construct, and 4 contained the pPZP211-PNPT-311-SAD2 construct.

To verify that the inserted synthetic SAD genes were inheritable and to gain an indication of the number of gene insertions, seeds from the seven fertile SAD transgenic cotton plants were planted into hydroponic rock wool slabs (Hummert, St. Louis, MO) that had been saturated with Peters Professional water-soluble fertilizer (5-11-26 HYDRO-SOL, supplemented with calcium nitrate and magnesium sulfate to provide a complete nutrient compliment; Hummert, St. Louis, MO). The hydroponic rock wool slabs were placed on benches in a greenhouse, and nutrients were maintained at optimal levels using a non-recycling hydroponic watering system. Plants were grown under greenhouse conditions ($28^{\circ}\text{C} \pm 5^{\circ}\text{C}$ air temperature) for 24 days. At this point, the plants were removed to a spray hood, sprayed with 2,4-D amine at the normal field rate of 1/2 lb/acre acid equivalents, maintained in the hood for 24 hours to allow the 2,4-D to volatilize, and then placed back in the greenhouse. The effect of the treatment was evaluated visually after 10-14 days, and the results are presented in Table 5 below.

TABLE 5

Transgenic Line	Number of plants	No Damage	Some symptoms	Severe damage	Ratio "Res"/Sens
311-22-1-4	43	16	16	11	3:1
311-22-1-5	47	6	18	23	1:1
311-22-1-8	56	14	28	14	3:1
512-21-1-5	56	8	34	14	3:1
512-21-4-3	30	7	20	3	9:1
512-21-4-5	41	5	24	12	2:1
512-24-4-4	63	7	21	35	1:1
2,4-D res control	55	7	33	15	3:1
2,4-D sens control	45	0	20	25	1:1

2,4-D res control = transgenic 2,4-D resistant cotton containing the naturally-occurring *tfdA* gene construct. (Bayley et al., Theoretical Applied Genetics, 83:645-649 (1992))

2,4-D sens control = Coker 312 (not transgenic) regenerated from somatic embryos in the same manner as those containing the SAD constructs

Some symptoms = some leaf wilt and minor leaf dessication

Severe damage = all leaves wilted and desiccation damage readily evident

The ratio "Res"/Sens was calculated as the number of plants that showed some resistance to 2,4-D treatment during the experiment divided by the combined number of plants that showed severe damage or death. The negative control of Coker 312 that had been regenerated from tissue culture did show some signs of resistance, so these ratios are not to be considered as definitive measures of Mendelian inheritance of the SAD gene. Nevertheless, all of the negative control plants did show 2,4-D-induced damage, whereas all of the transgenic lines that contain the SAD gene had individuals that exhibited no damage at all.

Five plants from each of the 7 lines that exhibited no damage when treated with 2,4-D 24 days after germination were chosen, and individual newly-formed leaf samples, one per plant, were taken for PCR testing, performed as described above. Each plant tested positive for the SAD construct by PCR. These plants were grown

for a further 14 days and then resprayed with 2,4-D amine at the normal field rate of 1/2 lb/acre acid equivalents. All 35 plants exhibited no damage following this treatment, whereas all negative controls did not survive this spray event. The 35 plants were grown to maturity, and seeds were collected.

The content of each of the references referred to hereinabove, including publications, patents, and published applications, are incorporated by reference herein.

SEQUENCE LISTING

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<120> Synthetic Herbicide Resistance Gene

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<160> 60

<170> PatentIn version 3.0

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65          70          75          80

Tyr Ala Glu Leu Ala Asp Ile Ser Asn Val Ser Leu Asp Gly Lys Val
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          100          105          110
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ctc agg gag gcg ctg ggc agc acc gaa gtg cgc gaa atc gag agg ctc 152
Leu Arg Glu Ala Leu Gly Ser Thr Glu Val Arg Glu Ile Glu Arg Leu
25 30 35

atg gac gag aag agc gtc ctc gtc ttc cgc ggc caa cca ctc tca cag 200
Met Asp Glu Lys Ser Val Leu Val Phe Arg Gly Gln Pro Leu Ser Gln
40 45 50

gat caa cag att gct ttt gct agg aat ttt gga cct ttg gag ggt gga	248
Asp Gln Gln Ile Ala Phe Ala Arg Asn Phe Gly Pro Leu Glu Gly Gly	
55 60 65	
ttc atc aag gtg aac cag cgc ccg tcc agg ttc aag tac gct gaa ctg	296
Phe Ile Lys Val Asn Gln Arg Pro Ser Arg Phe Lys Tyr Ala Glu Leu	
70 75 80	
gcc gac atc agc aac gtg tcc ctc gat ggg aag gtg gcc cag agg gac	344
Ala Asp Ile Ser Asn Val Ser Leu Asp Gly Lys Val Ala Gln Arg Asp	
85 90 95 100	
gct agg gaa gtt gtg ggc aac ttc gcc aac caa ctg tgg cac tcc gat	392
Ala Arg Glu Val Val Gly Asn Phe Ala Asn Gln Leu Trp His Ser Asp	
105 110 115	
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Ser Ser Phe Gln Gln Pro Ala Ala Arg Tyr Ser Met Leu Ser Ala Val	
120 125 130	
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Val Val Pro Pro Ser Gly Gly Asp Thr Glu Phe Cys Asp Met Arg Ala	
135 140 145	
gcg tac gac gcc ctc ccg agg gat ctg cag agc gag ctg gag ggc ctc	536
Ala Tyr Asp Ala Leu Pro Arg Asp Leu Gln Ser Glu Leu Glu Gly Leu	
150 155 160	
cgc gcg gag cac tac gcc ctc aac agc agg ttc ctc ctg ggg gac act	584
Arg Ala Glu His Tyr Ala Leu Asn Ser Arg Phe Leu Leu Gly Asp Thr	
165 170 175 180	
gac tac tcc gag gcc cag agg aac gcg atg cca cca gtg aac tgg ccc	632
Asp Tyr Ser Glu Ala Gln Arg Asn Ala Met Pro Pro Val Asn Trp Pro	
185 190 195	
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Leu Val Arg Thr His Ala Gly Ser Gly Arg Lys Phe Leu Phe Ile Gly	
200 205 210	

gcc cat gcc tcc cat gtg gag ggt ctc cct gtc gcg gag ggc cgc atg 728
 Ala His Ala Ser His Val Glu Gly Leu Pro Val Ala Glu Gly Arg Met
 215 220 225

ctc ctg gcc gag ctc ctg gag cac gcc acc caa cgc gag ttc gtc tac 776
 Leu Leu Ala Glu Leu Leu Glu His Ala Thr Gln Arg Glu Phe Val Tyr
 230 235 240

cgc cac agg tgg aat gtc ggc gac ctc gtc atg tgg gat aac cgc tgc 824
 Arg His Arg Trp Asn Val Gly Asp Leu Val Met Trp Asp Asn Arg Cys
 245 250 255 260

gtg ctg cac cgc ggc agg cgc tac gat atc agc gcg cgc agg gaa ctc 872
 Val Leu His Arg Gly Arg Arg Tyr Asp Ile Ser Ala Arg Arg Glu Leu
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 Arg Arg Ala Thr Thr Leu Asp Asp Ala Val Val
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agcatcgtaa gctgccctca 80

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<210> 37

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<210> 59
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cggttatccc acatgacgag 80

<210> 60
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WE CLAIM:

1. A DNA molecule comprising a synthetic DNA sequence,
the synthetic DNA sequence encoding an enzyme that degrades 2,4-dichlorophenoxyacetic acid to dichlorophenol, and
the synthetic DNA sequence comprising a natural microbial sequence that encodes the enzyme in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by a plant.
2. The DNA molecule of Claim 1 wherein all of the codons of the natural microbial sequence which are least preferred by the plant have been replaced by codons more preferred by the plant.
3. The DNA molecule of Claim 1 wherein at least 50% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.
4. The DNA molecule of Claim 3 wherein at least 80% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.
5. The DNA molecule of any one of Claims 1-4 wherein the natural microbial sequence is a natural bacterial sequence.
6. The DNA molecule of any one of Claims 1-4 wherein the codons of the natural microbial sequence have been replaced by codons more preferred by a dicotyledonous plant.
7. The DNA molecule of Claim 6 comprising the nucleotide sequence of SEQ ID NO:2.
8. The DNA molecule of any one of Claims 1-4 wherein the codons of the natural microbial sequence have been replaced by codons more preferred by a monocotyledonous plant.

9. The DNA molecule of Claim 8 comprising the nucleotide sequence of SEQ ID NO:3.
10. A DNA construct comprising a synthetic DNA sequence,
the synthetic DNA sequence being operatively linked to plant gene expression control sequences,
the synthetic DNA sequence encoding an enzyme that degrades 2,4-dichlorophenoxyacetic acid to dichlorophenol, and
the synthetic DNA sequence comprising a natural microbial sequence that encodes the enzyme in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by a plant.
11. The DNA construct of Claim 10 wherein all of the codons of the natural microbial sequence which are least preferred by the plant have been replaced by codons more preferred by the plant.
12. The DNA construct of Claim 10 wherein at least 50% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.
13. The DNA construct of Claim 12 wherein at least 80% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.
14. The DNA construct of any one of Claims 10-13 wherein the natural microbial sequence is a natural bacterial sequence.
15. The DNA construct of any one of Claims 10-13 wherein the codons of the natural microbial sequence have been replaced by codons more preferred by a dicotyledonous plant.
16. The DNA construct of Claim 15 comprising the nucleotide sequence of SEQ ID NO:2.

17. The DNA construct of Claim 15 comprising the nucleotide sequence of SEQ ID NO:4.
18. The DNA construct of any one of Claims 10-13 wherein the codons of the natural microbial sequence have been replaced by codons more preferred by a monocotyledonous plant.
19. The DNA construct of Claim 18 comprising the nucleotide sequence of SEQ ID NO:3.
20. The DNA construct of Claim 18 comprising the nucleotide sequence of SEQ ID NO:5.
21. The DNA construct of Claim 10 which is a vector.
22. The DNA construct of Claim 10 which is a plasmid.
23. The DNA construct of Claim 10 which is pProPClSV-SAD.
24. The DNA construct of Claim 10 which is pPZP211-PNPT-311-SAD.
25. The DNA construct of Claim 10 which is pPZP211-PNPT-512-SAD.
26. The DNA construct of Claim 10 wherein the expression control sequences comprise a Peanut Chlorotic Streak Virus promoter.
27. A transgenic plant or part of a plant comprising a synthetic DNA sequence,
the synthetic DNA sequence being operatively linked to plant gene expression control sequences,
the synthetic DNA sequence encoding an enzyme that degrades 2,4-dichlorophenoxyacetic acid to dichlorophenol, and
the synthetic DNA sequence comprising a natural microbial sequence that encodes the enzyme in which at least a plurality of

the codons of the natural microbial sequence have been replaced by codons more preferred by a plant.

28. The plant or plant part of Claim 27 wherein the expression control sequences comprise a Peanut Chlorotic Streak Virus promoter.

29. The plant or plant part of Claim 27 wherein all of the codons of the natural microbial sequence which are least preferred by the plant have been replaced by codons more preferred by the plant.

30. The plant or plant part of Claim 27 wherein at least 50% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.

31. The plant or plant part of Claim 30 wherein at least 80% of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.

32. The plant or plant part of any one of Claims 27-31 wherein the natural microbial sequence is a natural bacterial sequence.

33. The plant or plant part of Claim 27 which is a dicotyledonous plant or plant part and the codons of the natural microbial sequence have been replaced by codons more preferred by a dicotyledonous plant.

34. The plant or plant part of Claim 33 wherein the one or more cells comprise the nucleotide sequence of SEQ ID NO:2.

35. The plant or plant part of Claim 33 wherein the one or more cells comprise the nucleotide sequence of SEQ ID NO:4.

36. The plant or plant part of Claim 27 which is a monocotyledonous plant or plant part and the codons of the natural

microbial sequence have been replaced by codons more preferred by a monocotyledonous plant.

37. The plant or plant part of Claim 36 wherein the one or more cells comprise the nucleotide sequence of SEQ ID NO:3.

38. The plant or plant part of Claim 36 wherein the one or more cells comprise the nucleotide sequence of SEQ ID NO:5.

39. The plant or plant part of Claim 27 which is a cotton plant or plant part.

40. The plant part of Claim 27 which is a seed.

41. The plant part of Claim 27 which is a fruit.

42. A method of controlling weeds in a field containing a transgenic plant,

the method comprising applying an amount of an auxin herbicide to the field effective to control the weeds in the field,

the transgenic plant being tolerant to the auxin herbicide as a result of comprising a synthetic DNA sequence,

the synthetic DNA sequence being operatively linked to plant gene expression control sequences,

the synthetic DNA sequence encoding an enzyme that degrades 2,4-dichlorophenoxyacetic acid to dichlorophenol, and

the synthetic DNA sequence comprising a natural microbial sequence that encodes the enzyme in which at least a plurality of the codons of the natural microbial sequence have been replaced by codons more preferred by the plant.

43. The method of Claim 42 wherein the auxin herbicide is 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-D amine.

44. The method of Claim 42 wherein the plant is a monocotyledonous plant.

45. The method of Claim 42 wherein the plant is a dicotyledonous plant.

46. The method of Claim 42 wherein the plant is a cotton plant.

47. A method of selecting transformed plant cells comprising:
providing a population of plant cells;
transforming at least some of the plant cells in the population with the DNA construct of Claim 10; and
selecting the transformed plant cells by culturing the resulting population of plant cells in a culture medium containing an auxin herbicide at a concentration selected so that transformed plant cells proliferate and untransformed plant cells do not proliferate.

48. The method of Claim 47 wherein the auxin herbicide is 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-D amine.

49. A method of selecting transformed plants comprising:
providing a population of plants which may comprise one or more plants comprising a DNA construct according to Claim 10; and
selecting transformed plants by applying an amount of an auxin herbicide to the population of plants, the amount of herbicide being selected so that transformed plants grow and growth of untransformed plants is inhibited.

50. The method of Claim 49 wherein the auxin herbicide is 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-D amine.

1/2

Fig 1

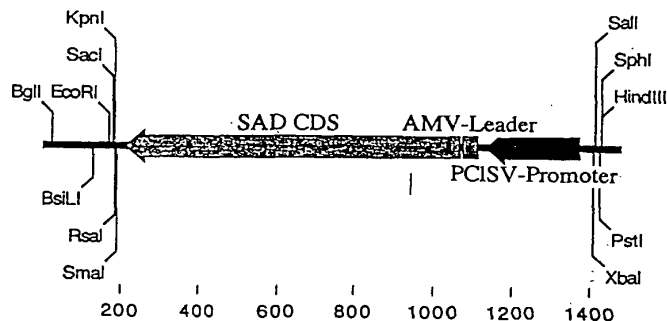


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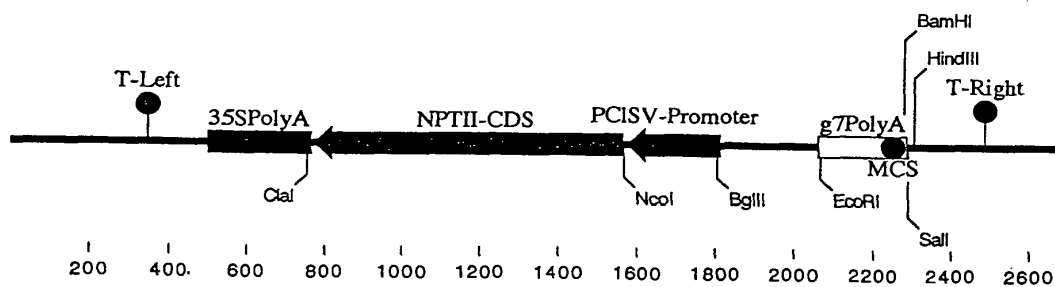
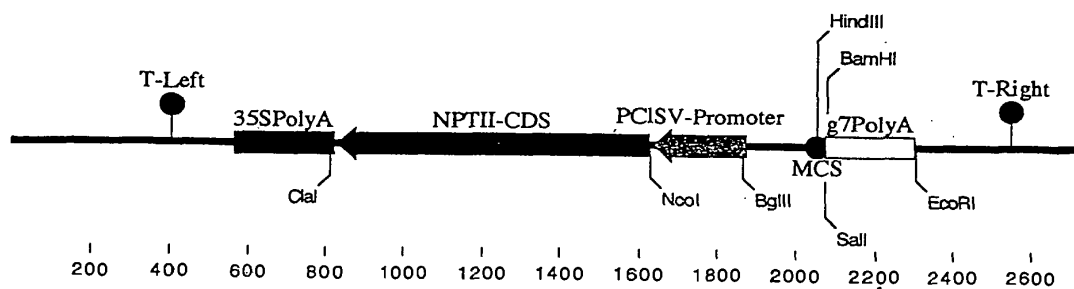


Fig 3



2/2

Fig 4

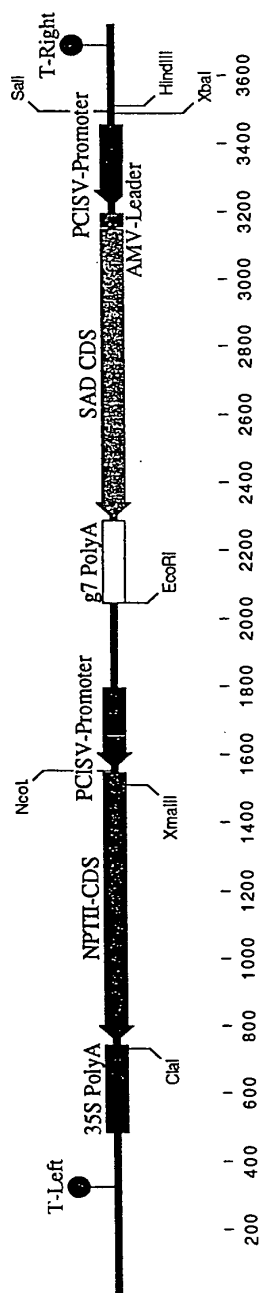
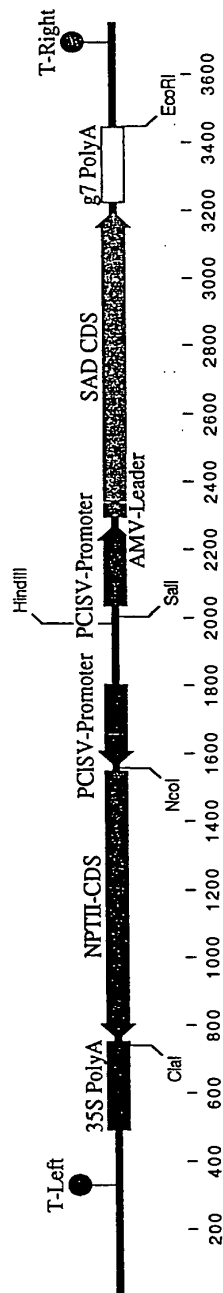


Fig 5



Sign in to NCBI

Nucleotide

Advanced Help

GenBank

Send to:

Delftia acidovorans plasmid pMC1, partial sequence

GenBank: AY327575.2

[FASTA](#) [Graphics](#)[Go to:](#)

LOCUS AY327575 61701 bp DNA linear BCT 25-JUL-2016

DEFINITION Delftia acidovorans plasmid pMC1, partial sequence.

ACCESSION AY327575 AF516749 AF516750

VERSION AY327575.2

KEYWORDS .

SOURCE Delftia acidovorans

ORGANISM Delftia acidovorans
Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Comamonadaceae; Delftia.

REFERENCE 1 (bases 1 to 61701)

AUTHORS Schleinitz,K.M., Kleinsteinuber,S., Vallaes,T. and Babel,W.

TITLE Localization and Characterization of Two Novel Genes Encoding
Stereospecific Dioxygenases Catalyzing
2(2,4-Dichlorophenoxy)propionate Cleavage in Delftia acidovorans
MC1

JOURNAL Appl. Environ. Microbiol. 70 (9), 5357-5365 (2004)

PUBMED [15345471](#)

REFERENCE 2 (bases 1 to 61701)

AUTHORS Schleinitz,K.M., Kleinsteinuber,S., Vallaes,T. and Babel,W.

TITLE Genetic background of enantiospecific 2,4-dichlorophenoxypropionate
cleavage in Delftia acidovorans MC1

JOURNAL Unpublished

REFERENCE 3 (bases 1 to 61701)

AUTHORS Schleinitz,K.M., Vallaes,T. and Kleinsteinuber,S.

TITLE Structural analysis of ISCR8, a subgroup of IS91-like elements

JOURNAL Unpublished

REFERENCE 4 (bases 1 to 61701)

AUTHORS Schleinitz,K.M.

TITLE Direct Submission

JOURNAL Submitted (31-MAY-2002) Environmental Microbiology, Centre for
Environmental Research, Permoserstrasse 15, Leipzig 04318, Germany

REFERENCE 5 (bases 1 to 61701)

AUTHORS Schleinitz,K.M.

TITLE Direct Submission

JOURNAL Submitted (20-JUN-2003) Environmental Microbiology, UFZ Centre for
Environmental Research, Permoserstrasse 15, Leipzig 04318, Germany

REMARK Nucleotide sequence updated by submitter

REFERENCE 6 (bases 1 to 61701)

AUTHORS Schleinitz,K.M.

TITLE Direct Submission

JOURNAL Submitted (03-SEP-2009) Environmental Microbiology, UFZ Centre for
Environmental Research, Permoserstrasse 15, Leipzig 04318, Germany

REMARK Sequence update by submitter

COMMENT On Sep 7, 2010 this sequence version replaced gi:22815752.

FEATURES

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CDS



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Related information

[Full text in PMC](#)[Gene](#)[Protein](#)[PubMed](#)[PubMed \(Weighted\)](#)[Taxonomy](#)

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Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Rebecca Ball/Mark Sweetin		
Attorney Docket Number:		14764-245772		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
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SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
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EFS ID:	32729990
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Rebecca Ball/Mark Sweetin
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1		245772_Response_NonFinal_052518.pdf	34897	yes	9
			389025ea4e6fb826116772ae4176ada3d0e7d2fb		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Applicant Arguments/Remarks Made in an Amendment		8	9	
	Claims		3	7	
	Amendment/Req. Reconsideration-After Non-Final Reject		1	2	
Warnings:					
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2		245772_Supp_IDS_052518.pdf	39839	yes	4
			1b36316cf9485f15b7ea60d605f129a52d58683		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Information Disclosure Statement (IDS) Form (SB08)		4	4	
	Transmittal Letter		1	3	
Warnings:					
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3	Non Patent Literature	Muller_Appl-Environ-Microbiol_2006.pdf	1331016	no	9
			ac103fac2526a856c6dbfb9b5f9375840a51cdd9		
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4	Non Patent Literature	Westendorf_Microbiol-Res_2002.pdf	140577	no	6
			4402afe8e2e5b858a39884e157545a08c8cbd58a		
Warnings:					
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5	Non Patent Literature	Wright_Natl-Acad-Sci_2010.pdf	521721	no	6
			940bc7b80d12e949795c2d914471c8ec46549b37		
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6	Foreign Reference	WO03034813A2.pdf	2492788	no	80
			40038b74463d2fe6578415b9549a0fe97181fa1b		
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7	Non Patent Literature	Genbank_AY327575_Schleinitz.pdf	281704	no	24
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National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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REMARKS

(I) CLAIM AMENDMENTS

Claims 1, 10, 21, and 24 have been amended to require that the polynucleotides that encode the respective proteins have SEQ ID NO: 3. Claim 27 has been cancelled without prejudice. Support for the amendments can be found in the originally filed claims and throughout the specification as filed. Applicant respectfully requests entry of the amendments.

(II) ALLOWABLE SUBJECT MATTER

Applicant thanks the Examiner for acknowledging that claims 9 and 27 depend from a rejected base claim and would be allowable if rewritten in independent form, and that the specific nucleotide sequence of SEQ ID NO: 3 is not obvious over the prior art. After entry of the amendments, claim 9 now depends from an allowable claim and claim 27 has been cancelled without prejudice.

(III) REJECTION UNDER 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-8, 10-22, 24 and 26 under 35 U.S.C. § 103(a), asserting that the claims are obvious over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al.*, Pallet *et al.* (U.S. Patent 7,250,561 B1), and Xu *et al.*, (U.S. Patent 6,664,384). Applicant notes the Office Action states the Xu *et al.* U.S. Patent number is 6,665,384, but Applicant believes the Examiner meant 6,664,384 as evidenced by the 892 Form provided January 12, 2017.

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Applicant obviates the Examiner's rejections under 35 U.S.C. §103(a) by the amendments to claims 1, 10, 21, and 24. The Examiner mentioned that the specific nucleotide sequence of SEQ ID NO: 3 would not have been obvious over the prior art. Applicant has amended claims 1, 10, 21, and 24 to require that the polynucleotides that encode the respective proteins have SEQ ID NO: 3. Claim 22 was already limited to a polynucleotide having SEQ ID NO: 3. As a result, all of the claims, including the dependent claims, should be allowable.

Withdrawal of the rejection of claims 1-8, 10-22, 24 and 26 under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

The foregoing amendments and remarks are believed to fully respond to the Examiner's rejections. The Applicant now believes the application is in condition for allowance.

Respectfully submitted,
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Indianapolis, Indiana 46204
317-231-7511

DMS 4860790v1

AMENDMENTS TO THE CLAIMS

A complete listing of the pending claims is provided pursuant to 37 C.F.R. § 1.121(c)(1). Please amend claims 1, 10, 21, and 24 as follows. Please cancel claim 27 without prejudice.

In the Claims

1. (Currently Amended) An expression cassette for expression in a plant cell, comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides, wherein ~~said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4~~ the polynucleotide that encodes said protein comprises SEQ ID NO:3.

2. (Original) The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.

3. (Previously Presented) The expression cassette of claim 1, wherein said plant promoter or said plant virus promoter is selected from a cassava vein mosaic virus promoter, a CaMV 35S promoter, a Figwort Mosaic Virus promoter, a rice actin promoter, a phaseolin promoter, an Arabidopsis thaliana Ubiquitin 10 promoter, a maize ubiquitin promoter, an Arabidopsis thaliana Act2 promoter, an Arabidopsis thaliana Ubiquitin 11 promoter, and an Arabidopsis thaliana Ubiquitin 3 promoter.

4. (Previously Presented) A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising the expression cassette of claim 1.

5. (Previously Presented) A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of an aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprises at least one plant that is stably transformed with the expression cassette of claim 1 and is able to grow in said effective amount of the aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette.

6. (Original) The method of claim 4, wherein said plurality of crop plants are dicots.

7. (Previously Presented) The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar beet plants, tomato plants, and sunflower plants.

8. (Previously Presented) The method of claim 4, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, and wherein said pyridyloxy auxin herbicide comprises one or more pyridyloxyacetate herbicides.

9. (Previously Presented) The method of claim 8, wherein said pyridyloxyacetate herbicide(s) is selected from the group consisting of triclopyr and fluroxypyr.

10. (Currently Amended) The method of claim 4, wherein said applying step comprises applying said herbicide to said crop plants within 14 days of planting a seed in said field, wherein said seed comprises an expression cassette comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides, ~~wherein said protein has at least 95% amino acid sequence identity with a sequence selected~~

~~from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 the polynucleotide that encodes~~
said protein comprises SEQ ID NO:3.

11. (Previously Presented) The method of claim 4, wherein said plurality of crop plants further comprises a second polynucleotide encoding a second protein that confers said crop plants with resistance to at least one other herbicide, wherein said second polynucleotide is heterologous, and said method further comprises applying said at least one other herbicide to least a portion of said field.

12. (Original) The method of claim 11, wherein said herbicides are applied sequentially or concurrently.

13. (Original) The method of claim 11, wherein said herbicides are applied from a tank mix.

14. (Original) The method of claim 11, wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispyribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyriithiobac, pyroxasulfone, pyroxsulam,

quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr, thidiazuron, thiencazone, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflurosulfuron, and tritosulfuron.

15. (Original) The method of claim 4, wherein said weeds are glyphosate-resistant.

16. (Original) The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.

17. (Previously Presented) The method of claim 4, wherein said herbicide is the phenoxy auxin herbicide.

18. (Original) The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.

19. (Previously Presented) A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising the expression cassette of claim 1.

20. (Previously Presented) The method of claim 19 wherein said volunteer plants are glyphosate-resistant and of a different species than said transgenic crop plants.

21. (Currently Amended) A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide has a sequence selected

~~from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5~~ the polynucleotide that encodes said protein comprises SEQ ID NO:3.

22. (Previous Presented) A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has SEQ ID NO:3.

23. (Original) The polynucleotide of claim 22 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

24. (Currently Amended) An isolated polynucleotide that is operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein that enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein ~~the polynucleotide that encodes said protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5~~ the polynucleotide that encodes said protein comprises SEQ ID NO:3.

25. (Cancelled)

26. (Previously Presented) The polynucleotide of claim 24 wherein said promoter is a cassava vein mosaic virus promoter.

27. (Cancelled)

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APPLICATION AS FILED – PART I										
(Column 1)		(Column 2)								
FOR		NUMBER FILED		NUMBER EXTRA		RATE (\$)		FEE (\$)		
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A		N/A		N/A				
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))		N/A		N/A		N/A				
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A		N/A		N/A				
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =		*		X \$ =				
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =		*		X \$ =				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).								
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))										
* If the difference in column 1 is less than zero, enter "0" in column 2.						TOTAL				
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AMENDMENT	05/25/2018	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 25	Minus	** 26	= 0	x \$100 =		0		
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	x \$460 =		0		
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE		0		
(Column 1)		(Column 2)		(Column 3)						
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)		ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =				
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =				
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
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NOTICE OF ALLOWANCE AND FEE(S) DUE

107540 7590 08/15/2018
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, INDIANA 46204
UNITED STATES OF AMERICA

EXAMINER

KRUSE, DAVID H

ART UNIT

PAPER NUMBER

1663

DATE MAILED: 08/15/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	11/15/2018

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

**Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885**

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

107540 7590 08/15/2018
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, INDIANA 46204
UNITED STATES OF AMERICA

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

	(Depositor's name)
	(Signature)
	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
KRUSE, DAVID H	1663	800-300000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1	_____
2	_____
3	_____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)

- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____	Date _____
Typed or printed name _____	Registration No. _____



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14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
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Barnes & Thornburg LLP (Dow)			KRUSE, DAVID H	
11 South Meridian Street			ART UNIT	
Indianapolis, INDIANA 46204			PAPER NUMBER	
UNITED STATES OF AMERICA			1663	
DATE MAILED: 08/15/2018				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.** Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b) (2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 14/491,197	Applicant(s) Wright et al.	
	Examiner DAVID H KRUSE	Art Unit 1663	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the Amendment filed 25 May 2018.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are 1-24 and 26. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. ____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☐ Examiner's Amendment/Comment
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date ____.	6. ☐ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material ____.	7. ☐ Other ____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date ____.	

/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	
--	--

Issue Classification 	Application/Control No. 14/491,197	Applicant(s)/Patent Under Reexamination Wright et al.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC						
Symbol					Type	Version
C12N	/	15	/	8274	F	2013-01-01
C12N	/	9	/	0069	I	2013-01-01
C12N	/	9	/	0071	I	2013-01-01
C12N	/	15	/	8275	I	2013-01-01
C12Y	/	113	/	11	A	2013-01-01

CPC Combination Sets							
Symbol				Type	Set	Ranking	Version
	/		/				

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	25	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	08 August 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office

Part of Paper No.: 20180808

Issue Classification 	Application/Control No. 14/491,197	Applicant(s)/Patent Under Reexamination Wright et al.
	Examiner DAVID H KRUSE	Art Unit 1663

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N	/	15	/ 82

NON-CLAIMED			
/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	25	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663	08 August 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office

Part of Paper No.: 20180808

Issue Classification 	Application/Control No. 14/491,197	Applicant(s)/Patent Under Reexamination Wright et al.
	Examiner DAVID H KRUSE	Art Unit 1663

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47															
CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	10	10	19	19										
2	2	11	11	20	20										
3	3	12	12	21	21										
4	4	13	13	22	22										
5	5	14	14	23	23										
6	6	15	15	24	24										
7	7	16	16		25										
8	8	17	17	25	26										
9	9	18	18		27										

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 25	
/DAVID H KRUSE/ Primary Examiner, Art Unit 1663 (Primary Examiner) _____ (Date) _____		08 August 2018 O.G. Print Claim(s) 1	O.G. Print Figure NONE

U.S. Patent and Trademark Office

Part of Paper No.: 20180808

Search Notes 	Application/Control No. 14/491,197	Applicant(s)/Patent Under Reexamination Wright et al.
	Examiner DAVID H KRUSE	Art Unit 1663

CPC - Searched*		
Symbol	Date	Examiner
C12N 9/0071	08/08/2018	/DK/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventor Name Search	12/29/2016	/DK/
EAST: USPAT, USPGPU, EPO, JPO, DERWENT	12/29/2016	/DK/
STN: AGRICOLA, BIOSIS, CAPLUS, EMBASE, CABA	12/29/2016	/DK/
Sequence Search of SEQ I DNO: 1-5 included interference search	12/9/2016	/DK/
Update EAST	6/15/2017	/DK/
Update EAST	01/24/2018	/DK/
Update EAST	08/08/2018	/DK/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
EAST	EAST	08/08/2018	/DK/

/DAVID H KRUSE/ Primary Examiner.Art Unit 1663	
--	--

Please type a plus sign (+) inside this box 

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i> Sheet 1 of 1	Complete if Known	
	Application Number:	14/491197
	Filing Date:	September 19, 2014
	First Named Inventor:	WRIGHT, Terry R.
	Group Art Unit:	1663
	Confirmation Number:	3336
	Examiner Name:	Kruse, David H.
		Attorney Docket Number: 14764-245772

U.S. PATENT DOCUMENTS						
Examiner Initials	Doc. No.	U.S. Patent Document		Name of Patentee or Applicant	Date of Publication	Filing Date If Appropriate
	B A	Application or Patent Number	Kind Code			
		4683195		Mullis et al.	07-28-1997	
	B B	4761373		Anderson et al.	08-02-1988	

FOREIGN PATENT DOCUMENTS								
Examiner Initials	Doc. No.	Foreign Patent Document			Name of Patentee or Applicant	Date of Publication	Translation	
		Office	Application or Patent Number	Kind Code			Yes	No* +
	B C	WO	2003/034813		U.S. Secretary of Agriculture	05-01-2003		

OTHER - NON PATENT LITERATURE DOCUMENTS							
Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.					Translation
							Yes No* +
	B D	Muller et al., "Purification and Characterization of Two Enantioselective α -Ketoglutarate-Dependent Dioxygenases, RdpA and SdpA, from <i>Sphingomonas herbicidovorans</i> MH," Appl. Environ. Microbiol., 72(7):4853-61 (2006).					
	B E	Schleinitz et al., "Delftia acidovorans plasmid pMC1, partial sequence," Appl. Environ. Microbiol. 70 (9), 5357-5365, Genbank accession no. AY327575 (2004).					
	B F	Westendorf et al., "The two enantiospecific dichlorprop/ α -ketoglutarate-dioxygenases from <i>Delftia acidovorans</i> MC1 - protein and sequence data of RdpA and SdpA," Microbiol. Res.157: 317-322 (2002).					
	B G	Wright et al., "Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes," Natl. Acad. Sci., 107(47): 20240-5 (2010).					

DMS 12002559v1

EXAMINER	/DAVID H KRUSE/	DATE CONSIDERED	08/08/2018
----------	-----------------	-----------------	------------

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

+ An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).

EAST Search History**EAST Search History (Prior Art)**

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	358	aryloxyalkanoate adj dioxygenase	USPAT	OR	OFF	2018/08/08 09:08
L2	14	L1 and (rdpA or sdpA)	USPAT	OR	OFF	2018/08/08 09:08
L3	85	cassava adj vein adj mosaic adj virus adj promoter	USPAT	OR	OFF	2018/08/08 09:08
L4	32	L3 and herbicide.clm.	USPAT	OR	OFF	2018/08/08 09:08

8/ 8/ 2018 9:09:05 AM**C:\Users\dkruse\Documents\EAST\Workspaces\14491197.wsp**

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Aug 15, 2018 03:28:44 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14491197	NOA	08/15/2018	14764-245772
	1449	08/15/2018	14764-245772

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Aug 21, 2018 01:39:30 PM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	NOA	08/15/2018	14764-245772
	1449	08/15/2018	14764-245772

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19 2014	Terry R. Wright	14764-245772	3336
107540 7590 08/27/2018 Barnes & Thornburg LLP (Dow) 11 South Meridian Street Indianapolis, IN 46204			EXAMINER KRUSE, DAVID H	
			ART UNIT 1663	PAPER NUMBER
			NOTIFICATION DATE 08/27/2018	DELIVERY MODE ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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www.uspto.gov

Application No. : 14491197
Applicant : Wright
Filing Date : 09/19/2014
Date Mailed : 08/27/2018

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

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/Kevin Danemark/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. **14491197**

NON-COMPLIANT ILLUSTRATIONS IN SPECIFICATION OR CLAIMS

☐ The illustration(s) in the location(s) referenced below do not come within the exceptions of 37 CFR 1.58(a). Please delete the illustration(s) from the specification or claims, provided the illustration(s) as part of the formal drawing(s) in accordance with 37 CFR 1.84, and amend the specification to provide a brief description of the drawings in accordance with 37 CFR 1.74. Please see the following item(s):

☐ Specification document coded dated , page(s) .

☐ Claims document coded dated , claim(s) .

☐ Amendment document coded dated .

☐ Other: .

☒ The chemical formula(s), mathematical formula(s), or table(s) in the location(s) referenced below contain shading (color/grayscale) and therefore do not comply with 37 CFR 1.52 and do not come within the exceptions of 37 CFR 1.58(a). Please delete chemical formula(s), mathematical formula(s), or table(s) with shading from the specification or claims, provide them as formal color drawing(s) in accordance with 37 CFR 1.84, including petition, fee, and amendment in accordance with 37 CFR 1.84(a)(2), and amend the specification to provide a brief description of the drawings in accordance with 37 CFR 1.74. Please see the following item(s):

☒ Specification document coded SPEC dated 09/19/2014, page(s) 99.

☐ Claims document coded dated , claim(s) .

☐ Amendment document coded dated .

☐ Other: .

Application No. **14491197**

**IDENTIFICATION OF APPLICATION DEFICIENCIES
IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012**

- ☒ Applicant must provide legible text for the following item(s).
- ☒ Specification filed 09/19/2014, page(s) 9 - table has illegible data.
- ☐ Claims filed , claim(s) .
- ☐ Other: .
- ☒ Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s). 104 - table contains truncated data
- ☐ The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).
- ☐ Applicant must provide an Abstract of the Disclosure.
- ☐ The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.
- ☐ Other:

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From: PAIR_eOfficeAction@uspto.gov
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Aug 27, 2018 03:58:50 AM

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11 South Meridian Street
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Application	Document	Mailroom Date	Attorney Docket No.
14491197	M327	08/27/2018	14764-245772

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**Courtesy Reminder for
Application Serial No: 14/491,197**

Attorney Docket No: 14764-245772

Customer Number: 107540

Date of Electronic Notification: 08/21/2018

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
107540 7590 08/27/2018 Barnes & Thornburg LLP (Dow) 11 South Meridian Street Indianapolis, IN 46204			EXAMINER KRUSE, DAVID H	
			ART UNIT 1663	PAPER NUMBER
			NOTIFICATION DATE 08/27/2018	DELIVERY MODE ELECTRONIC

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Application No. : 14491197
Applicant : Wright
Filing Date : 09/19/2014
Date Mailed : 08/27/2018

NOTICE TO FILE CORRECTED APPLICATION PAPERS

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Application No. **14491197**

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☐ Specification document coded dated , page(s) .

☐ Claims document coded dated , claim(s) .

☐ Amendment document coded dated .

☐ Other: .

☒ The chemical formula(s), mathematical formula(s), or table(s) in the location(s) referenced below contain shading (color/grayscale) and therefore do not comply with 37 CFR 1.52 and do not come within the exceptions of 37 CFR 1.58(a). Please delete chemical formula(s), mathematical formula(s), or table(s) with shading from the specification or claims, provide them as formal color drawing(s) in accordance with 37 CFR 1.84, including petition, fee, and amendment in accordance with 37 CFR 1.84(a)(2), and amend the specification to provide a brief description of the drawings in accordance with 37 CFR 1.74. Please see the following item(s):

☒ Specification document coded SPEC dated 09/19/2014, page(s) 99.

☐ Claims document coded dated , claim(s) .

☐ Amendment document coded dated .

☐ Other: .

Application No. **14491197**

**IDENTIFICATION OF APPLICATION DEFICIENCIES
IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012**

- ☒ Applicant must provide legible text for the following item(s).
- ☒ Specification filed 09/19/2014, page(s) 9 - table has illegible data.
- ☐ Claims filed , claim(s) .
- ☐ Other: .
- ☒ Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s). 104 - table contains truncated data
- ☐ The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).
- ☐ Applicant must provide an Abstract of the Disclosure.
- ☐ The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.
- ☐ Other:

Electronic Acknowledgement Receipt	
EFS ID:	34122816
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Adam Bobel/Sherri Cozart
Filer Authorized By:	Adam Bobel
Attorney Docket Number:	14764-245772
Receipt Date:	26-OCT-2018
Filing Date:	19-SEP-2014
Time Stamp:	11:33:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
------------------------	----

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Certification and Request for Missing Parts	Response_to_Notice_to_File_Corrected_Application_Papers_14764_245772.pdf	172151 5ebbd0598e1ab7e1c48c0116718d62fdd786c96bf	no	5

Warnings:

Information:					
2	Miscellaneous Incoming Letter	245772notice.pdf	130182	no	4
			7580b339d5370281b4cbe71ab89f527a121f9f8b		
Warnings:					
Information:					
Total Files Size (in bytes):				302333	
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

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<i>Customer No.</i>	107540	}	
		}	
<i>Art Unit:</i>	1663	}	
		}	
<i>Confirmation No.:</i>	3336	}	
		}	
<i>Application No.:</i>	14/491,197	}	
		}	
<i>Title:</i>	NOVEL HERBICIDE RESISTANCE GENES	}	<u>ELECTRONICALLY FILED ON:</u>
		}	<u>October 26, 2018</u>
		}	
<i>Inventor:</i>	Terry R. Wright et al.	}	
		}	
<i>Filed:</i>	September 19, 2014	}	
		}	
<i>Attorney</i>		}	
<i>Docket:</i>	14764-245772	}	
		}	
<i>Examiner:</i>	KRUSE, DAVID H	}	

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Notice to File Corrected Application Papers transmitted electronically on August 27, 2018, please consider the following:

Amendments to the Specification beginning on page 2 of this paper, and
Remarks beginning on page 5 of this paper.

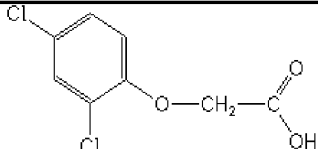
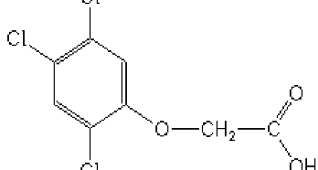
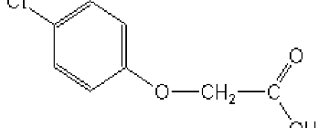
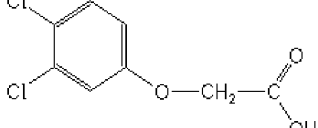
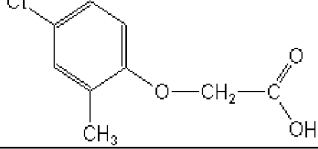
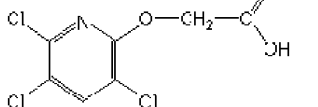
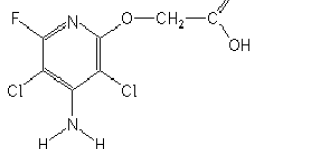
A copy of the **NOTICE TO FILE CORRECTED APPLICATION PAPERS** accompanies this paper.

AMENDMENTS TO THE SPECIFICATION

On page 99 of the specification, please replace Table 25 with the following table:

Table 25. T ₀ soybean response to 2,4-D leaf paint and 2,4-D spray application.														
Construct (pDAB#)	Gene	Promoter	Event	Leaf flip assay										
				NODE 2,4-D @ (18 HAT)										
				LEAF PAINTED	Node		Spray POST over the top with 2,4-D (g ae/ha)	Stage at appl (# nodes)	ELISA (ng/mL)	Southern Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT
N-1	N-2													
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100
					1=Flip				ND=Not determined					
					0=No Rip									

On page 9 of the specification, please replace Table 1 with the following table:

Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy 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-12 enzyme <i>in planta</i> as general plant esterases convert these esters to the active acids <i>in planta</i> . Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. 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 ac/ha)	Preferred use rate ranges (g ac/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	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

On page 104 of the specification, please replace Table 26 with the following table:

Table 26. Media for Cotton Transformation								
Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	30 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 2%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

REMARKS

In response to the Notice to File Corrected Application Papers transmitted electronically on August 27, 2018, Applicant hereby submits an amendment to the specification. The Notice to File Corrected Application Papers stated that Table 25 on page 99 contained improper shading (color/grayscale), Table 1 on page 9 had illegible data, and Table 26 on page 104 contained truncated data. The Applicant has amended the specification to remove the shading from Table 25 on page 99 and to make the data from Table 1 legible. With regard to Table 1 on page 9, the disclosure of grandparent application 12/091,896 has been expressly incorporated by reference in this application (see page 1 of the present application as filed). On page 9 of the specification in grandparent application 12/091,896, Table 1 appears without any illegible data. Table 1 on page 9 of the instant application has been replaced with Table 1 from the grandparent application. With regard to Table 26 on page 104, the disclosure of parent application 13/647,081 has been expressly incorporated by reference in this application (see page 1 of the present application as filed). On page 106 of the specification in parent application 13/647,081, Table 26 appears without any truncated data. Table 26 on page 104 of the instant application has been replaced with Table 26 from the parent application. No new matter has been added.

A copy of the Notice to File Corrected Application Papers accompanies this paper.

No fees are believed to be due with this paper. However, the Commissioner is authorized to charge any required fees, or credit any overpayment, to Barnes & Thornburg LLP Deposit Account No. 10-0435 with reference to our matter 14764-245772.

Respectfully submitted,
BARNES & THORNBURG LLP
/Adam S. Bobel/
Adam S. Bobel
Registration No. 70602
Attorney for Applicant

Indianapolis, Indiana
(317) 261-7942
adam.bobel@btlaw.com



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336
107540 7590 11/06/2018 Barnes & Thornburg LLP (Dow) 11 South Meridian Street Indianapolis, IN 46204			EXAMINER KRUSE, DAVID H	
			ART UNIT 1663	PAPER NUMBER
			NOTIFICATION DATE 11/06/2018	DELIVERY MODE ELECTRONIC

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Nov 07, 2018 04:21:01 AM

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	N271	11/06/2018	14764-245772

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Michele Lyons	(Depositor's name)
/Michele Lyons/	(Signature)
November 12, 2018	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	14764-245772	3336

TITLE OF INVENTION: NOVEL HERBICIDE RESISTANCE GENES

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
KRUSE, DAVID H	1663	800-300000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

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- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

BARNES & THORNBURG LLP

2 _____

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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

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Dow AgroSciences LLC

(B) RESIDENCE: (CITY and STATE OR COUNTRY)
Indianapolis IN USA

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- ☐ Publication Fee (No small entity discount permitted)
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- ☐ Applicant asserting small entity status. See 37 CFR 1.27
- ☐ Applicant changing to regular undiscounted fee status.

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Authorized Signature **/C. Philip Poirier/**

Date **11/12/18**

Typed or printed name **C. Philip Poirier**

Registration No. **43,006**

Electronic Patent Application Fee Transmittal				
Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Carl D. Corvin/Michele Lyons		
Attorney Docket Number:		14764-245772		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1000

Electronic Acknowledgement Receipt	
EFS ID:	34282062
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Carl D. Corvin/Michele Lyons
Filer Authorized By:	Carl D. Corvin
Attorney Docket Number:	14764-245772
Receipt Date:	12-NOV-2018
Filing Date:	19-SEP-2014
Time Stamp:	20:42:05
Application Type:	Utility under 35 USC 111(a)

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.H.K/

Sheet 1 of 1

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT				ATTY. DOCKET NO. 14764-245772		APPLICATION NO. 14/491,197	
				APPLICANT Terry R. Wright et al.			
				FILING DATE September 19, 2014		GROUP 1663	
U.S. PATENT DOCUMENTS							
*Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate
	AA	5,608,147	03-1997	Bryan J. Kaphammer			
	AB	7,205,561	04/2007 6-1998	Palit, et al. Chelvayohan, et al.			
	AC	6,645,497	11-2003	Malvar et al.			
	AD	8,283,522	10-2012	Wright, Terry R.			
	AE	8,916,752	12-2014	Wright, Terry R.			
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						
FOREIGN PATENT DOCUMENTS							
		Document Number	Date	Country	Class	Subclass	Translation Yes No
	AL	WO 2005/107437	11-2005	WO			
	AM	WO 2007/053482	05-2007	WO			
	AN						
	AO						
OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)							
	AP	SCHLEINITZ, et al., Localization and Characterization of two Novel Genes Encoding Stereospecific Diosxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in <i>Delftia acidovorans</i> MC1, Applied and Environmental Microbiology, Sept. 2004, Vol. 70, p. 5357-5365					
	AR	GRESSEL, Jonathan, Molecular biology of weed control, Transgenic Research 2000, Vol. 9, p. 355-382					
	AS	HEGG, Eric L. et al., Herbicide-Degrading α -Keto Acid-Dependent Enzyme TfdA: Metal Coordination Environment and Mechanistic Insights, Biochemistry, 1999, Vol. 38, p. 16714-16726					
	AT	PERLACK, Frederick J., Modification of the Coding Sequence Enhances Plant Expression of Insect Control Protein Genes, <i>Proc. Natl. Acad. Sci., USA</i> , Biochemistry, April 1991, Vol. 88, p. 3324-3328					
	AU	KASUGA, Mie, Improving Plant Drought, Salt, and Freezing Tolerance by Gene Transfer of a Single Stress-Inducible Transcription Factor, Nature Biotechnology, March 1999, Vol. 17, p. 287-291					
	AV	VERDAGUER, Bertrand, Isolation and Expression in Transgenic Tobacco and Rice Plants, of the Cassava Vein Mosaic Virus (CVMV) Promoter, Plant Molecular Biology, 1996, Vol. 31, p. 1129-1139.					
	AW	WESTENDORF, et al., Purification and Characterisation of the Enantiospecific Dioxygenases from <i>Delftia acidovorans</i> MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides, Acta Biotechnol. 2003, Vol. 23, p. 3-17					
	AX	FOURGOUX-NICOL et al., Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte, Plant Biology, 1999, Vol. 40, p. 857-872					
	AY	Iowa State University Extension 2005 Herbicide manual for Agricultural Professionals, p. 50-72					
	AZ	Halford et al., Weed Technology, 2001, Vol. 15, p. 737-744					
Examiner /DAVID H KRUSE/					Date Considered 06/15/2017		
*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.							

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT <i>(Use as many sheets as necessary)</i> Sheet 1 of 1	Complete if Known	
	Application Number:	14/491197
	Filing Date:	September 19, 2014
	First Named Inventor:	WRIGHT, Terry R.
	Group Art Unit:	1663
	Confirmation Number:	3336
	Examiner Name:	Kruse, David H.
		Attorney Docket Number: 14764-245772

Change(s) applied
to document,
/K.B./
11/6/2018

U.S. PATENT DOCUMENTS						
Examiner Initials	Doc. No.	U.S. Patent Document Application or Patent Number	Kind Code	Name of Patentee or Applicant	Date of Publication	Filing Date If Appropriate
	BA	4683195		Mullis et al.	07/1987	07-28-1997
	BB	4761373		Anderson et al.	08-02-1988	

FOREIGN PATENT DOCUMENTS								
Examiner Initials	Doc. No.	Office	Foreign Patent Document Application or Patent Number	Kind Code	Name of Patentee or Applicant	Date of Publication	Translation Yes	No*+
	BC	WO	2003/034813		U.S. Secretary of Agriculture	05-01-2003		

OTHER - NON PATENT LITERATURE DOCUMENTS							
Examiner Initials	Doc. No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number (s), publisher, city and/or country where published.				Translation Yes	
	BD	Muller et al., "Purification and Characterization of Two Enantioselective α -Ketoglutarate-Dependent Dioxygenases, RdpA and SdpA, from <i>Sphingomonas herbicidovorans</i> MH," Appl. Environ. Microbiol., 72(7):4853-61 (2006).					
	BE	Schleinitz et al., "Delftia acidovorans plasmid pMC1, partial sequence," Appl. Environ. Microbiol. 70 (9), 5357-5365, Genbank accession no. AY327575 (2004).					
	BF	Westendorf et al., "The two enantiospecific dichloroprop/ α -ketoglutarate-dioxygenases from <i>Delftia acidovorans</i> MC1 - protein and sequence data of RdpA and SdpA," Microbiol. Res.157: 317-322 (2002).					
	BG	Wright et al., "Robust crop resistance to broadleaf and grass herbicides provided by aryloxyalkanoate dioxygenase transgenes," Natl. Acad. Sci., 107(47): 20240-5 (2010).					

DMS 12002559v1

EXAMINER	/DAVID H KRUSE/	DATE CONSIDERED	08/08/2018
----------	-----------------	-----------------	------------

* A concise statement of relevance is being submitted in lieu of a translation. 37 CFR 1.98(a)(3).

+ An English-language equivalent/patent, or an English-language abstract, or an English-language version of the search report or action by a foreign patent office in a counterpart foreign application indicating the degree of relevance found by the foreign office is being submitted in lieu of a concise explanation of relevance under 37 CFR 1.98(a)(3).



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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	01/01/2019	10167483	14764-245772	3336

107540 7590 12/12/2018
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 527 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

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APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Terry R. Wright, Carmel, IN;
Dow AgroSciences LLC, Indianapolis, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, Zionsville, IN;
Donald Merlo, Carmel, IN;
Jayakumar Pon Samuel, Carmel, IN;
Gaofeng Lin, Zionsville, IN;

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IR 103 (Rev. 10/09)

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Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Dec 13, 2018 04:52:17 AM

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UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	ISSUE.NTF	12/12/2018	14764-245772

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