



(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION
(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2016/08/08
(87) Date publication PCT/PCT Publication Date: 2017/03/09
(85) Entrée phase nationale/National Entry: 2018/02/08
(86) N° demande PCT/PCT Application No.: US 2016/046041
(87) N° publication PCT/PCT Publication No.: 2017/039969
(30) Priorités/Priorities: 2015/09/01 (US62/212,716);
2016/04/18 (US62/323,852); 2016/08/04 (US15/228,993)

(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01),
A01N 43/90 (2006.01), *C12N 9/02* (2006.01)
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(54) Titre : METHODES ET COMPOSITIONS POUR LA TOLERANCE AUX HERBICIDES DES PLANTES
(54) Title: METHODS AND COMPOSITIONS FOR HERBICIDE TOLERANCE IN PLANTS

(57) **Abrégé/Abstract:**

The invention relates to biotechnology and provides novel recombinant DNA molecules and engineered proteins for conferring tolerance to protoporphyrinogen oxidase-inhibitor herbicides. The invention also provides herbicide tolerant transgenic plants, seeds, cells, and plant parts containing the recombinant DNA molecules, as well as methods of using the same.

(51) International Patent Classification:
C12N 15/82 (2006.01) C12N 9/02 (2006.01)
A01N 43/90 (2006.01)

(21) International Application Number:
PCT/US2016/046041

(22) International Filing Date:
8 August 2016 (08.08.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/212,716 1 September 2015 (01.09.2015) US
62/323,852 18 April 2016 (18.04.2016) US
15/228,993 4 August 2016 (04.08.2016) US

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

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

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHODS AND COMPOSITIONS FOR HERBICIDE TOLERANCE IN PLANTS

(57) Abstract: The invention relates to biotechnology and provides novel recombinant DNA molecules and engineered proteins for conferring tolerance to protoporphyrinogen oxidase-inhibitor herbicides. The invention also provides herbicide tolerant transgenic plants, seeds, cells, and plant parts containing the recombinant DNA molecules, as well as methods of using the same.

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TITLE

METHODS AND COMPOSITIONS FOR HERBICIDE TOLERANCE IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of United States Provisional Application No. 62/212,716, filed September 1, 2015, United States Provisional Application No. 62/323,852, filed April 18, 2016, and United States Non-Provisional Application No. 15/228,993, filed August 4, 2016, the disclosures of which are hereby incorporated by reference in their entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] The sequence listing that is contained in the file named MONS384WO_sequence_listing.txt, which is 204 kilobytes (measured in MS-Windows) and created on August 3, 2016, is filed herewith by electronic submission and incorporated herein by reference.

BACKGROUND

Field of the Invention

[0003] The present invention relates to the field of biotechnology. More specifically, the invention relates to recombinant DNA molecules encoding enzymes that provide tolerance to herbicides that inhibit protoporphyrinogen oxidase.

Related Art

[0004] Agricultural crop production often utilizes transgenic traits created using the methods of biotechnology. A heterologous gene, also known as a transgene, can be introduced into a plant to produce a transgenic trait. Expression of the transgene in the plant confers a trait, such as herbicide tolerance, on the plant. Examples of transgenic herbicide tolerance traits include glyphosate tolerance, glufosinate tolerance, and dicamba tolerance. With the increase of weed species resistant to the commonly used herbicides, new herbicide tolerance traits are needed in the field. Herbicides of particular interest include herbicides that inhibit protoporphyrinogen oxidase (PPO, EC 1.3.3.4), referred to as PPO herbicides. PPO herbicides provide control of a

spectrum of herbicide-resistant weeds, thus making a trait conferring tolerance to these herbicides particularly useful in a cropping system combined with one or more other herbicide-tolerance trait(s).

[0005] Protoporphyrinogen oxidase functions in both chlorophyll and heme biosynthesis pathways where it converts protoporphyrinogen IX to protoporphyrin IX. Following production of protoporphyrin IX, the chlorophyll and heme biosynthetic pathways diverge with different metal ions being incorporated (iron for heme and magnesium for chlorophyll). Segments of this pathway are conserved across prokaryotes and eukaryotes, and many of the PPO enzymes found across prokaryotes and eukaryotes are relatively similar. Some prokaryotes (*e.g.*, cyanobacteria) use this pathway for chlorophyll and heme production while other prokaryotes (*e.g.*, *Escherichia coli*) use this pathway for heme production.

[0006] Herbicide-insensitive protoporphyrinogen oxidases (iPPOs) have been isolated from a number of prokaryotes and eukaryotes. On a structural basis, it is believed that there are at least three distinct subclasses of PPO enzymes: HemY (M Hansson and L Hederstedt, "Cloning and characterization of the *Bacillus subtilis* hemEHY gene cluster, which encodes protoheme IX biosynthetic enzymes" *Journal of Bacteriology* 174(24):8081-8093 (1992)), HemG (A Sasarman, *et al.*, "Mapping of a new hem gene in *Escherichia coli* K12" *Microbiology* 113:297-303 (1979)), and HemJ (TO Boynton, *et al.*, "Discovery of a gene involved in a third bacterial protoporphyrinogen oxidase activity through comparative genomic analysis and functional complementation" *Applied and Environmental Microbiology* 77(14):4795-4801 (2011)). This invention provides novel recombinant iPPOs that are members of the HemY family. Despite over twenty years of research and the number of iPPOs identified to date, a transgenic crop plant comprising a recombinant iPPO has yet to be commercialized. A strong weed control platform depends, in part, on continued development of herbicide tolerance trait packages. Identifying and utilizing iPPOs to create transgenic crop traits therefore represents an advance to agriculture.

SUMMARY OF THE INVENTION

[0007] In one aspect, the invention provides a recombinant DNA molecule comprising a heterologous promoter operably linked to a nucleic acid sequence encoding a polypeptide that has at least 85% sequence identity to an amino acid sequence chosen from SEQ ID NOs:1-2 and

SEQ ID NOs:6-12, wherein the polypeptide has herbicide-insensitive protoporphyrinogen oxidase activity. In certain embodiments, the polypeptide has at least about 85% sequence identity, at least about 90% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity, or at least 99% sequence identity to an amino acid sequence chosen from among SEQ ID NOs:1-2 and SEQ ID NOs:6-12 and has herbicide-insensitive protoporphyrinogen oxidase activity. In some embodiments there is provided a recombinant DNA molecule, wherein the nucleic acid sequence is selected from the group consisting of SEQ ID NOs:26-27, 31-32, 36-46, and 47-48. In particular embodiments the recombinant DNA molecule encodes a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:1-2 and SEQ ID NOs:6-12. A recombinant polypeptide that comprises at least 85% sequence identity to the full length of an amino acid sequence chosen from among SEQ ID NOs:1-2 and SEQ ID NOs:6-12, wherein the polypeptide has herbicide-insensitive protoporphyrinogen oxidase activity is therefore provided by the invention.

[0008] In certain embodiments a heterologous promoter, for instance, a promoter functional in a plant cell, is operably linked to the nucleic acid sequence encoding a polypeptide that has at least 85% sequence identity to an amino acid sequence of the invention, for instance an amino acid sequence chosen from SEQ ID NOs:1-2 and SEQ ID NOs:6-12, wherein the polypeptide has herbicide-insensitive protoporphyrinogen oxidase activity. Such a resulting DNA molecule may further comprise a targeting sequence that functions to localize the polypeptide within a cell.

[0009] In one aspect, the invention provides a DNA construct comprising a recombinant DNA molecule of the invention. In one embodiment, such a DNA construct comprises, in operable linkage to a nucleic acid sequence of the invention, a targeting sequence that functions to localize the polypeptide within a cell. The DNA construct may be present in the genome of a transgenic plant, seed, or cell. In certain embodiments, the polypeptide confers herbicide tolerance to the cell, plant, seed, or plant part.

[0010] Another aspect of the invention provides a transgenic plant, seed, cell, or plant part comprising a recombinant DNA molecule of the invention or a recombinant polypeptide of the invention. The transgenic plant, seed, cell, or plant part may thus comprise, *i.e.* display, tolerance

to at least one PPO herbicide. In some embodiments, the transgenic plant, seed, cell, or plant part comprises an additional transgenic herbicide tolerance trait.

[0011] Another aspect of the invention provides a method for conferring herbicide tolerance to a plant, seed, cell, or plant part comprising: heterologously expressing a recombinant polypeptide of the invention in the plant, seed, cell, or plant part. In some embodiments of the method, the plant, seed, cell, or plant part comprises protoporphyrinogen oxidase activity conferred by the recombinant polypeptide. In some embodiments, the herbicide tolerance is to at least one PPO herbicide selected from the group consisting of acifluorfen, fomesafen, lactofen, fluoroglycofen-ethyl, oxyfluorfen, flumioxazin, azafenidin, carfentrazone-ethyl, sulfentrazone, fluthiacet-methyl, oxadiargyl, oxadiazon, pyraflufen-ethyl, saflufenacil and S-3100.

[0012] Another aspect of the invention relates to a method of plant transformation, comprising the steps of: a) introducing a recombinant DNA molecule of the invention into a plant cell; and b) regenerating a transgenic plant therefrom that comprises the recombinant DNA molecule. The method may further comprise the step of selecting a plant that is tolerant to at least one PPO herbicide. The method may also further comprise a step of crossing the regenerated plant with itself or with a second plant and collecting seed from the cross.

[0013] Yet another aspect of the invention provides a method for controlling weeds in a plant growth area, comprising contacting a plant growth area comprising the transgenic plant or seed with at least one PPO herbicide, wherein the transgenic plant or seed is tolerant to the PPO herbicide and wherein weeds are controlled in the plant growth area.

[0014] Also provided is a method of identifying a nucleotide sequence encoding a protein having protoporphyrinogen oxidase activity, the method comprising: a) transforming an *E. coli* strain having a gene knockout for the native *E. coli* PPO enzyme with a bacterial expression vector comprising a recombinant DNA molecule encoding a candidate herbicide tolerance protein; and b) growing said transformed *E. coli* using a heme-free bacterial medium, wherein growth using said bacterial medium identifies a protein having protoporphyrinogen oxidase activity.

[0015] Further provided by the invention is a method of identifying a nucleotide sequence encoding a protein having herbicide-insensitive protoporphyrinogen oxidase activity, the method

comprising: a) transforming an *E. coli* strain having a gene knockout for the native *E. coli* PPO enzyme with a bacterial expression vector comprising a recombinant DNA molecule encoding a recombinant protein; and b) growing said transformed *E. coli* using a bacterial medium containing at least one PPO herbicide, wherein growth of bacteria identifies a protein having herbicide-insensitive protoporphyrinogen oxidase activity.

[0016] Another aspect of the invention relates to a method of screening for a herbicide tolerance gene comprising: a) expressing a recombinant DNA molecule of the invention in a plant cell; and b) identifying a plant cell that displays tolerance to a PPO herbicide.

[0017] Further, the invention provides methods of screening for a herbicide tolerance gene comprising: a) expressing a recombinant DNA molecule of the invention in a bacterial cell lacking HemG, wherein the bacterial cell is grown in a heme-free medium in the presence of a PPO herbicide; and b) identifying a bacterial cell that displays tolerance to a PPO herbicide.

[0018] In another aspect, the invention provides a method of producing a plant tolerant to a PPO herbicide and at least one other herbicide comprising: a) obtaining a plant comprising a recombinant DNA molecule of the invention; b) crossing the transgenic plant with a second plant comprising tolerance to the at least one other herbicide, and c) selecting a progeny plant resulting from said crossing that comprises tolerance to a PPO herbicide and the at least one other herbicide is another aspect of the invention.

[0019] The invention also provides, in another aspect, a method for reducing the development of herbicide tolerant weeds comprising: a) cultivating in a crop growing environment a plant of the present invention that comprises tolerance to a PPO herbicide, for instance by comprising a DNA molecule of the present invention, and comprises tolerance to at least one other herbicide; and b) applying a PPO herbicide and at least one other herbicide to the crop growing environment, wherein the crop plant is tolerant to the PPO herbicide and the at least one other herbicide. In certain embodiments of the method, the PPO herbicide may be selected from the group consisting of acifluorfen, fomesafen, lactofen, fluoroglycofen-ethyl, oxyfluorfen, flumioxazin, azafenidin, carfentrazone-ethyl, sulfentrazone, fluthiacet-methyl, oxadiargyl, oxadiazon, pyraflufen-ethyl, saflufenacil and S-3100. In some embodiments of the method, the at least one other herbicide is selected from the group consisting of: an ACCase inhibitor, an ALS inhibitor, an EPSPS inhibitor, a synthetic auxin, a photosynthesis inhibitor, a

glutamine synthesis inhibitor, a HPPD inhibitor, a PPO inhibitor, and a long-chain fatty acid inhibitor. In particular embodiments, the ACCase inhibitor is an aryloxyphenoxy propionate or a cyclohexanedione; the ALS inhibitor is a sulfonylurea, imidazolinone, triazoloyrimidine, or a triazolinone; the EPSPS inhibitor is glyphosate; the synthetic auxin is a phenoxy herbicide, a benzoic acid, a carboxylic acid, or a semicarbazone; the photosynthesis inhibitor is a triazine, a triazinone, a nitrile, a benzothiadiazole, or a urea; the glutamine synthesis inhibitor is glufosinate; the HPPD inhibitor is an isoxazole, a pyrazolone, or a triketone; the PPO inhibitor is a diphenylether, a N-phenylphthalimide, an aryl triazinone, or a pyrimidinedione; or the long-chain fatty acid inhibitor is a chloroacetamide, an oxyacetamide, or a pyrazole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1. Assay results from herbicide bacterial screening system with PPO herbicides. Assay of *E. coli* HemG (H_N10) (SEQ ID NO:76), HemY PPO R1N473 (SEQ ID NO:13), HemY PPO R1N533 (SEQ ID NO:14); or HemY PPO R1N171 (SEQ ID NO:15) in the presence of acifluorfen, flumioxazin, lactofen, or fomesafen PPO herbicides.

[0021] Figure 2. Assay results from herbicide bacterial screening system with PPO herbicides. Assay of HemY R2N30 (SEQ ID NO:1), Hem Y R2N40 (SEQ ID NO:2), HemY R2N70 (SEQ ID NO:3), HemY R2N90 (SEQ ID NO:4), HemY R2N100 (SEQ ID NO:5), and negative control *Amaranthus tuberculatus* (WH) PPO (SEQ ID NO:80) in the presence of acifluorfen and S-3100 PPO herbicides

BRIEF DESCRIPTION OF THE SEQUENCES

[0022] SEQ ID NO:1 is the amino acid sequence of R2N30.

[0023] SEQ ID NO:2 is the amino acid sequence of R2N40.

[0024] SEQ ID NO:3 is the amino acid sequence of R2N70.

[0025] SEQ ID NO:4 is the amino acid sequence of R2N90.

[0026] SEQ ID NO:5 is the amino acid sequence of R2N100.

[0027] SEQ ID NO:6 is the amino acid sequence of a variant of SEQ ID NO:1 (R2N30).

- [0028] SEQ ID NOs:7-12 are the amino acid sequences of variants of SEQ ID NO:2 (R2N40).
- [0029] SEQ ID NO:13 is the amino acid sequence of R1N473.
- [0030] SEQ ID NO:14 is the amino acid sequence of R1N533.
- [0031] SEQ ID NO:15 is the amino acid sequence of R1N171.
- [0032] SEQ ID NO:16 is the amino acid sequence of R1N311.
- [0033] SEQ ID NO:17 is the amino acid sequence of R1N333.
- [0034] SEQ ID NO:18 is a truncated amino acid sequence of R1N473.
- [0035] SEQ ID NO:19 is a truncated amino acid sequence of R1N533.
- [0036] SEQ ID NO:20 is a truncated amino acid sequence of R1N171.
- [0037] SEQ ID NO:21 is a truncated amino acid sequence of R1N333.
- [0038] SEQ ID NO:22 is a truncated amino acid sequence of R1N473.
- [0039] SEQ ID NO:23 is a truncated amino acid sequence of R1N533.
- [0040] SEQ ID NO:24 is a truncated amino acid sequence of R1N171.
- [0041] SEQ ID NO:25 is a truncated amino acid sequence of R1N333.
- [0042] SEQ ID NOs:26-30 are the native bacterial nucleotide sequences encoding SEQ ID NOs:1 through SEQ ID NO:5, respectively.
- [0043] SEQ ID NOs:31-35 are nucleotide sequences encoding SEQ ID NOs:1-5, respectively, codon optimized for dicot expression.
- [0044] SEQ ID NOs:36-42 are nucleotide sequences encoding SEQ ID NOs:6-12, respectively, codon optimized for dicot expression.
- [0045] SEQ ID NOs:43-46 are nucleotide sequences encoding SEQ ID NO:9, codon optimized for dicot expression.
- [0046] SEQ ID NOs:47-51 are nucleotide sequences encoding SEQ ID NOs:1-5, respectively, codon optimized for monocot expression.
- [0047] SEQ ID NOs:52-56 are the native bacterial nucleotide sequences encoding SEQ ID NOs:13-17.
- [0048] SEQ ID NOs:57-62 are the nucleotide sequences encoding the amino acid sequences represented by SEQ ID NOs:13-17, codon optimized for dicot expression.
- [0049] SEQ ID NOs:63-67 are the nucleotide sequences encoding the amino acid sequences represented by SEQ ID NOs:13-17, codon optimized for monocot expression.

[0050] SEQ ID NOs:68-75 are the nucleotide sequences encoding the amino acid sequences represented by SEQ ID NOs:18-25, respectively, codon optimized for dicot expression.

[0051] SEQ ID NO:76 is the amino acid sequence of *E. coli* PPO enzyme HemG (protoporphyrinogen IX dehydrogenase; GenBank Accession No. WP_021498199).

[0052] SEQ ID NOs:77-79 are the nucleotide sequences encoding the amino acid sequence represented by SEQ ID NO:76.

[0053] SEQ ID NO:80 is the amino acid sequence of the wild-type protoporphyrinogen oxidase from *Amaranthus tuberculatus* (WH).

[0054] SEQ ID NO:81 is the nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO:80, codon optimized for bacterial *E. coli* expression.

DETAILED DESCRIPTION

[0055] The following descriptions and definitions are provided to better define the invention and to guide those of ordinary skill in the art in the practice of the invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

[0056] The invention provides novel, recombinant DNA molecules and proteins that encode herbicide-insensitive protoporphyrinogen oxidases (iPPOs). For instance, the invention provides in one embodiment vectors and expression cassettes encoding microbially derived iPPOs for expression in cells and plants. Methods for producing cells and plants tolerant to PPO herbicides are also provided. The invention further provides methods and compositions for using protein engineering and bioinformatic tools to obtain and improve iPPOs.

[0057] In specific aspects, the invention provides recombinant DNA molecules and proteins. As used herein, the term “recombinant” refers to a non-naturally occurring DNA, protein, cell, seed, or organism that is the result of genetic engineering and as such would not normally be found in nature. A “recombinant DNA molecule” is a DNA molecule comprising a DNA sequence that does not naturally occur in nature and as such is the result of human intervention, such as a DNA molecule comprised of at least two DNA molecules heterologous to each other. An example of a recombinant DNA molecule is a DNA molecule provided herein encoding herbicide-insensitive protoporphyrinogen oxidase operably linked to a heterologous regulatory or

other element, such as a heterologous promoter. A “recombinant protein” is a protein comprising an amino acid sequence that does not naturally occur and as such is the result of human intervention, such as an engineered protein or a chimeric protein. A recombinant cell, seed, or organism is a cell, seed, or organism comprising transgenic DNA, for example a transgenic cell, seed, plant, or plant part comprising a recombinant DNA molecule and therefore produced as a result of plant transformation.

[0058] As used herein, the term “genetic engineering” refers to the creation of a non-natural DNA, protein, or organism that would not normally be found in nature and therefore entails applying human intervention. Genetic engineering can be used to produce an engineered DNA, protein, or organism that was conceived of and created in the laboratory using one or more of the techniques of biotechnology such as molecular biology, protein biochemistry, bacterial transformation, and plant transformation. For example, genetic engineering can be used to create a chimeric gene comprising at least two DNA molecules heterologous to each other using one or more of the techniques of molecular biology, such as gene cloning, DNA ligation, and DNA synthesis. A chimeric gene may consist of two or more heterologous DNA molecules that are operably linked, such as a protein-coding sequence operably linked to a gene expression element such as a transit peptide-coding sequence or a heterologous promoter. Genetic engineering can be used to create an engineered protein whose amino acid sequence was created using one or more of the techniques of protein engineering, such as protein design using site-directed mutagenesis and directed evolution using random mutagenesis and DNA shuffling. An engineered protein may have one or more deletions, insertions, or substitutions relative to the coding sequence of the wild-type protein and each deletion, insertion, or substitution may consist of one or more amino acids. In another embodiment, an engineered protein may consist of two heterologous peptides that are operably linked, such as an enzyme operably linked to a transit peptide.

[0059] As used herein, “herbicide-insensitive” or “herbicide-insensitive protoporphyrinogen oxidase activity” means the ability of a protoporphyrinogen oxidase (PPO, EC 1.3.3.4) to maintain at least some of its protoporphyrinogen oxidase activity in the presence of one or more PPO herbicide(s). The term “protoporphyrinogen oxidase activity” means the ability to catalyze the six-electron oxidation (removal of electrons) of protoporphyrinogen IX to form protoporphyrin IX, that is, to catalyze the dehydrogenation of protoporphyrinogen to form

protoporphyrin. Enzymatic activity of a protoporphyrinogen oxidase can be measured by any means known in the art, for example, by an enzymatic assay in which the production of the product of protoporphyrinogen oxidase or the consumption of the substrate of protoporphyrinogen oxidase in the presence of one or more PPO herbicide(s) is measured via fluorescence, high performance liquid chromatography (HPLC), or mass spectrometry (MS). Another example of an assay for measuring enzymatic activity of a protoporphyrinogen oxidase is a bacterial assay, such as the assays described herein, whereby a recombinant protoporphyrinogen oxidase is expressed in a bacterial cell otherwise lacking PPO activity and the ability of the recombinant protoporphyrinogen oxidase to complement this knockout phenotype is measured. Herbicide-insensitivity may be complete or partial insensitivity to a particular herbicide, and may be expressed as a percent (%) tolerance or insensitivity to a particular PPO herbicide. As used herein, an “herbicide-insensitive protoporphyrinogen oxidase” or “iPPO” exhibits herbicide-insensitivity in the presence of one or more PPO herbicide(s).

[0060] As used herein, a “*hemG* knockout strain” means an organism or cell of an organism, such as *E. coli*, that lacks HemG activity to the extent that it is unable to grow on heme-free growth medium, or such that its growth is detectably impaired in the absence of heme relative to an otherwise isogenic strain comprising a functional HemG. A *hemG* knockout strain of, for instance, *E. coli* may be prepared in view of knowledge in the art, for instance in view of the *E. coli hemG* sequence (Ecogene Accession No. EG11485; Sasarman *et al.*, “Nucleotide sequence of the *hemG* gene involved in the protoporphyrinogen oxidase activity of *E. coli* K12” *Can J Microbiol* 39:1155-1161, 1993).

[0061] As used herein, the term “transgene” refers to a DNA molecule artificially incorporated into an organism’s genome because of human intervention, such as a plant transformation method. As used herein, the term “transgenic” means comprising a transgene, for example a “transgenic plant” refers to a plant comprising a transgene in its genome and a “transgenic trait” refers to a characteristic or phenotype conveyed or conferred by the presence of a transgene incorporated into the plant genome. Because of such genomic alteration, the transgenic plant is something distinctly different from the related wild-type plant and the transgenic trait is a trait not naturally found in the wild-type plant. Transgenic plants of the invention comprise the recombinant DNA molecules and engineered proteins provided by the invention.

[0062] As used herein, the term “heterologous” refers to the relationship between two or more items derived from different sources and thus not normally associated in nature. For example, a protein-coding recombinant DNA molecule is heterologous with respect to an operably linked promoter if such a combination is not normally found in nature. In another example, a protein-coding DNA molecule or a polypeptide may be heterologously expressed in a plant, seed, cell, or plant part if such protein-coding DNA molecule or polypeptide is not normally expressed in such a plant, seed, cell, or plant part in nature. A particular recombinant DNA molecule may be heterologous with respect to a cell, seed, or organism into which it is inserted when it would not naturally occur in that particular cell, seed, or organism. A particular polypeptide may be heterologous with respect to a cell, seed, or organism in which it is expressed when it would not naturally occur in that particular cell, seed, or organism.

[0063] As used herein, the term “isolated” refers to at least partially separating a molecule from other molecules typically associated with it in its natural state. In one embodiment, the term “isolated” refers to a DNA molecule that is separated from the nucleic acids that normally flank the DNA molecule in its natural state. For example, a DNA molecule encoding a protein that is naturally present in a bacterium would be an isolated DNA molecule if it was not within the DNA of the bacterium from which the DNA molecule encoding the protein is naturally found. Thus, a DNA molecule fused to or operably linked to one or more other DNA molecule(s) with which it would not be associated in nature, for example as the result of recombinant DNA or plant transformation techniques, is considered isolated herein. Such molecules are considered isolated even when integrated into the chromosome of a host cell or present in a nucleic acid solution with other DNA molecules.

[0064] As used herein, the term “protein-coding DNA molecule” refers to a DNA molecule comprising a nucleotide sequence that encodes a protein. A “protein-coding sequence” means a nucleic acid sequence that encodes a protein. A “sequence” means a sequential arrangement of nucleotides or amino acids. The boundaries of a protein-coding sequence may be determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A protein-coding molecule may comprise a nucleic acid sequence encoding an amino acid sequence. As used herein, “transgene expression”, “expressing a transgene”, “protein expression”, and “expressing a protein” mean the production of a protein through the process of transcribing a DNA molecule into messenger RNA (mRNA) and translating the mRNA into

polypeptide chains, which are ultimately folded into proteins. A protein-coding DNA molecule may be operably linked to a heterologous promoter in a DNA construct for use in expressing the protein in a cell transformed with the recombinant DNA molecule. As used herein, “operably linked” means two or more DNA molecules or two or more polypeptides linked in manner so that one may affect the function of the other. Operably-linked DNA molecules or operably-linked polypeptides may be part of a single contiguous molecule and may or may not be adjacent. For example, a promoter is operably linked with a protein-coding DNA molecule in a DNA construct where the two DNA molecules are so arranged that the promoter may affect the expression of the transgene.

[0065] As used herein, a “DNA construct” is a recombinant DNA molecule comprising two or more heterologous DNA sequences. DNA constructs are useful for transgene expression and may be comprised in vectors and plasmids. DNA constructs may be used in vectors for the purpose of transformation, that is the introduction of heterologous DNA into a host cell, to produce transgenic plants and cells, and as such may also be contained in the plastid DNA or genomic DNA of a transgenic plant, seed, cell, or plant part. As used herein, a “vector” means any recombinant DNA molecule that may be used for the purpose of bacterial or plant transformation. Recombinant DNA molecules as set forth in the sequence listing, can, for example, be inserted into a vector as part of a construct having the recombinant DNA molecule operably linked to a gene expression element that functions in a plant to affect expression of the engineered protein encoded by the recombinant DNA molecule. General methods useful for manipulating DNA molecules for making and using recombinant DNA constructs and plant transformation vectors are well known in the art and described in detail in, for example, handbooks and laboratory manuals including Michael R. Green and Joseph Sambrook, “Molecular Cloning: A Laboratory Manual” (Fourth Edition) ISBN:978-1-936113-42-2, Cold Spring Harbor Laboratory Press, NY (2012). The components for a DNA construct, or a vector comprising a DNA construct, include one or more gene expression elements operably linked to a transcribable nucleic acid sequence, such as the following: a promoter for the expression of an operably linked DNA, an operably linked protein-coding DNA molecule, and an operably linked 3’ untranslated region (UTR). Gene expression elements useful in practicing the present invention include, but are not limited to, one or more of the following type of elements:

promoter, 5' UTR, enhancer, leader, cis-acting element, intron, targeting sequence, 3' UTR, and one or more selectable marker transgenes.

[0066] The DNA constructs of the invention may include a promoter operably linked to a protein-coding DNA molecule provided by the invention, whereby the promoter drives expression of the recombinant protein molecule. Promoters useful in practicing the present invention include those that function in a cell for expression of an operably linked polynucleotide, such as a bacterial or plant promoter. Plant promoters are varied and well known in the art and include, for instance, those that are inducible, viral, synthetic, constitutive, temporally regulated, spatially regulated, and/or spatio-temporally regulated.

[0067] In one embodiment of the invention, a DNA construct provided herein includes a nucleic acid sequence encoding a targeting sequence that is operably linked to a heterologous nucleic acid sequence encoding a polypeptide molecule that has herbicide-insensitive protoporphyrinogen oxidase activity, whereby the targeting sequence facilitates localizing the polypeptide molecule within the cell. Targeting sequences are known in the art as signal sequences, targeting peptides, localization sequences, and transit peptides. An example of a targeting sequence is a chloroplast transit peptide (CTP), a mitochondrial targeting sequence (MTS), or a dual chloroplast and mitochondrial targeting peptide. By facilitating protein localization within the cell, the targeting sequence may increase the accumulation of recombinant protein, protect the protein from proteolytic degradation, and/or enhance the level of herbicide tolerance, and thereby reduce levels of injury in the transgenic cell, seed, or organism after herbicide application.

[0068] CTPs and other targeting molecules that may be used in connection with the present invention are known in the art and include, but are not limited to, the *Arabidopsis thaliana* EPSPS CTP (Klee *et al.*, *Mol Gen Genet.* 210:437-442, 1987), the *Petunia hybrida* EPSPS CTP (della-Cioppa *et al.*, *PNAS* 83:6873-6877, 1986), the maize cab-m7 signal sequence (Becker *et al.*, *Plant Mol Biol.* 20:49-60, 1992; PCT WO 97/41228), a mitochondrial pre-sequence (*e.g.* Silva Filho *et al.*, *Plant Mol Biol* 30:769-780, 1996), and the pea glutathione reductase signal sequence (Creissen *et al.*, *Plant J.* 8:167-175, 1995; PCT WO 97/41228).

[0069] Recombinant DNA molecules of the present invention may be synthesized and modified by methods known in the art, either completely or in part, where it is desirable to provide sequences useful for DNA manipulation (such as restriction enzyme recognition sites or

recombination-based cloning sites), plant-preferred sequences (such as plant-codon usage or Kozak consensus sequences), or sequences useful for DNA construct design (such as spacer or linker sequences). The present invention includes recombinant DNA molecules and engineered proteins having at least 70% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity, and at least 99% sequence identity to any of the recombinant DNA molecule or amino acid sequences provided herein, and having herbicide-insensitive protoporphyrinogen oxidase activity. As used herein, the term “percent sequence identity” or “% sequence identity” refers to the percentage of identical nucleotides or amino acids in a linear polynucleotide or amino acid sequence of a reference (“query”) sequence (or its complementary strand) as compared to a test (“subject”) sequence (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide or amino acid insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the Sequence Analysis software package of the GCG® Wisconsin Package® (Accelrys Inc., San Diego, CA), MEGAlign (DNASTar Inc., 1228 S. Park St., Madison, WI 53715), and MUSCLE (version 3.6) (RC Edgar, “MUSCLE: multiple sequence alignment with high accuracy and high throughput” *Nucleic Acids Research* 32(5):1792-7 (2004)) for instance with default parameters. An “identity fraction” for aligned segments of a test sequence and a reference sequence is the number of identical components that are shared by the two aligned sequences divided by the total number of components in the portion of the reference sequence segment being aligned, that is, the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more sequences may be to a full-length sequence or a portion thereof, or to a longer sequence. Engineered proteins may be produced by changing (that is, modifying) a wild-type protein to produce a new protein with modified characteristic(s) *e.g.* a particular cellular localization pattern, such as targeted to the chloroplast or mitochondria,

or a novel combination of useful protein characteristics, such as altered $V_{\max}$, K_m , K_i , IC_{50} , substrate specificity, inhibitor/herbicide specificity, substrate selectivity, the ability to interact with other components in the cell such as partner proteins or membranes, and protein stability, among others. Modifications may be made at specific amino acid positions in a protein and may be a substitution of the amino acid found at that position in nature (that is, in the wild-type protein) with a different amino acid. Engineered proteins provided by the invention thus provide a new protein with one or more altered protein characteristics relative to a similar protein found in nature. In one embodiment of the invention, an engineered protein has altered protein characteristics, such as those that result in decreased sensitivity to one or more herbicides as compared to a similar wild-type protein or improved ability to confer herbicide tolerance on a transgenic plant expressing the engineered protein to one or more herbicides. In one embodiment, the invention provides an engineered protein, and the recombinant DNA molecule encoding it, comprising at least one amino acid substitution selected from Table 1 and having at least about 70% sequence identity, about 80% sequence identity, about 85% sequence identity, about 90% sequence identity, about 95% sequence identity, about 96% sequence identity, about 97% sequence identity, about 98% sequence identity, and about 99% sequence identity to any of the engineered amino acid sequences provided herein, including but not limited to SEQ ID NOs: 1-2 and 6-12. Amino acid mutations may be made as a single amino acid substitution in the protein or in combination with one or more other mutation(s), such as one or more other amino acid substitution(s), deletions, or additions. Mutations may be made by any method known to those of skill in the art.

Table 1: Amino Acid Substitutions.

Residue	Conservative Substitutions	Residue	Conservative Substitutions
Ala	Ser	Leu	Ile; Val
Arg	Lys	Lys	Arg; Gln
Asn	Gln; His	Met	Leu; Ile
Asp	Glu	Phe	Met; Leu; Tyr
Gln	Asn	Ser	Thr; Gly
Cys	Ser	Thr	Ser; Val

Glu	Asp	Trp	Tyr
Gly	Pro	Tyr	Trp; Phe
His	Asn; Gln	Val	Ile; Leu
Ile	Leu; Val		

[0070] As used herein, “wild-type” means a naturally occurring similar, but not identical, version. A “wild-type DNA molecule” or “wild-type protein” is a naturally occurring version of the DNA molecule or protein, that is, a version of the DNA molecule or protein pre-existing in nature. An example of a wild-type protein useful for comparison with the engineered proteins provided by the invention is the protoporphyrinogen oxidase from *Arabidopsis thaliana*. A “wild-type plant” is a non-transgenic plant of the same type as the transgenic plant, and as such is genetically distinct from the transgenic plant comprising the herbicide tolerance trait. Examples of a wild-type plant useful for comparison with transgenic maize plants are non-transgenic LH244 maize (ATCC deposit number PTA-1173) and 01DKD2 inbred maize (I294213) (ATCC deposit number PTA-7859). For transgenic soybean plants an exemplary comparative line would be non-transgenic A3555 soy (ATCC deposit number PTA-10207), and for transgenic cotton plants an exemplary comparative line would be non-transgenic Coker 130 (Plant Variety Protection Number 8900252).

Transgenic Plants & Herbicides

[0071] One aspect of the invention includes transgenic plant cells, transgenic plant tissues, transgenic plants, and transgenic seeds that comprise the recombinant DNA molecules and engineered proteins provided by the invention. These cells, tissues, plants, and seeds comprising the recombinant DNA molecules and engineered proteins exhibit herbicide tolerance to one or more PPO herbicide(s), and, optionally, tolerance to one or more additional herbicide(s).

[0072] Suitable methods for transformation of host plant cells for use with the current invention include virtually any method by which DNA can be introduced into a cell (for example, where a recombinant DNA construct is stably integrated into a plant chromosome) and are well known in the art. An exemplary and widely utilized method for introducing a recombinant DNA construct into plants is the *Agrobacterium* transformation system, which is well known to those of skill in the art. Another exemplary method for introducing a recombinant

DNA construct into plants is insertion of a recombinant DNA construct into a plant genome at a pre-determined site by methods of site-directed integration. Site-directed integration may be accomplished by any method known in the art, for example, by use of zinc-finger nucleases, engineered or native meganucleases, TALE-endonucleases, or an RNA-guided endonuclease (for example a CRISPR/Cas9 system). Transgenic plants can be regenerated from a transformed plant cell by the methods of plant cell culture. A transgenic plant homozygous with respect to a transgene (that is, two allelic copies of the transgene) can be obtained by self-pollinating (selfing) a transgenic plant that contains a single transgene allele with itself, for example an R0 plant, to produce R1 seed. One fourth of the R1 seed produced will be homozygous with respect to the transgene. Plants grown from germinating R1 seed can be tested for zygosity, using a SNP assay, DNA sequencing, or a thermal amplification assay that allows for the distinction between heterozygotes and homozygotes, referred to as a zygosity assay.

[0073] As used herein, a “PPO inhibitor herbicide” or “PPO herbicide” is a chemical that targets and inhibits the enzymatic activity of a protoporphyrinogen oxidase (PPO), which catalyzes the dehydrogenation of protoporphyrinogen IX to form protoporphyrin IX, which is the precursor to heme and chlorophyll. Inhibition of protoporphyrinogen oxidase causes formation of reactive oxygen species, resulting in cell membrane disruption and ultimately the death of susceptible cells. PPO herbicides are well-known in the art and commercially available. Examples of PPO herbicides include, but are not limited to, diphenylethers (such as acifluorfen, its salts and esters, aclonifen, bifenox, its salts and esters, ethoxyfen, its salts and esters, fluoronitrofen, furyloxyfen, halosafen, chlomethoxyfen, fluoroglycofen, its salts and esters, lactofen, its salts and esters, oxyfluorfen, and fomesafen, its salts and esters); thiadiazoles (such as fluthiacet-methyl and thidiazimin); pyrimidinediones or phenyluracils (such as benzfendizone, butafenacil, ethyl [3-(2-chloro-4-fluoro-5-(1-methyl-6-trifluoromethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-3-yl)phenoxy]-2-pyridyloxy]acetate (CAS Registry Number 353292-31-6 and referred to herein as S-3100), flupropacil, saflufenacil, and tiafenacil); phenylpyrazoles (such as fluazolate, pyraflufen and pyraflufen-ethyl); oxadiazoles (such as oxadiargyl and oxadiazon); triazolinones (such as azafenidin, bencarbazone, carfentrazone, its salts and esters, and sulfentrazone); oxazolidinediones (such as pentoxazone); N-phenylphthalimides (such as cinidon-ethyl, flumiclorac, flumiclorac-pentyl, and flumioxazin); benzoxazinone derivatives (such as 1,5-dimethyl-6-thioxo-3-(2,2,7-trifluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H-1,4-

benzoxazin-6-yl)-1,3,5-triazinane-2,4-dione); flufenpyr and flufenpyr-ethyl; pyraclonil; and proflumizone. Protoporphyrinogen oxidases and cells, seeds, plants, and plant parts provided by the invention exhibit herbicide tolerance to one or more PPO herbicide(s).

[0074] Herbicides may be applied to a plant growth area comprising the plants and seeds provided by the invention as a method for controlling weeds. Plants and seeds provided by the invention comprise an herbicide tolerance trait and as such are tolerant to the application of one or more PPO herbicides. The herbicide application may be the recommended commercial rate (1X) or any fraction or multiple thereof, such as twice the recommended commercial rate (2X). Herbicide rates may be expressed as acid equivalent per pound per acre (lb ae/acre) or acid equivalent per gram per hectare (g ae/ha) or as pounds active ingredient per acre (lb ai/acre) or grams active ingredient per hectare (g ai/ha), depending on the herbicide and the formulation. The herbicide application comprises at least one PPO herbicide. The plant growth area may or may not comprise weed plants at the time of herbicide application. A herbicidally effective dose of PPO herbicide(s) for use in an area for controlling weeds may consist of a range from about 0.1X to about 30X label rate(s) over a growing season. The 1X label rate for some exemplary PPO herbicides is provided in Table 2. One (1) acre is equivalent to 2.47105 hectares and one (1) pound is equivalent to 453.592 grams. Herbicide rates can be converted between English and metric as: (lb ai/ac) multiplied by 1.12 = (kg ai/ha) and (kg ai/ha) multiplied by 0.89 = (lb ai/ac).

Table 2: Exemplary PPO Herbicides

PPO Herbicide	Chemical Family	1X Rate
acifluorfen	Diphenylethers	420 g ai/ha
fomesafen	Diphenylethers	420 g ai/ha
lactofen	Diphenylethers	7-220 g ai/ha
fluoroglycofen-ethyl	Diphenylethers	15- 40 g ai/ha
oxyfluorfen	Diphenylethers	0.28-2.24 kg ai/ha
flumioxazin	N-phenylphthalimide	70 g ai/ha
azafenidin	Triazolinone	240 g ai/ha
carfentrazone-ethyl	Triazolinone	4-36 g ai/ha
sulfentrazone	Triazolinone	0.1-0.42 kg ai/ha
fluthiacet-methyl	Thiadiazole	3-15 g ai/ha

oxadiargyl	Oxadiazole	50-150 g ai/ha
oxadiazon	Oxadiazole	2.24-4.48 kg ai/ha
pyraflufen-ethyl	Phenylpyrazole	6-12 g ai/ha
saflufenacil	Pyrimidine dione	25-50 g/ha
S-3100	Pyrimidine dione	5-80 g/ha

[0075] Herbicide applications may be sequentially or tank mixed with one, two, or a combination of several PPO herbicides or any other compatible herbicide. Multiple applications of one herbicide or of two or more herbicides, in combination or alone, may be used over a growing season to areas comprising transgenic plants of the invention for the control of a broad spectrum of dicot weeds, monocot weeds, or both, for example, two applications (such as a pre-planting application and a post-emergence application or a pre-emergence application and a post-emergence application) or three applications (such as a pre-planting application, a pre-emergence application, and a post-emergence application or a pre-emergence application and two post-emergence applications).

[0076] As used herein, “tolerance” or “herbicide tolerance” means a plant, seed, or cell’s ability to resist the toxic effects of an herbicide when applied. Herbicide tolerant crops can continue to grow and are unaffected or minimally affected by the presence of the applied chemical. As used herein, an “herbicide tolerance trait” is a transgenic trait imparting improved herbicide tolerance to a plant as compared to the wild-type plant. Contemplated plants which might be produced with an herbicide tolerance trait of the present invention could include, for instance, any plant including crop plants such as soybean (*e.g. Glycine max*), corn (maize), cotton (*Gossypium sp.*), and canola, among others.

[0077] The transgenic plants, progeny, seeds, plant cells, and plant parts of the invention may also contain one or more additional transgenic traits. Additional transgenic traits may be introduced by crossing a plant containing a transgene comprising the recombinant DNA molecules provided by the invention with another plant containing one or more additional transgenic trait(s). As used herein, “crossing” means breeding two individual plants to produce a progeny plant. Two transgenic plants may thus be crossed to produce progeny that contain the transgenic traits from each parent. As used herein “progeny” means the offspring of any generation of a parent plant, and transgenic progeny comprise a DNA construct provided by the

invention and inherited from at least one parent plant. Alternatively, additional transgenic trait(s) may be introduced by co-transforming a DNA construct for that additional transgenic trait(s) with a DNA construct comprising the recombinant DNA molecules provided by the invention (for example, with all the DNA constructs present as part of the same vector used for plant transformation) or by inserting the additional trait(s) into a transgenic plant comprising a DNA construct provided by the invention or *vice versa* (for example, by using any of the methods of plant transformation or gene editing on a transgenic plant or plant cell). Such additional transgenic traits include, but are not limited to, increased insect resistance, increased water use efficiency, increased yield performance, increased drought resistance, increased seed quality, improved nutritional quality, hybrid seed production, and herbicide tolerance, in which the trait is measured with respect to a wild-type plant. Exemplary additional herbicide tolerance traits may include transgenic or non-transgenic tolerance to one or more herbicides such as ACCase inhibitors (for example aryloxyphenoxy propionates and cyclohexanediones), ALS inhibitors (for example sulfonylureas, imidazolinones, triazoloyrimidines, and triazolinones) EPSPS inhibitors (for example glyphosate), synthetic auxins (for example phenoxy, benzoic acids, carboxylic acids, semicarbazones), photosynthesis inhibitors (for example triazines, triazinones, nitriles, benzothiadiazoles, and ureas), glutamine synthesis inhibitors (for example glufosinate), HPPD inhibitors (for example isoxazoles, pyrazolones, and triketones), PPO inhibitors (for example diphenylethers, N-phenylphthalimide, aryl triazinones, and pyrimidinediones), and long-chain fatty acid inhibitors (for example chloroacetamides, oxyacetamides, and pyrazoles), among others. Exemplary insect resistance traits may include resistance to one or more insect members within one or more of the orders of Lepidoptera, Coleoptera, Hemiptera, and Homoptera, among others. Such additional transgenic traits are known to one of skill in the art; for example, a list of such traits is provided by the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS).

[0078] A cell transformed with a polynucleotide of the present invention, such as an expression construct, may be selected for the presence of the polynucleotide or its encoded enzymatic activity before or after regenerating such a cell into a transgenic plant. Transgenic plants comprising such a polynucleotide may thus be selected for instance by identifying a transgenic plant that comprises the polynucleotide or the encoded enzymatic activity, and/or

displays an altered trait relative to an otherwise isogenic control plant. Such a trait may be, for example, tolerance to a PPO herbicide.

[0079] Transgenic plants and progeny that contain a transgenic trait provided by the invention may be used with any breeding methods that are known in the art. In plant lines comprising two or more transgenic traits, the transgenic traits may be independently segregating, linked, or a combination of both in plant lines comprising three or more transgenic traits. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of breeding methods that are used for different traits and crops are well known to those of skill in the art. To confirm the presence of the transgene(s) in a particular plant or seed, a variety of assays may be performed. Such assays include, for example, molecular biology assays, such as Southern and northern blotting, PCR, and DNA sequencing; biochemical assays, such as detecting the presence of a protein product, for example, by immunological means (ELISAs and western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole plant.

[0080] Introgression of a transgenic trait into a plant genotype is achieved as the result of the process of backcross conversion. A plant genotype into which a transgenic trait has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired transgenic trait may be referred to as an unconverted genotype, line, inbred, or hybrid.

[0081] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that the examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0082] The following examples are included to demonstrate embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes

can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit, and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein with the same or similar result achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

Example 1: Microbial Protoporphyrinogen Oxidase Discovery

[0083] Novel protoporphyrinogen oxidases were identified from microbial sequence databases using bioinformatic methods and a novel protoporphyrinogen oxidase bacterial screening system. Three sequences representing a diverse range of HemY microbial protoporphyrinogen oxidases and the sequence for the PPO herbicide sensitive HemY protoporphyrinogen oxidase from *Arabidopsis thaliana* were used to identify new putative protoporphyrinogen oxidase sequences from microbial sequence databases. The use of these four diverse sequences with bioinformatic tools permitted skewing of search results toward sequences that are more similar to microbial protoporphyrinogen oxidases than plant protoporphyrinogen oxidases in order to increase the likelihood of identifying PPO herbicide tolerant protoporphyrinogen oxidases.

[0084] Ninety-nine putative protoporphyrinogen oxidases of the HemY PPO family were identified using this method. The sequences encoding these putative HemY PPO enzymes were compared using phylogenetic tree mapping. Forty-four putative HemY PPO enzymes were selected for further analysis due to their representation of individual unique clustered members on the phylogenetic tree. The coding sequences for the forty-four selected putative HemY PPO enzymes were cloned into bacterial expression vectors for analysis in a *E. coli* hemG knockout screen described below.

[0085] A protoporphyrinogen oxidase bacterial screening system was created to test recombinant proteins for protoporphyrinogen oxidase activity. This screening system used a functional rescue assay in an *E. coli* strain that contained a gene knockout for the *E. coli* PPO enzyme (HemG; SEQ ID NO: 76). The *hemG* knockout *E. coli* strain showed minimal growth on classical bacterial media (*e.g.*, LB media), but growth rates recovered when the bacterial media was supplemented with free heme or when a recombinant protoporphyrinogen oxidase was

expressed in the *E. coli*. The *hemG* knockout *E. coli* strain could thus be used with recombinant protein expression to quickly and easily assay proteins for protoporphyrinogen oxidase activity.

[0086] The *hemG* knockout *E. coli* strain was transformed with the bacterial expression vectors containing the putative protoporphyrinogen oxidases and plated on LB media. Recombinant proteins were expressed in *E. coli* and growth rates were measured. Growth of the transformed *hemG* knockout *E. coli* strain on LB media indicated an amino acid sequence that confirmed as a functioning protoporphyrinogen oxidase. Using this assay, a large number of novel or engineered proteins can be screened to confirm and measure protoporphyrinogen oxidase activity. Ten of the forty-four putative novel PPO enzymes rescued the *hemG* knockout *E. coli* strain, confirming their activity as protoporphyrinogen oxidases, and were selected for further characterization. Table 3 provides the SEQ ID NOs corresponding to the ten selected HemY PPO variants, the *E. coli* HemG, and the *A. tuberculatus* PPO.

Table 3. SEQ ID NOs corresponding to HemY PPO variants

PPO	Protein	Bacterial DNA	Dicot codon optimized	Monocot codon optimized
R2N30	1, 6	26	31, 36	47
R2N40	2, 7, 8, 9, 10, 11, 12	27	32, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46	48
R2N70	3	28	33	49
R2N90	4	29	34	50
R2N100	5	30	35	51
R1N473	13, 18, 22	52	57, 68, 72	63
R1N533	14, 19, 23	53	58, 69, 73	64
R1N171	15, 20, 24	54	59, 70, 74	65
R1N311	16	55	60	66
R1N333	17, 21, 25	56	61, 62, 71, 75	67
<i>E. coli</i> HemG	76	77	78	79
<i>A. tuberculatus</i>	80	81	n/a	n/a

Example 2: Protoporphyrinogen Oxidase Inhibitor Insensitivity

[0087] Novel protoporphyrinogen oxidases that are tolerant to PPO herbicides were identified using an herbicide bacterial screening system. This screening system used a growth assay of the *hemG* knockout *E. coli* strain in LB liquid media supplemented with a PPO herbicide to identify protoporphyrinogen oxidases that were not sensitive to the PPO herbicide.

[0088] The *hemG* knockout *E. coli* strain was transformed with the bacterial expression vectors containing the confirmed protoporphyrinogen oxidases and cultured in LB liquid media. A saturating amount of the purified crystalline form of one of five different PPO herbicides (acifluorfen, flumioxazin, lactofen, fomesafen, and S-3100), representing three different PPO chemistry subclasses, was added to the media. Recombinant proteins were expressed and the *E. coli* growth rates were measured. Growth curves (OD600) were measured for the different variants in the presence and absence of the PPO herbicides at selected time-points (*e.g.*, eight hours). The growth of a transformed *hemG* knockout *E. coli* strain on LB media in the presence of a PPO herbicide indicates a protoporphyrinogen oxidase that is an herbicide-insensitive protoporphyrinogen oxidase (iPPO).

[0089] The novel putative protoporphyrinogen oxidases were used with this assay to test for insensitivity to PPO herbicides. Expression of *E. coli* HemG (SEQ ID NO: 76), HemY PPO R1N473 (SEQ ID NO: 13), HemY PPO R1N533 (SEQ ID NO: 14); or HemY PPO R1N171 (SEQ ID NO: 15) conferred normal growth rates on the *hemG* knockout *E. coli* strain in minimal media in the presence of acifluorfen, flumioxazin, lactofen, or fomesafen PPO herbicides, therefore indicating that these proteins were highly herbicide-insensitive protoporphyrinogen oxidases (iPPO). Data are provided in Figure 1. Bacteria transformed with constructs encoding HemY PPO R1 N311 (SEQ ID NO:16) or HemY PPO R1 N333 (SEQ ID NO:17) did not grow in the presence of any of acifluorfen, flumioxazin, lactofen, or fomesafen, indicating that these proteins did not confer tolerance to any of these four PPO herbicides. Expression of HemY R2N30 (SEQ ID NO:1) or Hem Y R2N40 (SEQ ID NO:2) conferred normal growth rates on the *hemG* knockout *E. coli* strain in minimal media in the presence of acifluorfen and S-3100, but at higher levels these two enzymes showed sensitivity to both herbicides. The growth rate was slower for the *hemG* knockout *E. coli* strain containing HemY R2N70 (SEQ ID NO:3), HemY R2N90 (SEQ ID NO:4), or HemY R2N100 (SEQ ID NO:5), but tolerance to acifluorfen and S-

3100 was much better than for the *hemG* knockout *E. coli* strain containing either the R2N30 (SEQ ID NO:1) and R2N40 (SEQ ID NO:2). Data are provided in Figure 2. The *hemG* knockout *E. coli* strain expressing the *A. tuberculatus* PPO (SEQ ID NO:80) was used as a negative control and was sensitive to all of the PPO herbicides. Using this assay, a large number of novel or engineered proteins can be screened to confirm protoporphyrinogen oxidase activity in the presence of PPO herbicide(s).

Example 3: Protoporphyrinogen Oxidase (PPO) Enzyme Assay

[0090] Novel protoporphyrinogen oxidases were characterized using an enzyme assay. This assay used recombinant HemY PPO proteins with plant plastid extract and PPO substrate to identify protoporphyrinogen oxidases that had activity in the presence of a PPO herbicide.

[0091] Plant plastid extract was produced used etioplasts and chloroplasts that were prepared from etiolated cotyledons (soybean, *Glycine max*), etiolated leaves/coleoptiles (corn, *Zea mays*) and unfolded apical leaves (*A. tuberculatus*) generally by the procedure described by Grossmann (2010). Soybean (A3555) and corn (LH244) seeds were placed between two sheets of moist germination paper (Anchor Paper Company, Saint Paul, Minnesota) in a beaker of water in continuous darkness for eight to ten days. *A. tuberculatus* plants were grown for 30 days in the greenhouse. Tissue was collected, placed between moist sheets of paper towels and ground to fine powder with a mortar and pestle in liquid nitrogen. Homogenization buffer (50 mM Tris-HCl, pH 7.4, 500 mM sucrose, 1 mM EDTA, 1 mM magnesium chloride, and 2 g/liter bovine serum albumin) was added to the frozen powder at 4:1 (ml homogenization buffer to g fresh weight tissue), mixed vigorously and filtered through four layers of pre-moistened Miracloth. The filtrate was centrifuged at 9299g for five minutes. The pellet was resuspended in homogenization buffer and centrifuged at 150g for two minutes. The supernatant solution was centrifuged at 4000g for fifteen minutes. All centrifugation steps were carried out at 4°C. The pellet (intact plastid fraction) was resuspended in 50 mM Tris-HCl (pH 7.4), 2 mM EDTA and 20% (v/v) glycerin and stored in aliquots at -80°C. Total protein in plastid preparations was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

[0092] Recombinant PPO enzymes were expressed in *E. coli* HemG knockout cell line and extracted for the enzyme assay. Bacterial cells from an overnight culture were used to inoculate 20 ml of fresh media. These cultures were allowed to grow for approximately 48 hrs at 20°C to a

dense culture. Bacterial cells were collected by centrifugation and the cell pellets stored at -80°C until enzyme assays were performed. Frozen bacterial pellets were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA & 1 mM MgCl_2) and sonicated (Sonics VibraCell™) for 30 seconds in an ice bath with a one-minute rest period between cycles. For *E. coli* transformed with a construct encoding *E. coli* HemG (SEQ ID NO: 76) the broken cells were centrifuged at 200g for 2 minutes at 4°C and the supernatant solution was used for PPO enzyme assays after dilution with extraction buffer. Frozen cell pellets prepared from bacteria transformed with a construct encoding R1N473 (SEQ ID NO:13), R1N171 (SEQ ID NO:15), R1N533 (SEQ ID NO:14), R2N30 (SEQ ID NO:1), R2N40 (SEQ ID NO:2), R2N90 (SEQ ID NO:4), or R2N100 (SEQ ID NO:5) were centrifuged at 9400g for 10 minutes at 4°C . The pellet fraction from this supernatant solution was prepared by ultracentrifugation at 100,000g for one hour at 4°C to 7°C and resuspended in extraction buffer for PPO assays. Total protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

[0093] The PPO substrate protoporphyrinogen IX (protogen) was prepared by reduction of commercially available protoporphyrin with sodium mercury amalgam as described by Jacobs and Jacobs (1999). Protoporphyrin (proto) was added to 0.01N potassium hydroxide in 20% ethanol and stirred in the dark until dissolved (about 40 minutes). A volume of 0.8 ml of proto was placed in a 2-ml polypropylene vial with a screw-top cap containing an O-ring, and about 1 g (a spatula tipful, oil drained off) of sodium mercury amalgam (Product Number 451908, Sigma-Aldrich, St. Louis, Missouri, stored under oil) was added. The tube was capped and mixed vigorously with a vortex mixer and vented about every 30 seconds by loosening the cap until the solution was no longer fluorescing red under a UV light (about five minutes). The reaction vial was flushed with argon and centrifuged briefly to pellet the remaining sodium amalgam. The supernatant solution was diluted 1:1 (v/v) with a solution of 0.1M DTT and 0.5M Tris-HCl, pH 7.5 and the vial flushed with argon. The protogen solution was split into smaller aliquots into 0.5 ml polypropylene capped tubes which were flushed with argon immediately after the aliquot was added. Capped tubes were covered with aluminum foil and stored at -80°C . For the enzyme assay, the thawed protogen aliquots were stored covered on ice, and used on the same day. The concentration of protogen in the preparation was calculated by subtracting the proto concentration, as measured by fluorescence HPLC (method described by Matsumoto, 1994), in the final protogen solution (typically about 1% of starting material) from the proto

concentration in the starting material and assuming no significant impurities in either sample. Protogen prepared and stored under these conditions was stable at least six months.

[0094] A PPO enzyme assay was conducted to measure PPO activity using the plant plastid extract and bacterial extract preparations with the PPO substrate. PPO activity was measured generally as described by Grossmann (2010). Ten microliters of either plastid extract (40 μ g total protein) or bacterial extract (1.1 μ g total protein for *E. coli* HemG (SEQ ID NO: 76); or 45 to 70 μ g total protein for R1N473 (SEQ ID NO:13), R1N171 (SEQ ID NO:15), R1N533 (SEQ ID NO:14), R2N30 (SEQ ID NO:1), R2N40 (SEQ ID NO:2), R2N90 (SEQ ID NO:4), or R2N100 (SEQ ID NO:5)) was added to assay buffer (100 mM Tris-HCl, pH 7.4, 5 mM DTT, 1 mM EDTA and 0.085% (v/v) Tween 80) with buffer or with S-3100 (added as a two-microliter volume from a 100X stock solution prepared in acetone). Analytical-grade S-3100 was provided by Sumitomo Chemical Company. All assays were run in a final concentration of 1% (v/v) acetone. The extracts (plastid or bacterial), buffer, and S-3100 were incubated at 30°C (plant extracts) or 37°C (bacterial extracts) for five minutes before addition of two microliters of protogen to initiate the assay. All assays were done in a 96-well black polystyrene microtiter plate (Costar® 3925, Corning, Inc., Corning, New York) at a final volume of 200 microliters. After protogen addition (3 μ M for IC₅₀ measurements; variable for K_m measurements) to all wells, the plate was incubated at 30°C (plant extracts) or 37°C (bacterial extracts) before initiating data collection. Fluorescence over time was measured at 30°C (plant extracts) or 37°C (bacterial extracts) with excitation and emission wavelengths of 405 nm and 630 nm, respectively, in a SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California). An assay blank was run by adding heat-inactivated (five minutes at 100°C) extract to the assay mixture. PPO apparent K_m values were calculated using rectangular hyperbola curve-fitting using the SoftPro® kinetics software package (Molecular Devices, Sunnyvale, California). The S-3100 IC₅₀ values were determined graphically from the semi logarithmic plot of S-3100 concentration versus PPO activity.

[0095] Substrate (protoporphyrinogen) binding affinity was measured as the K_m. Enzyme activity sensitivity to the PPO herbicide S-3100 was measured as the concentration giving 50% inhibition of control activity (IC₅₀). The K_m for the plant PPO enzymes (*A. tuberculatus*, soybean, or corn) and the bacterial PPO enzymes (*E. coli* HemG (SEQ ID NO: 76), R1N473 (SEQ ID NO:13), R1N171 (SEQ ID NO:15), R1N533 (SEQ ID NO:14), R2N30 (SEQ ID NO:1),

R2N40 (SEQ ID NO:2), R2N90 (SEQ ID NO:4), or R2N100 (SEQ ID NO:5)) were similar, ranging from 0.7 μM to 2.0 μM . Each of the three plant PPO enzymes were sensitive to S-3100 with an IC_{50} of 0.003 to 0.009 μM . The bacterial PPO enzymes R2N30 (SEQ ID NO:1) and R2N40 (SEQ ID NO:2) had an IC_{50} of 0.02 μM and 0.04 μM , respectively, and were 10-fold less sensitive to the herbicide than the plant PPO enzymes. The bacterial PPO enzymes *E. coli* HemG (SEQ ID NO: 76), R1N473 (SEQ ID NO:13), R1N171 (SEQ ID NO:15), R1N533 (SEQ ID NO:14), R2N90 (SEQ ID NO:4), and R2N100 (SEQ ID NO:5) had an IC_{50} of greater than 100 μM and were measured as insensitive to the herbicide. Data are provided in Table 4.

Table 4: PPO Enzymatic Activity

Source	K_m , μM	S-3100 IC_{50} , μM
<i>A. tuberculatus</i>	0.7	0.009
Soybean	1.8	0.004
Corn	2.0	0.003
<i>E. coli</i> HemG	1.6	> 100
R1N473	1.2	> 100
R1N171	0.2	> 100
R1N533	0.4	> 100
R2N30	0.8	0.02
R2N40	0.8	0.04
R2N90	2.8	> 100
R2N100	0.4	> 100

Example 4: Enzymatic Optimization of protoporphyrinogen oxidases

[0096] Protein optimization may be used to improve or alter the enzymatic properties of protoporphyrinogen oxidases. One or more methods of protein engineering may be used to optimize the enzymes. Non-limiting examples of protein engineering approaches include Alanine-Scanning Mutations; Homology-Scanning Mutations; Pro/Gly Scanning Mutations; Region Swaps or Mutations; and combinations of these various techniques (*see*, M Lehmann and M Wyss, *Current Opinion in Biotechnology* 12(4):371-375 (2001); B Van den Burg and VGH

Eijsink, Current Opinion in Biotechnology 13(4):333-337 (2002); and Weiss *et al.*, Proceedings of the National Academy of Sciences USA 97(16):8950–8954 (2000)). Engineered protoporphyrinogen oxidase nucleic acid sequences may be synthesized and cloned into a bacterial expression vector and used to transform the *hemG* knockout *E. coli* strain for the initial high-throughput bacterial rescue screen as described in Example 1. The engineered proteins that rescue the *hemG* knockout *E. coli* strain may be screened for sensitivity to one or more PPO herbicide(s) using the bacterial growth assay as described in Example 2. The engineered proteins that exhibit tolerance to PPO herbicides in the second screen may then be expressed as recombinant protein in a bacterial expression system, and enzyme characterization may be done using the purified protein as described in Example 3. Engineered proteins that are insensitive to PPO herbicides may be selected for cloning into plant transformation vectors and this may be used to produce transgenic plants for *in planta* testing.

[0097] A library of randomly mutagenized R2N40 coding sequences were produced using a GeneMorph® II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The resulting library of mutagenized bacterial expression vectors was used to transform the *hemG* knockout *E. coli* strain and this was plated onto LB media plates containing acifluorfen. Bacterial colonies that grew on the herbicide medium were selected, the transformation plasmids were purified, and the mutant PPO genes were sequenced. Engineered HemY PPO R2N40 enzymes are provided as SEQ ID NO:7-12.

Example 5: Expression and Testing of PPO enzymes in Soybean Plants

[0098] The microbial HemY PPO enzymes were expressed in transgenic soybean plants, and the transgenic plants were analyzed for PPO herbicide tolerance. A set of constructs for high-throughput screening were produced with the same promoter element and 3' UTR operably linked to one of ten different cassettes encoding HemY PPO enzymes R1N171 (SEQ ID NO: 20); R1N473 (SEQ ID NO:18); R1N533 (SEQ ID NO:19); R2N30 (SEQ ID NO:1, 6); R2N40 (SEQ ID NO:2, 7); R2N40opt (SEQ ID NO:9, 10-12); R2N70 (SEQ ID NO:3); R2N90 (SEQ ID NO:4); R2N100 (SEQ ID NO:5); and R1N333 (SEQ ID NO:21) operably linked to one of 39 different transit peptides. For plant transformation, the nucleotide sequences encoding the HemY PPO enzymes were codon optimized for dicot expression. This permitted the side-by-side

comparison of the seven different HemY PPO enzymes with thirty-nine different targeting peptides using the same promoter and 3'UTR elements for gene expression

[0099] The plant transformation constructs were used to transform soybean excised embryos (germplasm A3555) using *A. tumefaciens* and standard methods known in the art. Four hundred explants were inoculated for each construct resulting in twelve containers per construct. A sterile PPO herbicide solution was used for herbicide tolerance testing. The herbicide solution consisted of 0.3 g of S-3100 in crop oil concentrate (5.0 mL) and 495 mL of deionized water. This was filtered through a 0.45 micron Nalgene® Rapid-Flow™ Tissue Culture Filter Unit and Surfactant-Free Cellulose Acetate membrane filter unit (VWR, Radnor, PA, USA). The resulting sterile solution was shaken before application.

[00100] At five weeks post-transformation, four of the twelve plant containers per construct were sprayed with two passes of the sterile PPO herbicide solution. The treated plantlets were then enclosed in the container and received at least 15 hours of light exposure post spray each day for four days. At the end of day four post application of S-3100, the treated plantlets were photographed and scored on a visual scale of green coloration (green coloration was representative of healthy photosynthetic plant tissue as compared to photo-bleached tissue) versus damage. The scoring values were 0 for poor tolerance, high damage, low green coloration; 1 for some tolerance, average damage, moderate green coloration; and 2 for good tolerance, low damage, high green coloration. The results of herbicide application of S-3100 at five weeks is presented in Table 5, where n.d. indicates the analysis was not conducted. The results indicated that in this high-throughput screening a number of constructs comprising HemY PPO enzymes R1N473 (SEQ ID NO:18); R1N533 (SEQ ID NO:19); R2N30 (SEQ ID NO:1, 6); R2N40 (SEQ ID NO:2, 7); R2N40opt (SEQ ID NO:9, 10-12); and R2N70 (SEQ ID NO:3) provided tolerance to the PPO herbicide. The results indicated that in this high-throughput screening the HemY PPO enzymes R1N171 (SEQ ID NO:20); R2N90 (SEQ ID NO:4); R2N100 (SEQ ID NO:5); and R1N333 (SEQ ID NO:21) did not provide tolerance to the PPO herbicide.

Table 5. Tolerance score to S-3100 at 5 weeks for HemY PPO

Targeting Peptide	R1N 171	R1N 473	R1N 533	R2N 30	R2N 40	R2N 40 opt	R2N 70	R2N 90	R2N 100	R1N 333
TP1	0	2	0	2	n.d.	1	n.d.	n.d.	0	n.d.

TP2	0	0	2	n.d.	n.d.	n.d.	2	0	0	0
TP3	0	1	0	1	n.d.	n.d.	1	n.d.	0	0
TP4	n.d.	1	0	2	n.d.	0	0	n.d.	0	0
TP5	1	n.d.	n.d.	n.d.	1	1	n.d.	0	0	n.d.
TP6	n.d.	1	1	n.d.	n.d.	1	0	n.d.	0	0
TP7	0	1	0	1	n.d.	2	n.d.	n.d.	n.d.	0
TP8	1	1	n.d.	2	0	1	0	1	1	0
TP9	1	1	n.d.	2	0	0	n.d.	1	n.d.	1
TP10	1	1	1	2	n.d.	n.d.	n.d.	0	1	n.d.
TP11	0	0	0	2	n.d.	1	n.d.	n.d.	n.d.	0
TP12	0	1	0	1	n.d.	2	n.d.	1	0	1
TP13	0	0	0	n.d.	n.d.	0	n.d.	0	n.d.	0
TP14	n.d.	1	1	1	n.d.	n.d.	n.d.	0	1	0
TP15	1	1	1	2	n.d.	1	n.d.	n.d.	0	0
TP16	1	1	2	2	n.d.	1	0	0	n.d.	0
TP17	0	1	n.d.	1	n.d.	1	n.d.	n.d.	0	0
TP18	0	0	0	1	n.d.	0	n.d.	1	1	0
TP19	1	0	1	n.d.	2	1	n.d.	1	1	0
TP20	0	1	1	2	n.d.	1	n.d.	0	n.d.	n.d.
TP21	0	0	n.d.	1	n.d.	n.d.	0	1	0	0
TP22	n.d.	1	2	1	n.d.	n.d.	n.d.	0	0	0
TP23	1	1	1	1	n.d.	n.d.	n.d.	1	0	1
TP24	n.d.	0	2	2	n.d.	n.d.	n.d.	0	0	0
TP25	0	1	0	2	n.d.	1	n.d.	n.d.	0	0
TP26	1	0	1	1	n.d.	1	n.d.	n.d.	1	0
TP27	0	1	0	n.d.	n.d.	2	n.d.	0	0	1
TP28	0	2	0	2	n.d.	n.d.	n.d.	0	1	0
TP29	0	0	1	1	n.d.	0	n.d.	0	0	1
TP30	1	1	n.d.	1	n.d.	0	n.d.	0	0	0
TP31	1	1	1	1	0	0	1	0	1	1

TP32	0	1	1	1	n.d.	0	n.d.	0	0	0
TP33	0	2	0	n.d.	2	1	0	0	0	0
TP34	n.d.	n.d.	n.d.	0	1	n.d.	n.d.	n.d.	n.d.	1
TP35	0	0	1	1	1	1	n.d.	n.d.	n.d.	n.d.
TP36	n.d.	n.d.	0	1	1	n.d.	n.d.	n.d.	0	1
TP37	0	0	0	1	1	2	n.d.	n.d.	0	n.d.
TP38	n.d.	n.d.	2	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.
TP39	1	1	2	1	2	1	n.d.	n.d.	n.d.	n.d.

[00101] Plants in the non-sprayed containers corresponding to constructs having a high passing score of 2 and a few fails as negative controls were transplanted at approximately 7 weeks post transformation. The R0 plants were grown in a greenhouse under long-day nursery conditions (18 hr light at 80°F then 6 hr dark at 74°F) for approximately four weeks. At eleven weeks, the R0 plants were sprayed with two passes of the same herbicide solution (0.3 g of S-3100) described above. Herbicide injury ratings were collected seven days after treatment. Any injury rating of 30% or above was equivalent to non-transgenic soybean injury ratings. The results of the herbicide tolerance application at eleven weeks to the R0 plants are presented in Table 6, where n.d. indicates the analysis was not conducted. The results indicated that plants expressing a number of constructs comprising HemY PPO enzymes R2N30 (SEQ ID NO:1, 6); R2N40 (SEQ ID NO:2, 7); and R2N40opt (SEQ ID NO:9, 10-12) provided tolerance to the PPO herbicide with an injury rating below the non-transgenic control. Plants expressing the HemY PPO enzyme R2N30 (SEQ ID NO:1, 6) provided herbicide tolerance in 16 of the 19 constructs tested, with injury ratings for these constructs of 7% to 25%. Plants expressing the HemY PPO enzymes R2N40 (SEQ ID NO:2, 7) and R2N40opt (SEQ ID NO:9, 10-12) provided herbicide tolerance in 8 of the 11 constructs tested, with injury ratings for these constructs of 20% to 25%. The results indicated that in this high-throughput screening plants expressing the HemY PPO enzymes R1N171 (SEQ ID NO:20); R1N473 (SEQ ID NO:18); R1N533 (SEQ ID NO:19); R2N70 (SEQ ID NO:3); and R1N333 (SEQ ID NO:21) had an injury rating of 30% or above equivalent to non-transgenic control injury ratings and thus did not provide tolerance to the PPO herbicide.

Table 6. Tolerance score to S-3100 at 11 weeks for HemY PPO

Targeting Peptide	R1N 171	R1N 473	R1N 533	R2 N30	R2 N40	R2 N40opt	R2 N70	R1N 333
TP1	n.d.	30	n.d.	17	n.d.	20	n.d.	n.d.
TP2	n.d.	n.d.	40	n.d.	n.d.	n.d.	30	n.d.
TP3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP4	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP6	n.d.	35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP7	n.d.	n.d.	n.d.	25	n.d.	30	n.d.	n.d.
TP8	n.d.	n.d.	n.d.	35	n.d.	30	n.d.	35
TP9	n.d.	n.d.	n.d.	20	n.d.	25	n.d.	35
TP10	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP11	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP12	n.d.	n.d.	n.d.	n.d.	n.d.	35	n.d.	n.d.
TP13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP14	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	35
TP15	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP16	30	40	35	35	n.d.	n.d.	n.d.	n.d.
TP17	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP18	n.d.	n.d.	n.d.	15	n.d.	n.d.	n.d.	n.d.
TP19	n.d.	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.
TP20	n.d.	n.d.	n.d.	15	n.d.	20	n.d.	n.d.
TP21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP22	n.d.	n.d.	35	25	n.d.	n.d.	n.d.	n.d.
TP23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP24	n.d.	n.d.	35	15	n.d.	n.d.	n.d.	n.d.
TP25	n.d.	n.d.	n.d.	7	n.d.	n.d.	n.d.	n.d.
TP26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

TP27	n.d.	n.d.	n.d.	n.d.	n.d.	25	n.d.	n.d.
TP28	n.d.	35	n.d.	35	n.d.	n.d.	n.d.	n.d.
TP29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP30	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP33	n.d.	40	n.d.	n.d.	20	n.d.	n.d.	n.d.
TP34	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TP36	n.d.	n.d.	n.d.	25	n.d.	n.d.	n.d.	n.d.
TP37	n.d.	n.d.	n.d.	n.d.	n.d.	25	n.d.	n.d.
TP38	n.d.	n.d.	35	n.d.	25	n.d.	n.d.	n.d.
TP39	n.d.	30	35	n.d.	n.d.	n.d.	n.d.	n.d.

What is claimed is:

Claim 1. A recombinant DNA molecule comprising a heterologous promoter operably linked to a nucleic acid molecule encoding a protein that has at least 85% sequence identity to amino acid sequence selected from the group consisting of: SEQ ID NOs:1-2 and SEQ ID NOs:6-12, wherein the protein has herbicide-insensitive protoporphyrinogen oxidase activity.

Claim 2. The recombinant DNA molecule of claim 1, wherein the nucleic acid molecule comprises a sequence selected from the group consisting of SEQ ID NOs:26-27, 31-32, 36-46, and 47-48.

Claim 3. The recombinant DNA molecule of claim 1, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NOs:1-2 and SEQ ID NOs:6-12.

Claim 4. The recombinant DNA molecule of claim 1, wherein the heterologous promoter is functional in a plant cell.

Claim 5. The recombinant DNA molecule of claim 4, wherein the nucleic acid molecule is operably linked to a DNA molecule encoding a targeting sequence that functions to localize an operably linked protein within a cell.

Claim 6. A DNA construct comprising the recombinant DNA molecule of claim 1.

Claim 7. The DNA construct of claim 6, wherein the recombinant DNA molecule further comprises an operably linked DNA molecule encoding a targeting sequence that functions to localize the protein within a cell.

Claim 8. The DNA construct of claim 7, wherein the protein confers PPO herbicide tolerance to said cell.

Claim 9. The DNA construct of claim 6, wherein the DNA construct is present in the genome of a transgenic plant, seed, or cell.

Claim 10. A recombinant polypeptide that comprises at least 85% sequence identity to the full length of a sequence chosen from SEQ ID NOs:1-2 and SEQ ID NOs:6-12, wherein the recombinant polypeptide has herbicide-insensitive protoporphyrinogen oxidase activity.

Claim 11. A transgenic plant, seed, cell, or plant part comprising the recombinant DNA molecule of claim 1.

Claim 12. The transgenic plant, seed, cell, or plant part of claim 11, wherein the transgenic plant, seed, cell, or plant part comprises an additional transgenic herbicide tolerance trait.

Claim 13. The transgenic plant, seed, cell, or plant part of claim 11, defined as comprising herbicide tolerance to at least one PPO herbicide.

Claim 14. A transgenic plant, seed, cell, or plant part comprising the recombinant polypeptide of claim 10.

Claim 15. A method for conferring PPO herbicide tolerance to a plant, seed, cell, or plant part comprising: heterologously expressing in said plant, seed, cell, or plant part the recombinant polypeptide of claim 10.

Claim 16. The method of claim 15, wherein said plant, seed, cell, or plant part comprises protoporphyrinogen oxidase activity conferred by the recombinant polypeptide.

Claim 17. The method of claim 15, wherein the herbicide tolerance is to at least one PPO herbicide selected from the group consisting of: acifluorfen, fomesafen, lactofen, fluoroglycofen-ethyl, oxyfluorfen, flumioxazin, azafenidin, carfentrazone-ethyl, sulfentrazone, fluthiacet-methyl, oxadiargyl, oxadiazon, pyraflufen-ethyl, saflufenacil and S-3100.

Claim 18. A method of plant transformation, comprising the steps of:

- a) introducing the recombinant DNA molecule of claim 1 into a plant cell; and
- b) regenerating a plant therefrom that comprises the recombinant DNA molecule.

Claim 19. The method of claim 18, further comprising the step of selecting a plant that is tolerant to at least one PPO herbicide.

Claim 20. The method of claim 18, further comprising the step of crossing the regenerated plant with itself or with a second plant and collecting seed from the cross.

Claim 21. A method for controlling weeds in a plant growth area, comprising contacting a plant growth area comprising the transgenic plant or seed of claim 11 with at least one PPO herbicide,

wherein the transgenic plant or seed is tolerant to the PPO herbicide and wherein at least a first weed is controlled in the plant growth area by the PPO herbicide.

Claim 22. A method of identifying a nucleotide sequence encoding a protein having protoporphyrinogen oxidase activity, the method comprising:

- a) transforming an *E. coli* strain having a gene knockout for the native *E. coli* PPO enzyme with a bacterial expression vector comprising a recombinant DNA molecule encoding a candidate protein; and
- b) growing said transformed *E. coli* using a heme-free bacterial medium, wherein growth using said bacterial medium identifies a protein having protoporphyrinogen oxidase activity.

Claim 23. A method of identifying a nucleotide sequence encoding a protein having herbicide-insensitive protoporphyrinogen oxidase activity, the method comprising:

- a) transforming an *E. coli* strain having a gene knockout for the native *E. coli* PPO enzyme with a bacterial expression vector comprising a recombinant DNA molecule encoding a recombinant protein; and
- b) growing said transformed *E. coli* using a bacterial medium containing at least one PPO herbicide, wherein growth of bacteria identifies a protein having herbicide-insensitive protoporphyrinogen oxidase activity.

Claim 24. A method of screening for a herbicide tolerance gene comprising:

- a) expressing the recombinant DNA molecule of claim 1 in a plant cell; and
- b) identifying a plant cell that displays tolerance to a PPO herbicide.

Claim 25. A method of screening for a herbicide tolerance gene comprising:

- a) expressing a recombinant DNA molecule of claim 1 in a bacterial cell lacking HemG, wherein the bacterial cell is grown in a heme-free medium in the presence of a PPO herbicide; and
- b) identifying a bacterial cell that displays tolerance to a PPO herbicide.

Claim 26. A method of producing a plant tolerant to a PPO herbicide and at least one other herbicide comprising:

- a) obtaining a plant according to claim 11; and
- b) crossing the transgenic plant with a second plant comprising tolerance to the at least one other herbicide, and
- c) selecting a progeny plant resulting from said crossing that comprises tolerance to a PPO herbicide and the at least one other herbicide.

Claim 27. A method for reducing the development of herbicide tolerant weeds comprising:

- a) cultivating in a crop growing environment a plant according to claim 12; and
- b) applying a PPO herbicide and at least one other herbicide to the crop growing environment, wherein the crop plant is tolerant to the PPO herbicide and the at least one other herbicide.

Claim 28. The method of claim 27, wherein the PPO herbicide is selected from the group consisting of acifluorfen, fomesafen, lactofen, fluoroglycofen-ethyl, oxyfluorfen, flumioxazin, azafenidin, carfentrazone-ethyl, sulfentrazone, fluthiacet-methyl, oxadiargyl, oxadiazon, pyraflufen-ethyl, saflufenacil and S-3100.

Claim 29. The method of claim 27, wherein the at least one other herbicide is selected from the group consisting of: an ACCase inhibitor, an ALS inhibitor, an EPSPS inhibitor, a synthetic auxin, a photosynthesis inhibitor, a glutamine synthesis inhibitor, a HPPD inhibitor, a PPO inhibitor, and a long-chain fatty acid inhibitor.

Claim 30. The method of claim 29, wherein the ACCase inhibitor is an aryloxyphenoxy propionate or a cyclohexanedione; the ALS inhibitor is a sulfonylurea, imidazolinone, triazoloyrimidine, or a triazolinone; the EPSPS inhibitor is glyphosate; the synthetic auxin is a phenoxy herbicide, a benzoic acid, a carboxylic acid, or a semicarbazone; the photosynthesis inhibitor is a triazine, a triazinone, a nitrile, a benzothiadiazole, or a urea; the glutamine synthesis inhibitor is glufosinate; the HPPD inhibitor is an isoxazole, a pyrazolone, or a triketone; the PPO inhibitor is a diphenylether, a N-phenylphthalimide, an aryl triazinone, or a pyrimidinedione; or the long-chain fatty acid inhibitor is a chloroacetamide, an oxyacetamide, or a pyrazole.

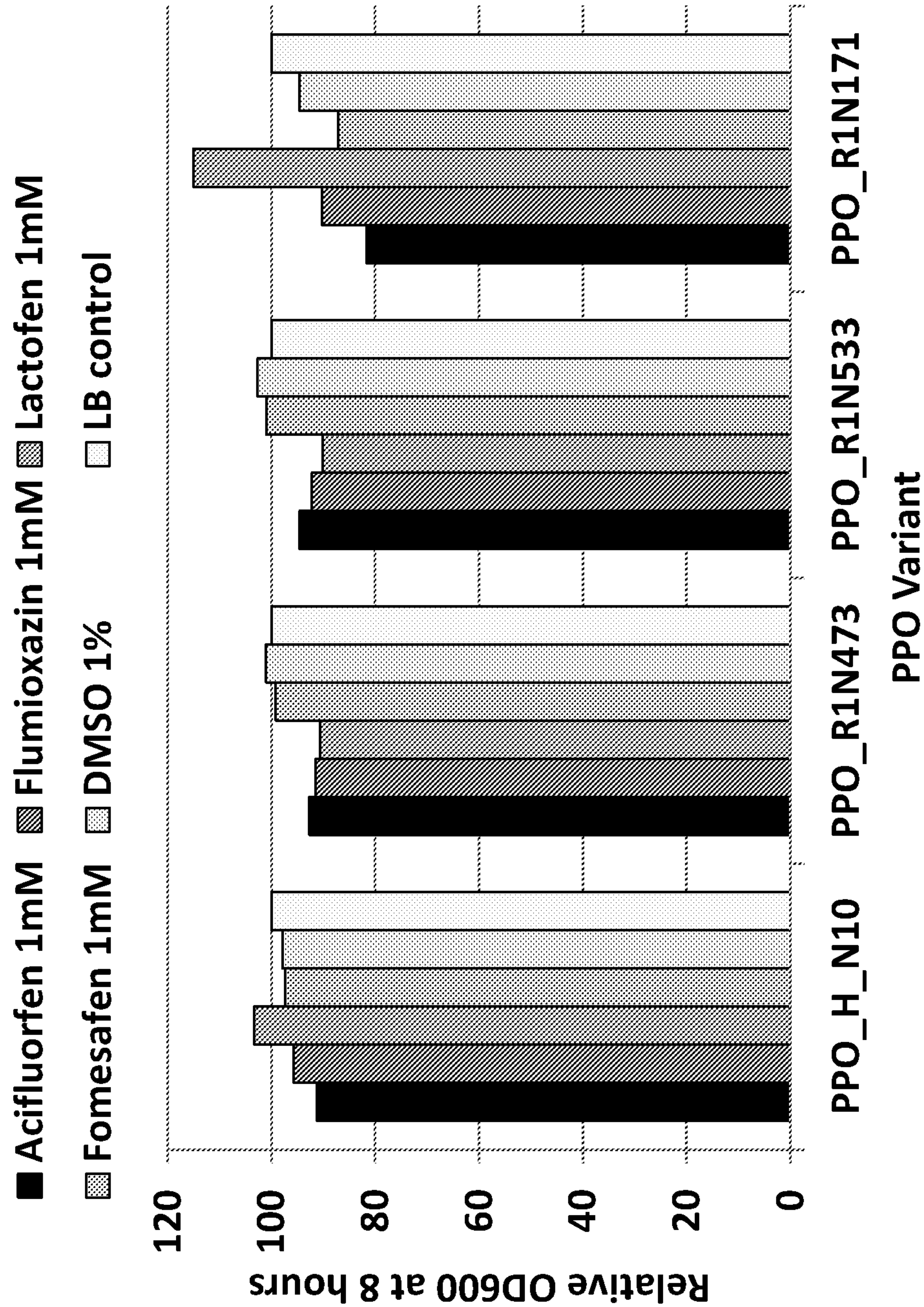


Figure 1

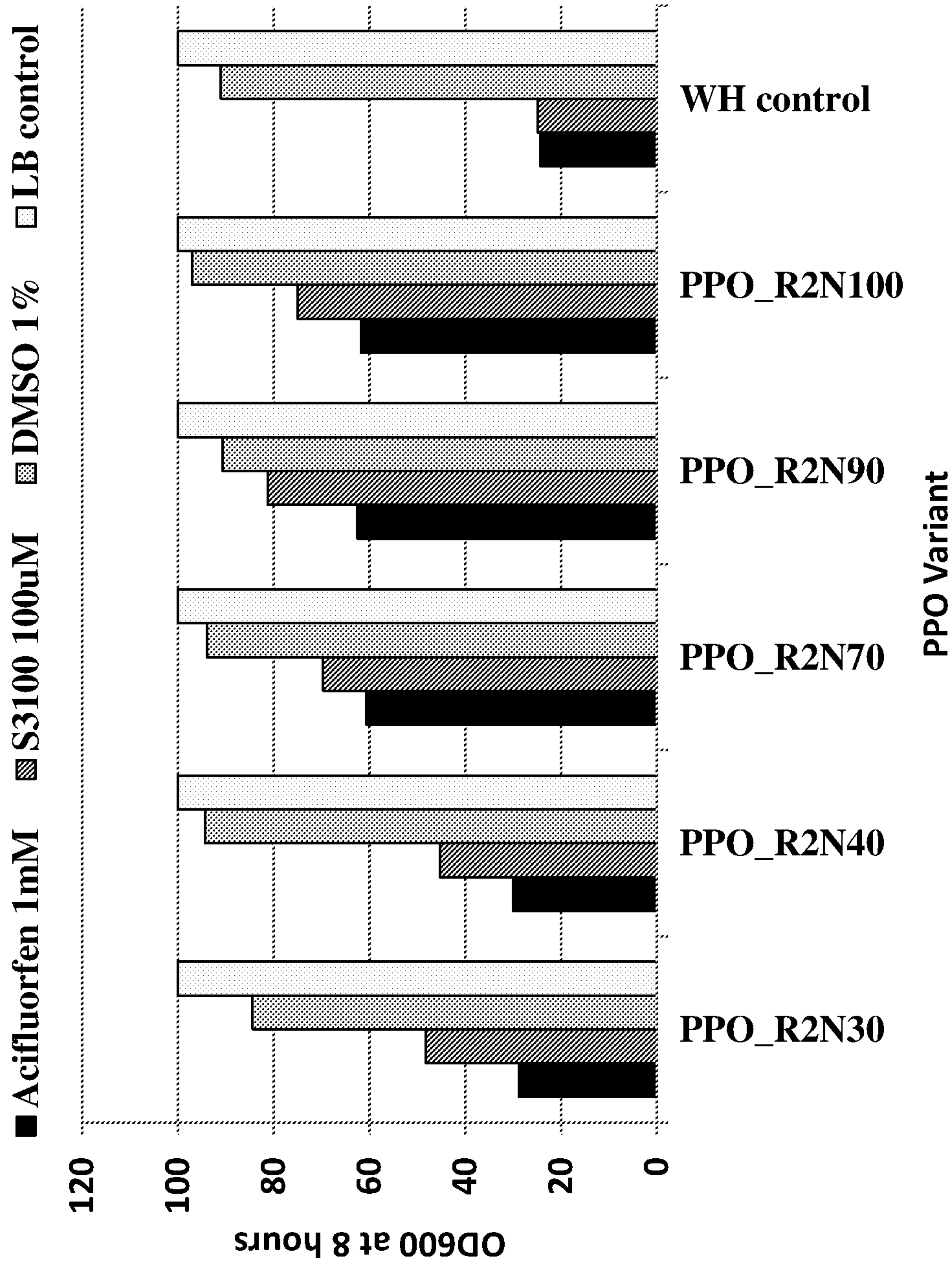


Figure 2