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(54) **Title:** HPPD VARIANTS AND METHODS OF USE

(57) **Abstract:** Compositions and methods for conferring herbicide tolerance to bacteria, plants, plant cells, tissues and seeds are provided. Compositions include polynucleotides encoding herbicide tolerance polypeptides, vectors comprising those polynucleotides, and host cells comprising the vectors. The nucleotide sequences of the invention can be used in DNA constructs or expression cassettes for transformation and expression in organisms, including microorganisms and plants. Compositions also include transformed bacteria, plants, plant cells, tissues, and seeds. In particular, isolated polynucleotides encoding HPPD inhibitor tolerance polypeptides are provided. Additionally, amino acid sequences corresponding to the polynucleotides are encompassed.



HPPD VARIANTS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial Nos. 61/701,037, filed September 14, 2012; 61/766,057, filed February 18, 2013; and
5 61/790,404, filed March 15, 2013 the contents of which is herein incorporated by reference in its entirety.

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FIELD OF THE INVENTION

This invention relates to plant molecular biology, particularly novel HPPD polypeptides that confer improved tolerance to HPPD inhibitor herbicides.

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BACKGROUND OF THE INVENTION

The 4-hydroxyphenylpyruvate dioxygenases (HPPDs) are enzymes which catalyze the reaction in which para-hydroxyphenylpyruvate (abbreviated herein as HPP), a tyrosine degradation product, is transformed into homogentisate (abbreviated herein as HG), the precursor in plants of tocopherol and plastoquinone (Crouch N.P.
25 et al. (1997), Tetrahedron, 53, 20, 6993-7010, Fritze et al. (2004), Plant Physiology 134:1388-1400). Tocopherol acts as a membrane-associated antioxidant. Plastoquinone, firstly acts as an electron carrier between PSII and the cytochrome b6/f complex and secondly, is a redox cofactor for phytoene desaturase, which is involved in the biosynthesis of carotenoids.

30 Up to now, more than 1000 nucleic acid sequences from various organisms present in the NCBI database were annotated as coding for a putative protein having an HPPD domain. But for most of those, it has not been proven that the protein would have an HPPD enzymatic activity either in an *in vitro* assay or in an *in planta*

approach, nor that such HPPD protein can confer herbicide tolerance to HPPD inhibitor herbicides when expressed in a plant. Several HPPD proteins and their primary sequences have been described in the state of the art, in particular the HPPD proteins of bacteria such as *Pseudomonas* (Rüetschi et al., Eur. J. Biochem., 205, 459-466, 1992, WO 96/38567), *Kordia* (WO2011076889) *Synechococcus* (WO2011076877), and *Rhodococcus* (WO2011076892), of protists such as *Blepharisma* (WO2011076882), of euryarchaeota such as *Picrophilus* (WO2011076885) of plants such as *Arabidopsis* (WO 96/38567, GENBANK® AF047834), carrot (WO 96/38567, GENBANK® 87257), *Avena sativa* (WO 02/046387, WO 11/068567), wheat (WO 02/046387), *Brachiaria platyphylla* (WO 02/046387), *Cenchrus echinatus* (WO 02/046387), *Lolium rigidum* (WO 02/046387), *Festuca arundinacea* (WO 02/046387), *Setaria faberi* (WO 02/046387), *Eleusine indica* (WO 02/046387), *Sorghum* (WO 02/046387, WO 12/021785), corn (WO 12/021785), *Coccicoides* (GENBANK® COITRP), of *Coptis japonica* (WO 06/132270), *Chlamydomonas reinhardtii* (ES 2275365) / WO2011/145015, or of mammals such as mouse or pig.

Inhibition of HPPD leads to uncoupling of photosynthesis, deficiency in accessory light-harvesting pigments and, most importantly, to destruction of chlorophyll by UV-radiation and reactive oxygen species (bleaching) due to the lack of photo protection normally provided by carotenoids (Norris et al. (1995), Plant Cell 7: 2139-2149). Bleaching of photosynthetically active tissues leads to growth inhibition and plant death.

Some molecules which inhibit HPPD, and which inhibit transformation of the HPP into homogentisate while binding specifically to the enzyme, have proven to be very effective herbicides.

At present, most commercially available HPPD inhibitor herbicides belong to one of these chemical families:

1) the triketones, e.g. sulcotrione [i.e. 2-[2-chloro-4-(methylsulfonyl)benzoyl]-1,3-cyclohexanedione], mesotrione [i.e. 2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione]; tembotrione [i.e. 2-[2-chloro-4-(methylsulfonyl)-3-[(2,2,2-trifluoroethoxy)methyl] benzoyl]-1,3-cyclohexanedione]; tefuryltrione [i.e. 2-[2-chloro-4-(methylsulfonyl)-3-[(tetrahydro-2-furanyl)methoxy]methyl]benzoyl]-1,3-

cyclohexanedione]]]; bicyclopyrone [i.e. 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]bicyclo[3.2.1]oct-3-en-2-one]; Benzobicyclon [i.e. 3-(2-chloro-4-mesylbenzoyl)-2-phenylthiobicyclo[3.2.1]oct-2-en-4-one];

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2) the diketonitriles, e.g. 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)-propane-1,3-dione and 2-cyano-1-[4-(methylsulphonyl)-2-trifluoromethylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione;

10 3) the isoxazoles, e.g. isoxaflutole [i.e. (5-cyclopropyl-4-isoxazolyl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone]. In plants, isoxaflutole is rapidly metabolized in DKN, a diketonitrile compound which exhibits the HPPD inhibitor property;

15 4) the pyrazolines, e.g. topramezone [i.e. [3-(4,5-dihydro-3-isoxazolyl)-2-methyl-4-(methylsulfonyl) phenyl](5-hydroxy-1-methyl-1H-pyrazol-4-yl)methanone], and pyrasulfotole [i.e. (5-hydroxy-1,3-dimethylpyrazol-4-yl)(2-mesyl-4-trifluoromethylphenyl)methanone]; pyrazofen [i.e. 2-[4-(2,4-dichlorobenzoyl)-1,3-dimethylpyrazol-5-yl]oxy]acetophenone];

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5) N-(1,2,5-oxadiazol-3-yl)benzamides (WO 2011/035874); and

6) N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides (WO2012/028579).

25 These HPPD-inhibiting herbicides can be used against grass and/or broad leaf weeds in field of crop plants that display metabolic tolerance, such as maize (*Zea mays*), rice (*Oryza Sativa*) and wheat (*Triticum aestivum*) in which they are rapidly degraded (Schulz et al. (1993), FEBS letters, 318, 162-166; Mitchell et al. (2001), Pest Management Science, Vol 57, 120-128; Garcia et al. (2000), Biochem., 39, 7501-
30 7507; Pallett et al. (2001), Pest Management Science, Vol 57, 133-142). In order to extend the scope of use of these HPPD-inhibiting herbicides, several efforts have been developed in order to confer to plants, particularly plants without or with an underperforming metabolic tolerance, a tolerance level acceptable under agronomic field conditions.

Besides the attempt of by-passing HPPD-mediated production of homogentisate (US 6,812,010), overexpressing the sensitive enzyme so as to produce quantities of the target enzyme in the plant which are sufficient in relation to the herbicide has been performed (WO96/38567). Overexpression of HPPD resulted in better pre-emergence tolerance to the diketonitrile derivative (DKN) of isoxaflutole (IFT), but tolerance was not sufficient for tolerance to post-emergence treatment (Matringe et al. (2005), Pest Management Science 61: 269-276).

A third strategy was to mutate the HPPD in order to obtain a target enzyme which, while retaining its properties of catalyzing the transformation of HPP into homogentisate, is less sensitive to HPPD inhibitors than is the native HPPD before mutation.

This strategy has been successfully applied for the production of plants tolerant to 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)-propane-1,3-dione and to 2-cyano-1-[4-(methylsulphonyl)-2-trifluoromethylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione (EP496630), two HPPD-inhibiting herbicides belonging to the diketonitriles family (WO 99/24585). Pro215Leu, Gly336Glu, Gly336Ile, and more particularly Gly336Trp (positions of the mutated amino acid are indicated with reference to the *Pseudomonas* HPPD) were identified as mutations which are responsible for an increased tolerance to treatment with these diketonitrile herbicides.

More recently, introduction of a *Pseudomonas* HPPD gene into the plastid genome of tobacco and soybean has shown to be more effective than nuclear transformation, conferring tolerance to post-emergence application of isoxaflutole (Dufourmantel et al. (2007), Plant Biotechnol J.5(1):118-33).

In WO 04/024928, the inventors sought to increase the prenylquinone biosynthesis (e.g., synthesis of plastoquinones, tocopherols) in the cells of plants by increasing the flux of the HPP precursor into the cells of these plants. This has been done by connecting the synthesis of said precursor to the "shikimate" pathway by overexpression of a PDH enzyme. They have also noted that the transformation of plants with a gene encoding a PDH enzyme and a gene encoding an HPPD enzyme makes it possible to increase the tolerance of said plants to HPPD inhibitors.

In the patent application WO 2009/144079, nucleic acid sequences encoding an hydroxyphenylpyruvate dioxygenase (HPPD) with specific mutations at position

336 of the *Pseudomonas fluorescens* HPPD protein and its use for obtaining plants which are tolerant to HPPD inhibitor herbicides was disclosed.

In WO 2002/046387, several domains of HPPD proteins originating from plants have been identified that may be relevant to confer tolerance to various HPPD inhibitor herbicides but neither in planta nor biochemical data have been shown to confirm the impact of the as described domain functions.

In WO 2008/150473, the combination of two distinct tolerance mechanisms – a modified *Avena sativa* gene coding for a mutant HPPD enzyme and a CYP450 Maize monooxygenase (*nsf1* gene) – was exemplified in order to obtain an improved tolerance to HPPD inhibitor herbicides, but no data have been disclosed demonstrating the synergistic effects based on the combination of both proteins.

Further, in US20110173718, a method to generate plants tolerant to HPPD inhibitors by overexpressing not only a gene coding for a tolerant HPPD, as for example from *Avena sativa*, but also in combination with several plant genes coding for an HST (homogentisate solanesyltransferase) protein is disclosed. However, the level of tolerance to some selected HPPD inhibitor herbicides was rather limited.

In WO2011094199 and US20110185444, the tolerance of several hundred of soybean wild type lines to the HPPD inhibitor isoxaflutole was evaluated. Very few lines displayed reasonable level of tolerance to the herbicides. The putative QTL (quantitative trait loci) responsible for the tolerance was identified. In this region of the genome, a gene coding for an ABC transporter was identified as being the main trait responsible for the improved tolerance to the HPPD inhibitor herbicide observed. However, transgenic plants expressing the identified genes did not display any improvement in tolerance to the tested HPPD inhibitor herbicides.

In WO2010/085705, several mutants of the *Avena sativa* HPPD were disclosed. It was shown that some of the variants displayed improved tolerance in vitro to the triketone “mesotrione”, however, only very few mutants were expressed in tobacco plants. Additionally, none of the tobacco plants expressing these mutants displayed improved tolerance to mesotrione or isoxaflutole compared to tobacco plants expressing the wild type *Avena sativa* HPPD gene.

US 2012/0042413 describes polypeptides having HPPD activity but also showing a certain insensitivity to at least one HPPD inhibitor and further suggests a certain set of mutations at different positions of HPPD enzymes and finally discloses

biochemical data as well as tolerance levels of plants containing few of such mutated HPPDs.

In EP 21453012, several mutants of HPPD have been described; however, the improved tolerance of the described mutants was not demonstrated in planta against
5 several HPPD inhibitor herbicides.

SUMMARY OF INVENTION

Compositions and methods for conferring tolerance to HPPD inhibitor herbicides are provided. Compositions include HPPD polypeptides that are tolerant to
10 HPPD inhibitor herbicides, and isolated, recombinant or chimeric nucleic acid molecules encoding such polypeptides, vectors and host cells comprising those nucleic acid molecules. Compositions also include the antibodies to those polypeptides. The nucleotide sequences can be used in DNA constructs or expression cassettes for transformation and expression in organisms, including microorganisms
15 and plants. The nucleotide sequences may be synthetic sequences that have been designed for expression in an organism including, but not limited to, a microorganism or a plant.

The compositions include nucleic acid molecules encoding herbicide tolerant polypeptides, including nucleic acid molecules encoding an HPPD protein having one
20 or more amino acid substitutions at the positions corresponding to amino acid positions 188, 189, 215, 335, 336, 339, and 340 of SEQ ID NO:1, including the HPPD protein set forth in any of SEQ ID NO:1, 2, 63, 64, or 65, wherein one or more amino acid substitutions at the positions corresponding to amino acid positions 188, 189, 215, 335, 336, 339, and 340 of SEQ ID NO:1 have been introduced, and including
25 any nucleic acid sequence encoding the amino acid sequences set forth in any of SEQ ID NO:3-59, as well as variants and fragments thereof. The invention further comprises the herbicide tolerant HPPD protein encoded by the nucleic acid molecules, as well as compositions comprising the HPPD protein.

Compositions also comprise transformed bacteria, plants, plant cells, tissues,
30 and seeds that are tolerant to the HPPD inhibitor herbicides by the introduction of the nucleic acid sequence of the invention into the genome of the bacteria, plants, plant cells, tissues, and seeds. Where the organism is a plant, the introduction of the sequence allows for HPPD inhibitor herbicides to be applied to plants to selectively kill HPPD inhibitor sensitive weeds or other untransformed plants, but not the

transformed organism. The sequences can additionally be used as a marker for selection of plant cells growing in the presence of one or more HPPD inhibitor herbicides.

Methods for identifying an HPPD enzyme with HPPD inhibitor tolerance activity are additionally provided.

The compositions and methods of the invention are useful for the production of organisms with enhanced tolerance to HPPD inhibitor herbicides. These organisms and compositions comprising the organisms are desirable for agricultural purposes. Plants or seeds comprising the nucleic acid sequence of the invention can be grown in a field and harvested to obtain a plant product. The compositions of the invention are also useful for generating altered or improved proteins that have HPPD inhibitor herbicide tolerance activity, or for detecting the presence of HPPD inhibitor herbicide tolerant proteins or nucleic acids in products or organisms.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an alignment of amino acid sequence of HPPDs from microbial and plant species, including *Pseudomonas fluorescens* (SEQ ID NO:1), *Avena sativa* (SEQ ID NO:63), a variant of the HPPD from *Avena sativa* (SEQ ID NO:64), *Zea mays* (SEQ ID NO:65), *Streptomyces avermitilis* (SEQ ID NO:69), *Arabidopsis thaliana* (SEQ ID NO:66), *Hordeum vulgare* (SEQ ID NO:67), *Daucus carota* (SEQ ID NO:68), *Mycosphaerella graminicola* (SEQ ID NO:70), and *Coccidioides immitis* (SEQ ID NO:71).

DETAILED DESCRIPTION OF THE INVENTION

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to

the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

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Overview

Several efforts have been developed in order to confer to plants an agronomically-acceptable level of tolerance to a broad range of HPPD inhibitor herbicides, including by-passing HPPD-mediated production of homogentisate (US 6,812,010), overexpressing the sensitive enzyme so as to produce quantities of the target enzyme in the plant which are sufficient in relation to the herbicide (WO96/38567), and mutating the HPPD in order to obtain a target enzyme which, while retaining its properties of catalyzing the transformation of HPP into homogentisate, is less sensitive to HPPD inhibitors than is the native HPPD before mutation.

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Despite these successes obtained for the development of plants showing tolerance to several HPPD inhibitors herbicides described above, it is still necessary to develop and/or improve the tolerance of plants to newer or to several different HPPD inhibitors, particularly HPPD inhibitors belonging to the classes of the triketones (e.g. sulcotrione, mesotrione, tembotrione, benzobicyclon and bicyclopyrone), the pyrazolinates (e.g., topramezone and pyrasulfotole), N-(1,2,5-Oxadiazol-3-yl)benzamides (WO 2011/035874), and N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides (WO2012/028579).

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Thus, the present invention provides improved compositions and methods for regulating HPPD inhibitor herbicide tolerance. HPPD inhibitor herbicides like those of the class of N (1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolinates, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione, have an outstanding herbicidal activity against a broad spectrum of economically important monocotyledonous and dicotyledonous annual harmful

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plants. The active substances also act efficiently on perennial harmful plants which produce shoots from rhizomes, wood stocks or other perennial organs and which are difficult to control. Within the meaning of the present invention, "herbicide" is understood as being a herbicidally active substance on its own or such a substance which is combined with an additive which alters its efficacy, such as, for example, an agent which increases its activity (a synergistic agent) or which limits its activity (a safener). The herbicide may further comprise solid or liquid adjuvants or carriers that are ordinarily employed in formulation technology (e.g. natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, emulsifiers, growth promoting agents, and the like), as well as one or more additional herbicides and/or one or more pesticides (e.g., insecticides, virucides, microbicides, amoebicides, pesticides, fungicides, bacteriocides, nematocides, molluscicides, and the like).

The methods involve transforming organisms with nucleotide sequences encoding an HPPD inhibitor tolerance gene of the invention or otherwise introducing such HPPD inhibitor tolerance genes in organisms not containing them (e.g., by mating, cell fusion, or by crossing organisms containing an introduced HPPD inhibitor gene of the invention with organisms not containing it and obtaining progeny containing such gene). The nucleotide sequences of the invention are useful for preparing plants that show increased tolerance to HPPD inhibitor herbicides, particularly increased tolerance to HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolinates, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione. The HPPD inhibitor herbicide tolerance gene of the invention may also show tolerance towards the "coumarone-derivative herbicides" (described in WO2009/090401 , WO2009/090402, WO2008/071918, WO2008/009908). In this regard, any one of the HPPD inhibitor herbicide tolerance genes of the invention can also be expressed in a plant also expressing a chimeric homogentisate solanesyltransferase (HST) gene or a mutated HST gene as described in WO2011/145015, WO2013/064987,

WO2013/064964, or WO2010/029311, to obtain plants tolerant to HST inhibitor herbicides. As used herein, a "coumarone-derivative herbicide" or "HST inhibitor herbicide" encompasses compounds which fall under the IUPAC nomenclature of 5H-thiopyrano[4,3-b]pyridin-8-ol, 5H-thiopyrano[3,4-b]pyrazin-8-ol, oxathiino[5,6-b]pyridin-4-ol, and oxathiino[5, 6-b]pyrazin-4-ol.

Thus, by "HPPD inhibitor herbicide tolerance" gene of the invention is intended a gene encoding a protein that confers upon a cell or organism the ability to tolerate a higher concentration of an HPPD inhibitor herbicide than such cell or organism that does not express the protein, or to tolerate a certain concentration of an HPPD inhibitor herbicide for a longer time than such cell or organism that does not express the protein, or that confers upon a cell or organism the ability to perform photosynthesis, grow, and/or reproduce with less damage or growth inhibition observed than such cell or organism not expressing such protein. An "HPPD inhibitor tolerance protein" includes a protein that confers upon a cell or organism the ability to tolerate a higher concentration of HPPD inhibitor herbicide than such cell or organism that does not express the protein, or to tolerate a certain concentration of HPPD inhibitor herbicide for a longer period of time than such cell or organism that does not express the protein, or that confers upon a cell or organism the ability to perform photosynthesis, grow, and/or reproduce with less damage or growth inhibition observed than such cell or organism not expressing such protein. By "tolerate" or "tolerance" is intended either to survive a particular HPPD inhibitor herbicide application, or the ability to carry out essential cellular functions such as photosynthesis, protein synthesis or respiration and reproduction in a manner that is not readily discernable from untreated cells or organisms, or the ability to have no significant difference in yield or even improved yield for plants treated with HPPD inhibitor herbicide compared to such plants not treated with such herbicide (but where weeds have been removed or prevented by a mechanism other than application of the HPPD inhibitor herbicide, such as the methods described in WO2011/100302, which is herein incorporated by reference in its entirety).

In addition to conferring upon a cell HPPD inhibitor tolerance, the HPPD nucleic acid sequences of the invention encode polypeptides having HPPD activity, i. e., catalyzing the reaction in which para-hydroxyphenylpyruvate (pHPP) is transformed into homogentisate. Preferentially, the catalytic activity of the HPPD protein of the present invention, when tested in vitro, does not differ from that of a

reference HPPD protein by more than 90%, more than 70%, or more than 50%, when assayed under identical conditions and in the absence of the HPPD inhibitor herbicides described above. In some embodiments, the catalytic activity is improved relative to the reference HPPD protein. The catalytic activity of an HPPD enzyme
5 may be defined by various methods well-known in the art. WO 2009/144079 describes various suitable screening methods.

HPPD enzymes that exhibit enhanced tolerance to an HPPD inhibitor herbicide may do so by virtue of exhibiting, relative to the reference HPPD: a) a higher K_m value for the natural substrate, 4-hydroxyphenylpyruvate; b) a lower k_{cat}
10 value for converting 4-hydroxyphenylpyruvate to homogentisate; c) a lower value of the rate constant, k_{on} , governing formation of an enzyme:HPPD inhibitor herbicide complex; d) an increased value of the rate constant, k_{off} , governing dissociation of an enzyme:HPPD inhibitor herbicide complex; and/ or e) as a result of changes in one or both of c) and d), an increased value of the equilibrium constant, K_i (also called K_d),
15 governing dissociation of an enzyme:HPPD inhibitor herbicide complex.

The enzymatic activity of HPPD proteins can be measured by any method that makes it possible either to measure the decrease in the amount of the HPP or O_2 substrates, or to measure the accumulation of any of the products derived from the enzymatic reaction, i.e. homogentisate or CO_2 . In particular, the HPPD activity can be
20 measured by means of the method described in WO2009/144079; Garcia et al. (1997), Biochem. J. 325, 761-769; Garcia et al. (1999), Plant Physiol. 119, 1507-1516; or in WO2012/021785, which are incorporated herein by reference.

For the purposes of the present invention, a "reference" HPPD protein (or HPPD gene) is any HPPD protein or nucleic acid against which the HPPD protein or
25 HPPD nucleic acid of the invention is being compared. This reference HPPD can be a native plant, bacterial, or animal HPPD, or can be a mutated HPPD that is known in the art. Such reference HPPD can be used to determine whether the HPPD protein or nucleic acid of the invention has a particular property of interest (e.g., improved, comparable or decreased HPPD inhibitor herbicide tolerance or HPPD enzyme
30 activity; improved, comparable or decreased expression in a host cell; improved, comparable or decreased protein stability, and the like).

In various embodiments herein, the HPPD inhibitor herbicide tolerant nucleic acid (including isolated, recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleic acid, HPPD polypeptides and

compositions thereof encoded by the nucleic acid, as well as methods of using the nucleic acid for increasing tolerance of a plant to HPPD inhibitor herbicides, particularly increased tolerance to HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolinates, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione) encodes a an HPPD protein that has been modified to contain one or more amino acid substitutions, including 2, 3, 4, 5, 6, or 7 amino acid substitutions, at the positions corresponding to amino acid positions 188, 189, 215, 335, 336, 339, and/or 340 of SEQ ID NO:1. By “corresponding to” is intended the nucleotide or amino acid position relative to that position in SEQ ID NO:1 when two (or more) sequences are aligned using standard alignment algorithms described elsewhere herein. A representative alignment of SEQ ID NO:1 with HPPD amino acid sequences from various microbial and plant species is shown in Figure 1. For example, amino acid positions 188, 189, 215, 335, 336, 339, and 340 of SEQ ID NO:1 correspond to amino acid positions 241, 242, 271, 412, 413, 416, and 417, respectively, of the HPPD from *Avena sativa* (SEQ ID NO:63); to amino acid positions 235, 236, 265, 406, 407, 410, and 411, respectively, of the HPPD from *Hordeum vulgare* (SEQ ID NO:67) and to amino acid positions 242, 243, 272, 413, 414, 417, and 418, respectively, of the HPPD from *Zea mays* (SEQ ID NO:65). Accordingly, depending on the length of the concerned HPPD amino acid sequence, having either additional or fewer residues than the sequence of SEQ ID NO:1, the corresponding position can be located at a position different from positions 188, 189, 215, 335, 336, 339, and 340 in such concerned HPPD protein.

In one embodiment, the HPPD of the present invention has been modified to comprise one or more amino acid substitution(s) selected from the group consisting of:

- (a) a tryptophan, glycine, or serine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1;
- (b) a serine, cysteine, or arginine at the amino acid position corresponding to amino acid position 189 of SEQ ID NO:1;

(c) a proline, serine, histidine, alanine, glycine, or glutamine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1;

(d) a serine or tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

5 (e) a threonine, alanine, or serine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1;

(f) a glutamine, alanine, valine, or glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; and

(g) a leucine at the amino acid position corresponding to amino acid position
10 215 of SEQ ID NO:1.

In another embodiment, the HPPD has been modified to comprise amino acid substitution(s) of:

(a) a proline at the amino acid position corresponding to amino acid
15 position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position
20 corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

25 (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) a proline at the amino acid position corresponding to amino acid
30 position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In specific embodiments, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:1, wherein the HPPD has been modified to comprise amino acid substitution(s) of:

10 (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

20 (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

25 (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

30 (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%,

at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:1 and wherein said HPPD comprises the amino acid substitution(s) of:

- 5 (a) a tryptophan at amino acid position 188 and a tryptophan at amino acid position 336; or
- (b) a proline at amino acid position 335.

 In yet another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:63, wherein the HPPD has been modified to comprise amino acid substitution(s) of:

- 15 (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- 20 (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- 25 (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- 30

(f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In yet another embodiment, the HPPD of the invention has at least 53%, at
5 least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:64, wherein the HPPD has been modified to comprise amino acid substitution(s) of:

10 (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to
15 amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position
20 corresponding to amino acid position 336 of SEQ ID NO:1;

(d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding
25 to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) a proline at the amino acid position corresponding to amino acid
30 position 335 of SEQ ID NO:1.

In yet another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%,

at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:65, wherein the HPPD has been modified to comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:57, or is encoded by a nucleotide sequence having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence set forth herein as SEQ ID NO:60. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1; an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:58, or is encoded by a nucleotide sequence having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence set forth herein as SEQ ID NO:61. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1; an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 53%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:59, or is encoded by a nucleotide sequence having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence set forth herein as SEQ ID NO:62. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1; an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 85% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:1, wherein the HPPD has been modified to comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

5 (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

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In another embodiment, the HPPD of the invention has at least 85% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:1 and wherein said HPPD comprises the amino acid substitution(s) of:

(a) a tryptophan at amino acid position 188 and a tryptophan at amino acid position 336; or

(b) a proline at amino acid position 335.

25 In another embodiment, the HPPD of the invention has at least 85% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:57. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

(a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

5 (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

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In another embodiment, the HPPD of the invention has at least 85% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:58. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

(a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

30 (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

In another embodiment, the HPPD of the invention has at least 85% sequence identity to the amino acid sequence set forth herein as SEQ ID NO:59. The HPPD of this embodiment may further comprise amino acid substitution(s) of:

- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1; an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (f) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1.

Any HPPD sequence can be modified to contain one or more of the substitutions disclosed herein. For example, the HPPD of the invention also encompasses any naturally-occurring bacterial, plant, or animal HPPD enzymes that has been modified to contain one or more of the substitutions described *supra*.

5 In arriving at the HPPD protein of the current invention, a starting amino acid sequence of an existing protein has to be modified by man by replacing at least one amino acid as defined in the present application, which is most conveniently done by modifying the DNA encoding such protein by replacing a certain codon by another codon encoding another amino acid.

10 Exemplary HPPD sequences that can be modified according to the present invention include those from bacteria, for example, of the *Pseudomonas* sp. type, for example *Pseudomonas fluorescens*, or otherwise cyanobacteria of the *Synechocystis* genus. The sequence can also be of plant origin, in particular derived from dicotyledonous plants, umbelliferous plants, or otherwise monocotyledonous plants.

15 Advantageous examples which may be cited are plants such as tobacco, *Arabidopsis*, *Daucus carotta*, *Zea mays* (corn), wheat, barley, *Avena sativa*, *Brachiaria platyphylla*, *Cenchrus echinatus*, *Lolium rigidum*, *Festuca arundinacea*, *Setaria faberi*, *Eleusine indica*, *Sorghum*, *Cenchrus echinatus*, *festuca arundinacea*. The coding sequences, and the way of isolating and cloning them, are known in the art or described elsewhere
20 herein (e.g., SEQ ID NO:63-76). In a particular embodiment of the invention, the HPPD that can be modified according to the present invention is from a bacterial origin, particularly from *Pseudomonas* sp., more particularly from *Pseudomonas fluorescens*, *Rhodococcus* sp., *Blepharisma japonicum*, *Synechococcus* sp., *Picrophilus torridus*, *Kordia algicida* or from a plant origin, including from
25 *Arabidopsis thaliana*, *Sorghum bicolor*, *Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare*, *Lolium rigidum*, or *Avena sativa*.

For the purposes of the present invention, the HPPD of the invention may also comprise further modifications, for example, wherein some amino acids (e.g., 1 to 10 amino acids) have been replaced, added or deleted for cloning purposes, to make a
30 transit peptide fusion, and the like, which retains HPPD activity, i.e. the property of catalyzing the conversion of para-hydroxyphenylpyruvate to homogentisate, or can be any HPPD that can be further improved. For example, the HPPD that can be further improved by the modifications described herein can be the variant HPPD derived from *Pseudomonas fluorescens* set forth herein as SEQ ID NO:2, the variant HPPD

from *Avena sativa* set forth herein as SEQ ID NO:64, the variant HPPD sequences set forth in any of SEQ ID NO:3-326, 383-389, 393, 395, and 397-459 in WO2012/021785, which is herein incorporated by reference in its entirety; the HPPD sequences set forth in any of SEQ ID NO:2-14 and 20-50 of WO2011/068567, which is herein incorporated by reference in its entirety; the the HPPD sequences set forth in any of SEQ ID NO:15-26 of WO2010/085705, which is herein incorporated by reference in its entirety; an HPPD having one or more of the substitutions described in WO09/144079 or United States Patent 6,245,968, each of which is herein incorporated by reference in its entirety; an HPPD having one or more of the substitutions described in Tables 1, 2, 5, or 6 of WO2010/085705; and/or an HPPD having one or more of the substitutions described in Table 1 of WO2011/068567.

In some embodiments, the nucleotide sequence of the invention (including isolated, recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleic acid sequence, amino acid sequences and compositions thereof encoded by the nucleic acid sequence, as well as methods of using the nucleic acid sequence for increasing tolerance of a plant to HPPD inhibitor herbicides, particularly increased tolerance to HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione) encodes the amino acid sequence set forth in any one of SEQ ID NO:3-59, and fragments and variants thereof that encode a HPPD inhibitor herbicide tolerance polypeptide. Thus, in this embodiment, the HPPD of the invention comprises the amino acid sequence set forth in any of SEQ ID NO:3-59, and fragments and variants thereof, that confer tolerance to HPPD inhibitor herbicides in a host cell.

A. Methods for measuring HPPD inhibitor tolerance

Any suitable method for measuring tolerance to HPPD inhibitor herbicides can be used to evaluate the HPPD sequences of the invention. Tolerance can be measured

by monitoring the ability of a cell or organism to survive a particular HPPD inhibitor herbicide application, or the ability to carry out essential cellular functions such as photosynthesis, protein synthesis or respiration and reproduction in a manner that is not readily discernable from untreated cells or organisms, or the ability to have no significant difference in yield or even improved yield for plants treated with HPPD inhibitor herbicide compared to such plants not treated with such herbicide (but where weeds have been removed or prevented by a mechanism other than application of the HPPD inhibitor herbicide). In some embodiments, tolerance can be measured according to a visible indicator phenotype of the cell or organism transformed with a nucleic acid comprising the gene coding for the respective HPPD protein, or in an *in vitro* assay of the HPPD protein, in the presence of different concentrations of the various HPPD inhibitors. Dose responses and relative shifts in dose responses associated with these indicator phenotypes (formation of brown color, growth inhibition, bleaching, herbicidal effect etc) are conveniently expressed in terms, for example, of GR50 (concentration for 50% reduction of growth) or MIC (minimum inhibitory concentration) values where increases in values correspond to increases in inherent tolerance of the expressed HPPD, in the normal manner based upon plant damage, meristematic bleaching symptoms etc. at a range of different concentrations of herbicides. These data can be expressed in terms of, for example, GR50 values derived from dose/response curves having "dose" plotted on the x-axis and "percentage kill", "herbicidal effect", "numbers of emerging green plants" etc. plotted on the y-axis where increased GR50 values correspond to increased levels of inherent tolerance of the expressed HPPD. Herbicides can suitably be applied pre-emergence or post emergence.

In various embodiments, tolerance level of the nucleic acid or gene encoding an HPPD protein according to the invention, or the HPPD protein of the invention can be screened via transgenesis, regeneration, breeding and spray testing of a test plant such as tobacco, or a crop plant such as soybean or cotton. In line with the results obtained by such screening, such plants are more tolerant, desirably tolerant to at least 2 times the normal dose recommended for field applications, even more preferably tolerant up to 4 times the normal dose recommended for field applications, to HPPD inhibitors (e.g., HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-

Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, 5 topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione) than such plants that do not contain any exogenous gene encoding an HPPD protein, or than plants that contain a gene comprising a reference HPPD-encoding DNA, for example, an *Arabidopsis thaliana* HPPD-encoding DNA, under control of the same promoter as the nucleic acid encoding the HPPD protein of the invention. Accordingly, the term 10 “capable of increasing the tolerance of a plant to at least one herbicide acting on HPPD” denotes a tolerance by the plant expressing the HPPD of the invention to at least 2x, or 3x, or 4x, or greater, the normal field dose of the HPPD inhibitor herbicide as compared to a plant only expressing its endogenous HPPD or a plant expressing a reference HPPD enzyme. In this regard, the term “herbicide acting on HPPD” is not 15 limited to substances which are known and/or used as herbicides but to any substances which inhibit the catalytic activity of HPPD proteins.

Alternatively, at the quantitative level data like pI_{50} (pI_{50} -value means the log value of the concentration of inhibitor necessary to inhibit 50% of the enzyme activity in molar concentration) can be obtained for the HPPD protein of the invention and 20 compared to a reference HPPD sequence in presence or absence of any respective HPPD inhibitor herbicide.

A specific, although non-limiting, type of assay that can be used to evaluate the HPPD sequences of the invention is a colorimetric assay. In this assay, a YT-broth-type culture medium with 1% agarose, 5mM L-Tyrosine and 42mM Succinate, 25 which contains the selection agent for the vector pSE420 (Invitrogen, Karlsruhe, Germany) or a modified version of pSE420 (pSE420(RI)NX) is poured into deep well plates. *E. coli* culture in the exponential growth phase which contains the vector pSE420-HPPDx (HPPDx means any gene coding for a putative HPPD enzyme/protein) is applied to each well. After 16 hours at 37°C, the wells which do 30 not contain the culture medium, those which have been seeded with an *E. coli* culture containing the empty vector pSE420 are transparent, or those which have been seeded with an *E. coli* culture containing a vector pSE420-HPPDx containing a gene coding for an inactive HPPD are transparent, while the wells seeded with an *E. coli* culture containing the vector pSE420-HPPDx coding for an active HPPD are brown. It has

been previously demonstrated that this test reflects the HPPD activity, whatever the origin of this activity is, and allows the identification of HPPD activities (US 6,768,044), i.e. at a qualitative level.

5 *B. Methods of introducing mutations into HPPD sequences*

In the mutated HPPD protein encoded by the nucleic acid of the invention at least one amino acid has been replaced as defined above.

The replacement can be effected in the nucleic acid sequence which encodes the reference HPPD as defined above by any means which is appropriate for
10 replacing, in the said sequence, the codon which encodes the amino acid to be replaced with the codon which corresponds to the amino acid which is to replace it, with the said codons being widely described in the literature and well known to the skilled person.

Several molecular biological methods can be used to achieve this replacement.

15 A useful method for preparing a mutated nucleic acid sequence according to the invention and the corresponding protein comprises carrying out site-directed mutagenesis on codons encoding one or more amino acids which are selected in advance. The methods for obtaining these site-directed mutations are well known to the skilled person and widely described in the literature (in particular: Directed
20 Mutagenesis: A Practical Approach, 1991, Edited by M.J. McPHERSON, IRL PRESS), or are methods for which it is possible to employ commercial kits (for example the QUIKCHANGE™ lightning mutagenesis kit from Qiagen). After the site-directed mutagenesis, it is useful to select the cells which contain a mutated HPPD which is less sensitive to an HPPD inhibitor by using an appropriate screening
25 aid. Appropriate screening methods to achieve this have been described above.

Alternatively, a DNA sequence encoding the reference HPPD can be modified *in silico* to encode an HPPD protein having one or more of the substitutions recited herein, and then synthesized *de novo*. The nucleotide sequence encoding the mutated HPPD protein can be introduced into a host cell as described elsewhere herein.

30

A. Isolated polynucleotides, and variants and fragments thereof

In some embodiments, the present invention comprises isolated or recombinant, polynucleotides. An “isolated” or “recombinant” polynucleotide or polypeptide/protein, or biologically active portion thereof, as defined herein is no

longer present in its original, native organism, such as when contained in a heterologous host cell or in a transgenic plant cell, seed or plant.. In one embodiment, an “isolated” polynucleotide is free of sequences (for example, protein encoding or regulatory sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5’ and 3’ ends of the nucleic acid) in the genomic DNA of the organism from which the polynucleotide is derived. The term “recombinant” encompasses polynucleotides or polypeptides that have been manipulated with respect to the native polynucleotide or polypeptide, such that the polynucleotide or polypeptide differs (e.g., in chemical composition or structure) from what is occurring in nature. In another embodiment, an “isolated” or “recombinant” polynucleotide is free of internal sequences (i.e. introns) that naturally occur in the genomic DNA of the organism from which the polynucleotide is derived. A typical example of such polynucleotide is a so-called Complementary DNA (cDNA). For example, in various embodiments, the isolated HPPD inhibitor herbicide tolerance-encoding polynucleotide can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequence that naturally flanks the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. Nucleic acid molecules of the invention include those that encode the HPPD of the invention, including, for example, the nucleotide sequences set forth in any of SEQ ID NO:60-62.

The present invention further contemplates variants and fragments of any nucleic acid sequence encoding the amino acid sequences set forth in any of SEQ ID NO:3-59. A “fragment” of a polynucleotide may encode a biologically active portion of a polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed elsewhere herein. Polynucleotides that are fragments of a polynucleotide comprise at least about 15, 20, 50, 75, 100, 200, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, contiguous nucleotides, or up to the number of nucleotides present in a full-length polynucleotide disclosed herein depending upon the intended use (e.g., an HPPD nucleic acid described herein). By “contiguous” nucleotides is intended nucleotide residues that are immediately adjacent to one another.

Fragments of the polynucleotides of the present invention generally will encode polypeptide fragments that retain the biological activity of the full-length HPPD inhibitor herbicide tolerance protein; i.e., herbicide-tolerance activity. By “retains herbicide tolerance activity” is intended that the fragment will have at least

about 30%, at least about 50%, at least about 70%, at least about 80%, 85%, 90%, 95%, 100%, 110%, 125%, 150%, 175%, 200%, 250%, at least about 300% or greater of the herbicide tolerance activity of the full-length HPPD inhibitor herbicide tolerance protein disclosed herein as SEQ ID NO:2. Methods for measuring herbicide tolerance activity are well known in the art and exemplary methods are described herein. In a non-limiting example, a fragment of the invention will be tolerant to the same dose of an HPPD inhibitor herbicide, or tolerant to 2x, 3x, 4x, or higher dose of an HPPD inhibitor herbicide, or the fragments will be as or more tolerant based on PI_{50} or K_i between the fragment and SEQ ID NO:2.

10 A fragment of a polynucleotide that encodes a biologically active portion of a polypeptide of the invention will encode at least about 150, 175, 200, 250, 300, 350 contiguous amino acids, or up to the total number of amino acids present in a full-length polypeptide of the invention. In a non-limiting example, a fragment of a polynucleotide that encodes a biologically active portion of a polypeptide of the invention comprises one or more of amino acid positions 188, 189, 215, 335, 336, 339 and 340 of SEQ ID NO:2.

The invention also encompasses variant polynucleotides as described *supra*. “Variants” of the polynucleotide also include those sequences that encode the HPPD of the invention but that differ conservatively because of the degeneracy of the genetic code, as well as those that are sufficiently identical. Variants of the present invention will retain HPPD enzyme activity and HPPD herbicide inhibitor tolerance. The term “sufficiently identical” is intended a polypeptide or polynucleotide sequence that has at least about 60% or 65% sequence identity, about 70% or 75% sequence identity, about 80% or 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity compared to a reference sequence using one of the alignment programs using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of polypeptides encoded by two polynucleotides by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

30 Bacterial genes quite often possess multiple methionine initiation codons in proximity to the start of the open reading frame. Often, translation initiation at one or more of these start codons will lead to generation of a functional protein. These start codons can include ATG codons. However, bacteria such as *Bacillus* sp. also

recognize the codon GTG as a start codon, and proteins that initiate translation at GTG codons contain a methionine at the first amino acid. Furthermore, it is not often determined *a priori* which of these codons are used naturally in the bacterium. Thus, it is understood that use of one of the alternate methionine codons may lead to

5 generation of variants that confer herbicide tolerance. These herbicide tolerance proteins are encompassed in the present invention and may be used in the methods of the present invention. Naturally occurring allelic variants can be identified with the use of well-known molecular biology techniques, such as polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also

10 include synthetically derived polynucleotides that have been generated, for example, by using site-directed or other mutagenesis strategies but which still encode the polypeptide having the desired biological activity.

The skilled artisan will further appreciate that changes can be introduced by further mutation of the polynucleotides of the invention thereby leading to further

15 changes in the amino acid sequence of the encoded polypeptides, without altering the biological activity of the polypeptides. Thus, variant isolated polynucleotides can be created by introducing one or more additional nucleotide substitutions, additions, or deletions into the corresponding polynucleotide encoding the HPPD of the invention, such that 1-5, 1-10, or 1-15 amino acid substitutions, additions or deletions, or 1, 2, 3,

20 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acid substitutions, additions or deletions, are introduced into the encoded polypeptide. Further mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis, or gene shuffling techniques. Such variant polynucleotides are also encompassed by the present invention.

25 Variant polynucleotides can be made by introducing mutations randomly along all or part of the coding sequence, such as by saturation mutagenesis or permutational mutagenesis, and the resultant mutants can be screened for the ability to confer herbicide tolerance activity to identify mutants that retain activity.

Additional methods for generating variants include subjecting a cell

30 expressing a protein disclosed herein (or library thereof) to a specific condition that creates a stress to the activity of the protein. Specific conditions can include (but are not limited to) changes in temperature, changes in pH, and changes in the concentrations of substrates or inhibitors. The protein library can be subjected to

these conditions during the time of protein expression (e.g., in *E. coli* or other host) or following creation of a protein extract, or following protein purification.

The functional or enzymatic activity of the protein library that has been subjected to a stress condition can then be compared to the reference protein to
5 identify proteins with improved properties. This activity comparison can be carried out as part of a growth screen or alternatively as part of an enzymatic assay that quantifies the activity of the protein. The properties that can be identified as improved can include HPPD inhibitor herbicide tolerance, changes in kinetic constants (including K_m , K_i , V_{max}), protein stability, protein thermostability, or
10 protein temperature optimum.

C. Isolated Proteins and Variants and Fragments Thereof

Herbicide tolerance polypeptides are also encompassed within the present invention. A herbicide tolerance polypeptide includes preparations of polypeptides
15 having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-herbicide tolerance polypeptide (also referred to herein as a “contaminating protein”). In the present invention, “herbicide tolerance protein” is intended an HPPD polypeptide disclosed herein. Fragments, biologically active portions, and variants thereof are also provided, and may be used to practice the methods of the present invention.

20 “Fragments” or “biologically active portions” include polypeptide fragments comprising a portion of an amino acid sequence encoding an herbicide tolerance protein and that retains herbicide tolerance activity. A biologically active portion of an herbicide tolerance protein can be a polypeptide that is, for example, 10, 25, 50, 100 or more amino acids in length. Such biologically active portions can be prepared
25 by recombinant techniques and evaluated for herbicide tolerance activity.

By “variants” is intended proteins or polypeptides having an amino acid sequence that is at least about 60%, 65%, about 70%, 75%, about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to any of SEQ ID NO:3-59, wherein said variant has HPPD enzyme activity and HPPD inhibitor
30 herbicide tolerance. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of polypeptides encoded by two polynucleotides by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

For example, conservative amino acid substitutions may be made at one or more nonessential amino acid residues. A “nonessential” amino acid residue is a residue that can be altered from the reference sequence of a polypeptide without altering the biological activity, whereas an “essential” amino acid residue is required for biological activity. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Amino acid substitutions may be made in nonconserved regions that retain function. In general, such substitutions would not be made for conserved amino acid residues, or for amino acid residues residing within a conserved motif, where such residues are essential for polypeptide activity. However, one of skill in the art would understand that functional variants may have minor conserved or nonconserved alterations in the conserved residues.

Antibodies to the HPPD of the present invention, or to variants or fragments thereof, are also encompassed. Methods for producing antibodies are well known in the art (see, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; U.S. Patent No. 4,196,265).

Thus, one aspect of the invention concerns antibodies, single-chain antigen binding molecules, or other proteins that specifically bind to one or more of the protein or peptide molecules of the invention and their homologs, fusions or fragments. In a particularly preferred embodiment, the antibody specifically binds to a protein having the amino acid sequence set forth in SEQ ID NO:3-59 or a fragment thereof. In another embodiment, the antibody specifically binds to a fusion protein comprising an amino acid sequence selected from the amino acid sequence set forth in SEQ ID NO:3-59, or a fragment thereof.

Antibodies of the invention may be used to quantitatively or qualitatively detect the protein or peptide molecules of the invention, or to detect post translational

modifications of the proteins. As used herein, an antibody or peptide is said to "specifically bind" to a protein or peptide molecule of the invention if such binding is not competitively inhibited by the presence of non-related molecules.

5 *D. Gene stacking*

In the commercial production of crops, it is desirable to eliminate under reliable pesticidal management unwanted plants (i.e., "weeds") from a field of crop plants. An ideal treatment would be one which could be applied to an entire field but which would eliminate only the unwanted plants while leaving the crop plants
10 unaffected. One such treatment system would involve the use of crop plants which are tolerant to an herbicide so that when the herbicide is sprayed on a field of herbicide-tolerant crop plants, the crop plants would continue to thrive while non-herbicide-tolerant weeds are killed or severely damaged. Ideally, such treatment systems would take advantage of varying herbicide properties so that weed control could provide the
15 best possible combination of flexibility and economy. For example, individual herbicides have different longevities in the field, and some herbicides persist and are effective for a relatively long time after they are applied to a field while other herbicides are quickly broken down into other and/or non-active compounds. An ideal treatment system would allow the use of different herbicides so that growers could
20 tailor the choice of herbicides for a particular situation.

While a number of herbicide-tolerant crop plants are presently commercially available, an issue that has arisen for many commercial herbicides and herbicide/crop combinations is that individual herbicides typically have incomplete spectrum of activity against common weed species. For most individual herbicides which have
25 been in use for some time, populations of herbicide resistant weed species and biotypes have become more prevalent (see, e.g., Tranel and Wright (2002) *Weed Science* 50: 700-712; Owen and Zelaya (2005) *Pest Manag. Sci.* 61: 301-311). Transgenic plants which are tolerant to more than one herbicide have been described (see, e.g., W02005/012515). However, improvements in every aspect of crop
30 production, weed control options, extension of residual weed control, and improvement in crop yield are continuously in demand.

The HPPD protein or nucleotide sequence of the invention is advantageously combined in plants with other genes which encode proteins or RNAs that confer useful agronomic properties to such plants. Among the genes which encode proteins

or RNAs that confer useful agronomic properties on the transformed plants, mention can be made of the DNA sequences encoding proteins which confer tolerance to one or more herbicides that, according to their chemical structure, differ from HPPD inhibitor herbicides, and others which confer tolerance to certain insects, those which
5 confer tolerance to certain diseases, DNAs that encodes RNAs that provide nematode or insect control, and the like.

Such genes are in particular described in published PCT Patent Applications WO 91/02071 and WO95/06128 and in U.S. Patents 7,923,602 and US Patent Application Publication No. 20100166723, each of which is herein incorporated by reference in its
10 entirety.

Among the DNA sequences encoding proteins which confer tolerance to certain herbicides on the transformed plant cells and plants, mention can be made of a bar or PAT gene or the *Streptomyces coelicolor* gene described in WO2009/152359 which confers tolerance to glufosinate herbicides, a gene encoding a suitable EPSPS
15 which confers tolerance to herbicides having EPSPS as a target, such as glyphosate and its salts (US 4,535,060, US 4,769,061, US 5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,310,667, US 5,312,910, US 5,627,061, US 5,633,435), a gene encoding glyphosate-n-acetyltransferase (for example, US 8,222,489, US 8,088,972, US 8,044,261, US 8,021,857, US 8,008,547, US 7,999,152,
20 US 7,998,703, US 7,863,503, US 7,714,188, US 7,709,702, US 7,666,644, US 7,666,643, US 7,531,339, US 7,527,955, and US 7,405,074), or a gene encoding glyphosate oxydoreductase (for example, US 5,463,175).

Among the DNA sequences encoding a suitable EPSPS which confer tolerance to the herbicides which have EPSPS as a target, mention will more
25 particularly be made of the gene which encodes a plant EPSPS, in particular maize EPSPS, particularly a maize EPSPS which comprises two mutations, particularly a mutation at amino acid position 102 and a mutation at amino acid position 106 (WO 2004/074443), and which is described in Patent Application US 6566587, hereinafter named double mutant maize EPSPS or 2mEPSPS, or the gene which
30 encodes an EPSPS isolated from *Agrobacterium* and which is described by sequence ID No. 2 and sequence ID No. 3 of US Patent 5,633,435, also named CP4.

Among the DNA sequences encoding a suitable EPSPS which confer tolerance to the herbicides which have EPSPS as a target, mention will more particularly be made of the gene which encodes an EPSPS GRG23 from *Arthrobacter*

globiformis, but also the mutants GRG23 ACE1, GRG23 ACE2, or GRG23 ACE3, particularly the mutants or variants of GRG23 as described in WO2008/100353, such as GRG23(ace3)R173K of SEQ ID No. 29 in WO2008/100353.

In the case of the DNA sequences encoding EPSPS, and more particularly
5 encoding the above genes, the sequence encoding these enzymes is advantageously preceded by a sequence encoding a transit peptide, in particular the “optimized transit peptide” described in US Patent 5,510,471 or 5,633,448.

Exemplary herbicide tolerance traits that can be combined with the nucleic acid sequence of the invention further include at least one ALS (acetolactate synthase)
10 inhibitor (WO 2007/024782); a mutated Arabidopsis ALS/AHAS gene (U.S. Patent 6,855,533); genes encoding 2,4-D-monooxygenases conferring tolerance to 2,4-D (2,4-dichlorophenoxyacetic acid) by metabolism (U.S. Patent 6,153,401); and, genes encoding Dicamba monooxygenases conferring tolerance to dicamba (3,6-dichloro-2-methoxybenzoic acid) by metabolism (US 2008/0119361 and US
15 2008/0120739).

In various embodiments, the HPPD of the invention is stacked with one or more herbicide tolerant genes, including one or more additional HPPD inhibitor herbicide tolerant genes, and/or one or more genes tolerant to glyphosate and/or glufosinate. In one embodiment, the HPPD of the invention is combined with
20 2mEPSPS and bar.

Among the DNA sequences encoding proteins concerning properties of tolerance to insects, mention will more particularly be made of the Bt proteins widely described in the literature and well known to those skilled in the art. Mention will also be made of proteins extracted from bacteria such as *Photorhabdus* (WO 97/17432 &
25 WO 98/08932).

Among such DNA sequences encoding proteins of interest which confer novel properties of tolerance to insects, mention will more particularly be made of the Bt Cry or VIP proteins widely described in the literature and well known to those skilled in the art. These include the Cry1F protein or hybrids derived from a Cry1F protein
30 (e.g., the hybrid Cry1A-Cry1F proteins described in US 6,326,169; US 6,281,016; US 6,218,188, or toxic fragments thereof), the Cry1A-type proteins or toxic fragments thereof, preferably the Cry1Ac protein or hybrids derived from the Cry1Ac protein (e.g., the hybrid Cry1Ab-Cry1Ac protein described in US 5,880,275) or the Cry1Ab or Bt2 protein or insecticidal fragments thereof as described in EP451878, the

Cry2Ae, Cry2Af or Cry2Ag proteins as described in WO02/057664 or toxic fragments thereof, the Cry1A.105 protein described in WO 2007/140256 (SEQ ID No. 7) or a toxic fragment thereof, the VIP3Aa19 protein of NCBI accession ABG20428, the VIP3Aa20 protein of NCBI accession ABG20429 (SEQ ID No. 2 in
 5 WO 2007/142840), the VIP3A proteins produced in the COT202 or COT203 cotton events (WO 2005/054479 and WO 2005/054480, respectively), the Cry proteins as described in WO01/47952, the VIP3Aa protein or a toxic fragment thereof as described in Estruch et al. (1996), Proc Natl Acad Sci U S A. 28;93(11):5389-94 and US 6,291,156, the insecticidal proteins from *Xenorhabdus* (as described in
 10 WO98/50427), *Serratia* (particularly from *S. entomophila*) or *Photorhabdus* species strains, such as Tc-proteins from *Photorhabdus* as described in WO98/08932 (e.g., Waterfield et al., 2001, Appl Environ Microbiol. 67(11):5017-24; Ffrench-Constant and Bowen, 2000, Cell Mol Life Sci.; 57(5):828-33). Also any variants or mutants of any one of these proteins differing in some (1-10, preferably 1-5) amino acids from
 15 any of the above sequences, particularly the sequence of their toxic fragment, or which are fused to a transit peptide, such as a plastid transit peptide, or another protein or peptide, is included herein.

In various embodiments, the HPPD sequence of the invention can be combined in plants with one or more genes conferring a desirable trait, such as
 20 herbicide tolerance, insect tolerance, drought tolerance, nematode control, water use efficiency, nitrogen use efficiency, improved nutritional value, disease resistance, improved photosynthesis, improved fiber quality, stress tolerance, improved reproduction, and the like.

Particularly useful transgenic events which may be combined with the genes
 25 of the current invention in plants of the same species (e.g., by crossing or by re-transforming a plant containing another transgenic event with a chimeric gene of the invention), include Event 531/ PV-GHBK04 (cotton, insect control, described in WO2002/040677), Event 1143-14A (cotton, insect control, not deposited, described in WO 06/128569); Event 1143-51B (cotton, insect control, not deposited, described
 30 in WO 06/128570); Event 1445 (cotton, herbicide tolerance, not deposited, described in US-A 2002-120964 or WO 02/034946); Event 17053 (rice, herbicide tolerance, deposited as PTA-9843, described in WO 10/117737); Event 17314 (rice, herbicide tolerance, deposited as PTA-9844, described in WO 10/117735); Event 281-24-236 (cotton, insect control - herbicide tolerance, deposited as PTA-6233, described in WO

05/103266 or US-A 2005-216969); Event 3006-210-23 (cotton, insect control - herbicide tolerance, deposited as PTA-6233, described in US-A 2007-143876 or WO 05/103266); Event 3272 (corn, quality trait, deposited as PTA-9972, described in WO 06/098952 or US-A 2006-230473); Event 33391 (wheat, herbicide tolerance, deposited as PTA-2347, described in WO2002/027004), Event 40416 (corn, insect control - herbicide tolerance, deposited as ATCC PTA-11508, described in WO 11/075593); Event 43A47 (corn, insect control - herbicide tolerance, deposited as ATCC PTA-11509, described in WO 11/075595); Event 5307 (corn, insect control, deposited as ATCC PTA-9561, described in WO 10/077816); Event ASR-368 (bent grass, herbicide tolerance, deposited as ATCC PTA-4816, described in US-A 2006-162007 or WO 04/053062); Event B16 (corn, herbicide tolerance, not deposited, described in US-A 2003-126634); Event BPS-CV127-9 (soybean, herbicide tolerance, deposited as NCIMB No. 41603, described in WO 10/080829); Event BLR1 (oilseed rape, restoration of male sterility, deposited as NCIMB 41193, described in WO2005/074671), Event CE43-67B (cotton, insect control, deposited as DSM ACC2724, described in US-A 2009-217423 or WO 06/128573); Event CE44-69D (cotton, insect control, not deposited, described in US-A 2010-0024077); Event CE44-69D (cotton, insect control, not deposited, described in WO 06/128571); Event CE46-02A (cotton, insect control, not deposited, described in WO 06/128572); Event COT102 (cotton, insect control, not deposited, described in US-A 2006-130175 or WO 04/039986); Event COT202 (cotton, insect control, not deposited, described in US-A 2007-067868 or WO 05/054479); Event COT203 (cotton, insect control, not deposited, described in WO 05/054480);); Event DAS21606-3 / 1606 (soybean, herbicide tolerance, deposited as PTA-11028, described in WO2012/033794), Event DAS40278 (corn, herbicide tolerance, deposited as ATCC PTA-10244, described in WO 11/022469); Event DAS-44406-6 / pDAB8264.44.06.1 (soybean, herbicide tolerance, deposited as PTA-11336, described in WO2012/075426), Event DAS-14536-7 /pDAB8291.45.36.2 (soybean, herbicide tolerance, deposited as PTA-11335, described in WO2012/075429), Event DAS-59122-7 (corn, insect control - herbicide tolerance, deposited as ATCC PTA 11384 , described in US-A 2006-070139); Event DAS-59132 (corn, insect control - herbicide tolerance, not deposited, described in WO 09/100188); Event DAS68416 (soybean, herbicide tolerance, deposited as ATCC PTA-10442, described in WO 11/066384 or WO 11/066360); Event DP-098140-6 (corn, herbicide tolerance, deposited as ATCC PTA-8296, described in US-A 2009-

137395 or WO 08/112019); Event DP-305423-1 (soybean, quality trait, not deposited, described in US-A 2008-312082 or WO 08/054747); Event DP-32138-1 (corn, hybridization system, deposited as ATCC PTA-9158, described in US-A 2009-0210970 or WO 09/103049); Event DP-356043-5 (soybean, herbicide tolerance, deposited as ATCC PTA-8287, described in US-A 2010-0184079 or WO 08/002872); Event EE-1 (brinjal, insect control, not deposited, described in WO 07/091277); Event FI117 (corn, herbicide tolerance, deposited as ATCC 209031, described in US-A 2006-059581 or WO 98/044140); Event FG72 (soybean, herbicide tolerance, deposited as PTA-11041, described in WO2011/063413), Event GA21 (corn, herbicide tolerance, deposited as ATCC 209033, described in US-A 2005-086719 or WO 98/044140); Event GG25 (corn, herbicide tolerance, deposited as ATCC 209032, described in US-A 2005-188434 or WO 98/044140); Event GHB119 (cotton, insect control - herbicide tolerance, deposited as ATCC PTA-8398, described in WO 08/151780); Event GHB614 (cotton, herbicide tolerance, deposited as ATCC PTA-6878, described in US-A 2010-050282 or WO 07/017186); Event GJ11 (corn, herbicide tolerance, deposited as ATCC 209030, described in US-A 2005-188434 or WO 98/044140); Event GM RZ13 (sugar beet, virus resistance , deposited as NCIMB-41601, described in WO 10/076212); Event H7-1 (sugar beet, herbicide tolerance, deposited as NCIMB 41158 or NCIMB 41159, described in US-A 2004-172669 or WO 04/074492); Event JOPLIN1 (wheat, disease tolerance, not deposited, described in US-A 2008-064032); Event LL27 (soybean, herbicide tolerance, deposited as NCIMB41658, described in WO 06/108674 or US-A 2008-320616); Event LL55 (soybean, herbicide tolerance, deposited as NCIMB 41660, described in WO 06/108675 or US-A 2008-196127); Event LLcotton25 (cotton, herbicide tolerance, deposited as ATCC PTA-3343, described in WO 03/013224 or US-A 2003-097687); Event LLRICE06 (rice, herbicide tolerance, deposited as ATCC 203353, described in US 6,468,747 or WO 00/026345); Event LLRice62 (rice, herbicide tolerance, deposited as ATCC 203352, described in WO2000/026345), Event LLRICE601 (rice, herbicide tolerance, deposited as ATCC PTA-2600, described in US-A 2008-2289060 or WO 00/026356); Event LY038 (corn, quality trait, deposited as ATCC PTA-5623, described in US-A 2007-028322 or WO 05/061720); Event MIR162 (corn, insect control, deposited as PTA-8166, described in US-A 2009-300784 or WO 07/142840); Event MIR604 (corn, insect control, not deposited, described in US-A 2008-167456 or WO 05/103301); Event MON15985 (cotton,

insect control, deposited as ATCC PTA-2516, described in US-A 2004-250317 or WO 02/100163); Event MON810 (corn, insect control, not deposited, described in US-A 2002-102582); Event MON863 (corn, insect control, deposited as ATCC PTA-2605, described in WO 04/011601 or US-A 2006-095986); Event MON87427 (corn, 5 pollination control, deposited as ATCC PTA-7899, described in WO 11/062904); Event MON87460 (corn, stress tolerance, deposited as ATCC PTA-8910, described in WO 09/111263 or US-A 2011-0138504); Event MON87701 (soybean, insect control, deposited as ATCC PTA-8194, described in US-A 2009-130071 or WO 09/064652); Event MON87705 (soybean, quality trait - herbicide tolerance, deposited as ATCC 10 PTA-9241, described in US-A 2010-0080887 or WO 10/037016); Event MON87708 (soybean, herbicide tolerance, deposited as ATCC PTA-9670, described in WO 11/034704); Event MON87712 (soybean, yield, deposited as PTA-10296, described in WO2012/051199), Event MON87754 (soybean, quality trait, deposited as ATCC PTA-9385, described in WO 10/024976); Event MON87769 (soybean, quality trait, 15 deposited as ATCC PTA-8911, described in US-A 2011-0067141 or WO 09/102873); Event MON88017 (corn, insect control - herbicide tolerance, deposited as ATCC PTA-5582, described in US-A 2008-028482 or WO 05/059103); Event MON88913 (cotton, herbicide tolerance, deposited as ATCC PTA-4854, described in WO 04/072235 or US-A 2006-059590); Event MON88302 (oilseed rape, herbicide 20 tolerance, deposited as PTA-10955, described in WO2011/153186), Event MON88701 (cotton, herbicide tolerance, deposited as PTA-11754, described in WO2012/134808), Event MON89034 (corn, insect control, deposited as ATCC PTA-7455, described in WO 07/140256 or US-A 2008-260932); Event MON89788 (soybean, herbicide tolerance, deposited as ATCC PTA-6708, described in US-A 25 2006-282915 or WO 06/130436); Event MS11 (oilseed rape, pollination control - herbicide tolerance, deposited as ATCC PTA-850 or PTA-2485, described in WO 01/031042); Event MS8 (oilseed rape, pollination control - herbicide tolerance, deposited as ATCC PTA-730, described in WO 01/041558 or US-A 2003-188347); Event NK603 (corn, herbicide tolerance, deposited as ATCC PTA-2478, described in 30 US-A 2007-292854); Event PE-7 (rice, insect control, not deposited, described in WO 08/114282); Event RF3 (oilseed rape, pollination control - herbicide tolerance, deposited as ATCC PTA-730, described in WO 01/041558 or US-A 2003-188347); Event RT73 (oilseed rape, herbicide tolerance, not deposited, described in WO 02/036831 or US-A 2008-070260); Event SYHT0H2 / SYN-000H2-5 (soybean,

herbicide tolerance, deposited as PTA-11226, described in WO2012/082548), Event T227-1 (sugar beet, herbicide tolerance, not deposited, described in WO 02/44407 or US-A 2009-265817); Event T25 (corn, herbicide tolerance, not deposited, described in US-A 2001-029014 or WO 01/051654); Event T304-40 (cotton, insect control - herbicide tolerance, deposited as ATCC PTA-8171, described in US-A 2010-077501 or WO 08/122406); Event T342-142 (cotton, insect control, not deposited, described in WO 06/128568); Event TC1507 (corn, insect control - herbicide tolerance, not deposited, described in US-A 2005-039226 or WO 04/099447); Event VIP1034 (corn, insect control - herbicide tolerance, deposited as ATCC PTA-3925., described in WO 03/052073), Event 32316 (corn, insect control-herbicide tolerance, deposited as PTA-11507, described in WO 11/084632), Event 4114 (corn, insect control-herbicide tolerance, deposited as PTA-11506, described in WO 11/084621), event EE-GM3 / FG72 (soybean, herbicide tolerance, ATCC Accession N° PTA-11041, WO2011/063413A2), event DAS-68416-4 (soybean, herbicide tolerance, ATCC Accession N° PTA-10442, WO2011/066360A1), event DAS-68416-4 (soybean, herbicide tolerance, ATCC Accession N° PTA-10442, WO2011/066384A1), event DP-040416-8 (corn, insect control, ATCC Accession N° PTA-11508, WO2011/075593A1), event DP-043A47-3 (corn, insect control, ATCC Accession N° PTA-11509, WO2011/075595A1), event DP-004114-3 (corn, insect control, ATCC Accession N° PTA-11506, WO2011/084621A1), event DP-032316-8 (corn, insect control, ATCC Accession N° PTA-11507, WO2011/084632A1), event MON-88302-9 (oilseed rape, herbicide tolerance, ATCC Accession N° PTA-10955, WO2011/153186A1), event DAS-21606-3 (soybean, herbicide tolerance, ATCC Accession No. PTA-11028, WO2012/033794A2), event MON-87712-4 (soybean, quality trait, ATCC Accession N°. PTA-10296, WO2012/051199A2), event DAS-44406-6 (soybean, stacked herbicide tolerance, ATCC Accession N°. PTA-11336, WO2012/075426A1), event DAS-14536-7 (soybean, stacked herbicide tolerance, ATCC Accession N°. PTA-11335, WO2012/075429A1), event SYN-000H2-5 (soybean, herbicide tolerance, ATCC Accession N°. PTA-11226, WO2012/082548A2), event DP-061061-7 (oilseed rape, herbicide tolerance, no deposit N° available, WO2012071039A1), event DP-073496-4 (oilseed rape, herbicide tolerance, no deposit N° available, US2012131692), event 8264.44.06.1 (soybean, stacked herbicide tolerance, Accession N° PTA-11336, WO2012075426A2), event 8291.45.36.2 (soybean, stacked herbicide tolerance,

Accession N°. PTA-11335, WO2012075429A2), event SYHT0H2 (soybean, ATCC
Accession N°. PTA-11226, WO2012/082548A2), event MON88701 (cotton, ATCC
Accession N° PTA-11754, WO2012/134808A1), event KK179-2 (alfalfa, ATCC
Accession N° PTA-11833, WO2013003558A1), event pDAB8264.42.32.1 (soybean,
5 stacked herbicide tolerance, ATCC Accession N° PTA-11993, WO2013010094A1),
event MZDT09Y (corn, ATCC Accession N° PTA-13025, WO2013012775A1).

E. Polynucleotide Constructs

The polynucleotides encoding the HPPD polypeptides of the present invention
10 may be modified to obtain or enhance expression in plant cells. The polynucleotides
encoding the polypeptides identified herein may be provided in expression cassettes
for expression in the plant of interest. A “plant expression cassette” includes a DNA
construct, including a recombinant DNA construct, that is capable of resulting in the
expression of a polynucleotide in a plant cell. The cassette can include in the 5'-3'
15 direction of transcription, a transcriptional initiation region (i.e., promoter,
particularly a heterologous promoter) operably-linked to one or more polynucleotides
of interest, and/or a translation and transcriptional termination region (i.e., termination
region) functional in plants. The cassette may additionally contain at least one
additional polynucleotide to be introduced into the organism, such as a selectable
20 marker gene. Alternatively, the additional polynucleotide(s) can be provided on
multiple expression cassettes. Such an expression cassette is provided with a plurality
of restriction sites for insertion of the polynucleotide(s) to be under the transcriptional
regulation of the regulatory regions.

In a further embodiment, the present invention relates to a chimeric gene
25 comprising a coding sequence comprising heterologous the nucleic acid of the
invention operably linked to a plant-expressible promoter and optionally a
transcription termination and polyadenylation region. “Heterologous” generally refers
to the polynucleotide or polypeptide that is not endogenous to the cell or is not
endogenous to the location in the native genome in which it is present, and has been
30 added to the cell by infection, transfection, microinjection, electroporation,
microprojection, or the like. By “operably linked” is intended a functional linkage
between two polynucleotides. For example, when a promoter is operably linked to a
DNA sequence, the promoter sequence initiates and mediates transcription of the
DNA sequence. It is recognized that operably linked polynucleotides may or may not

be contiguous and, where used to reference the joining of two polypeptide coding regions, the polypeptides are expressed in the same reading frame.

The promoter may be any polynucleotide sequence which shows transcriptional activity in the chosen plant cells, plant parts, or plants. The promoter
 5 may be native or analogous, or foreign or heterologous, to the plant host and/or to the DNA sequence of the invention. Where the promoter is “native” or “analogous” to the plant host, it is intended that the promoter is found in the native plant into which the promoter is introduced. Where the promoter is “foreign” or “heterologous” to the DNA sequence of the invention, it is intended that the promoter is not the native or
 10 naturally occurring promoter for the operably linked DNA sequence of the invention. 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 (1987) *Nucleic Acids Res.*
 15 15:2343-2361. Also, the location of the promoter relative to the transcription start may be optimized. See, e.g., Roberts *et al.* (1979) *Proc. Natl. Acad. Sci. USA*, 76:760-764. 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
 20 (PCISV) promoter (U.S. Pat. No. 5,850,019); the 35S promoter from cauliflower mosaic virus (CaMV) (Odell *et al.* (1985) *Nature* 313:810-812); promoters of *Chlorella* virus methyltransferase genes (U.S. Pat. No. 5,563,328) and the full-length transcript promoter from figwort mosaic virus (FMV) (U.S. Pat. No. 5,378,619); the promoters from such genes as rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171
 25 and U.S. Patent 5,641,876); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730 and U.S. Patent 5,510,474); maize H3 histone (Lepetit *et al.* (1992) *Mol. Gen. Genet.* 231:276-285 and Atanassova *et al.* (1992) *Plant J.* 2(3):291-300);
 30 *Brassica napus* ALS3 (PCT application WO 97/41228); a plant ribulose-biscarboxylase/oxygenase (RuBisCO) small subunit gene; the circovirus (AU 689 311) or the Cassava vein mosaic virus (CsVMV, US 7,053,205); and promoters of various *Agrobacterium* genes (see U.S. Pat. Nos. 4,771,002; 5,102,796; 5,182,200; and 5,428,147).

Suitable inducible promoters for use in plants include: the promoter from the ACE1 system which responds to copper (Mett *et al.* (1993) *PNAS* 90:4567-4571); the promoter of the maize In2 gene which responds to benzenesulfonamide herbicide safeners (Hershey *et al.* (1991) *Mol. Gen. Genetics* 227:229-237 and Gatz *et al.* (1994) *Mol. Gen. Genetics* 243:32-38); and the promoter of the Tet repressor from Tn10 (Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237). Another 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.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421) 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.* (2000) *Plant J.*, 24:265-273). Other inducible promoters for use in plants are described in EP 332104, PCT WO 93/21334 and PCT WO 97/06269 which are herein incorporated by reference in their entirety. Promoters composed of portions of other promoters and partially or totally synthetic promoters can also be used. See, e.g., Ni *et al.* (1995) *Plant J.* 7:661-676 and PCT WO 95/14098 describing such promoters for use in plants.

In one embodiment of this invention, a promoter sequence specific for particular regions or tissues of plants can be used to express the HPPD proteins of the invention, such as promoters specific for seeds (Datla, R. *et al.*, 1997, *Biotechnology Ann. Rev.* 3, 269-296), especially the napin promoter (EP 255 378 A1), the phaseolin promoter, the glutenin promoter, the helianthinin promoter (WO 92/17580), the albumin promoter (WO 98/45460), the oleosin promoter (WO 98/45461), the SAT1 promoter or the SAT3 promoter (PCT/US98/06978).

Use may also be made of an inducible promoter advantageously chosen from the phenylalanine ammonia lyase (PAL), HMG-CoA reductase (HMG), chitinase, glucanase, proteinase inhibitor (PI), PR1 family gene, nopaline synthase (nos) and vspB promoters (US 5 670 349, Table 3), the HMG2 promoter (US 5 670 349), the apple beta-galactosidase (ABG1) promoter and the apple aminocyclopropane carboxylate synthase (ACC synthase) promoter (WO 98/45445). Multiple promoters can be used in the constructs of the invention, including in succession.

The promoter may include, or be modified to include, one or more enhancer elements. In some embodiments, the promoter may 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 PCISV enhancer element (U.S. Pat. No. 5,850,019), the CaMV 35S enhancer element (U.S. Pat. Nos. 5,106,739 and 5,164,316) and the FMV enhancer element (Maiti *et al.* (1997) *Transgenic Res.* 6:143-156); the translation activator of the tobacco mosaic virus (TMV) described in Application WO 87/07644, or of the tobacco etch virus (TEV) described by Carrington & Freed 1990, J. Virol. 64: 1590-1597, for example, or introns such as the adh1 intron of maize or intron 1 of rice actin. See also PCT WO 96/23898, WO2012/021794, WO2012/021797, WO2011/084370, and WO2011/028914.

Often, such constructs can contain 5' and 3' untranslated regions. Such constructs may contain a "signal sequence" or "leader sequence" to facilitate co-translational or post-translational transport of the peptide of interest to certain intracellular structures such as the chloroplast (or other plastid), endoplasmic reticulum, or Golgi apparatus, or to be secreted. For example, the construct can be engineered to contain a signal peptide to facilitate transfer of the peptide to the endoplasmic reticulum. By "signal sequence" is intended a sequence that is known or suspected to result in cotranslational or post-translational peptide transport across the cell membrane. In eukaryotes, this typically involves secretion into the Golgi apparatus, with some resulting glycosylation. By "leader sequence" is intended any sequence that, when translated, results in an amino acid sequence sufficient to trigger co-translational transport of the peptide chain to a sub-cellular organelle. Thus, this includes leader sequences targeting transport and/or glycosylation by passage into the endoplasmic reticulum, passage to vacuoles, plastids including chloroplasts, mitochondria, and the like. It may also be preferable to engineer the plant expression cassette to contain an intron, such that mRNA processing of the intron is required for expression.

By "3' untranslated region" is intended a polynucleotide located downstream of a coding sequence. Polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor are 3' untranslated regions. By "5' untranslated region" is intended a polynucleotide located upstream of a coding sequence.

Other upstream or downstream untranslated elements include enhancers. Enhancers are polynucleotides that act to increase the expression of a promoter region. Enhancers are well known in the art and include, but are not limited to, the SV40 enhancer region and the 35S enhancer element.

5 The termination region may be native with the transcriptional initiation region, may be native with the sequence of the present invention, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot
10 (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639; and European Patent Application EP 0 633 317 A1.

 In one aspect of the invention, synthetic DNA sequences are designed for a
15 given polypeptide, such as the polypeptides of the invention. Expression of the open reading frame of the synthetic DNA sequence in a cell results in production of the polypeptide of the invention. Synthetic DNA sequences can be useful to simply remove unwanted restriction endonuclease sites, to facilitate DNA cloning strategies, to alter or remove any potential codon bias, to alter or improve GC content, to remove
20 or alter alternate reading frames, and/or to alter or remove intron/exon splice recognition sites, polyadenylation sites, Shine-Delgarno sequences, unwanted promoter elements and the like that may be present in a native DNA sequence. It is also possible that synthetic DNA sequences may be utilized to introduce other improvements to a DNA sequence, such as introduction of an intron sequence,
25 creation of a DNA sequence that is expressed as a protein fusion to organelle targeting sequences, such as chloroplast transit peptides, apoplast/vacuolar targeting peptides, or peptide sequences that result in retention of the resulting peptide in the endoplasmic reticulum. Synthetic genes can also be synthesized using host cell-preferred codons for improved expression, or may be synthesized using codons at a
30 host-preferred codon usage frequency. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11; U.S. Patent Nos. 6,320,100; 6,075,185; 5,380,831; and 5,436,391, U.S. Published Application Nos. 20040005600 and 20010003849, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

In one embodiment, the polynucleotides of interest are targeted to the chloroplast for expression. In this manner, where the polynucleotide of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a polynucleotide encoding a transit peptide to direct the nucleotide of interest to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; and Shah *et al.* (1986) *Science* 233:478-481.

The polynucleotides of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the polynucleotides of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

This plant expression cassette can be inserted into a plant transformation vector. By “transformation vector” is intended a DNA molecule that allows for the transformation of a cell. Such a molecule may consist of one or more expression cassettes, and may be organized into more than one vector DNA molecule. For example, binary vectors are plant transformation vectors that utilize two non-contiguous DNA vectors to encode all requisite cis- and trans-acting functions for transformation of plant cells (Hellens and Mullineaux (2000) *Trends in Plant Science* 5:446-451). “Vector” refers to a polynucleotide construct designed for transfer between different host cells. “Expression vector” refers to a vector that has the ability to incorporate, integrate and express heterologous DNA sequences or fragments in a foreign cell.

The plant transformation vector comprises one or more DNA vectors for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that comprise more than one contiguous DNA segment. These vectors are often referred to in the art as binary vectors. Binary vectors as well as vectors with helper plasmids are most often used for *Agrobacterium*-mediated transformation, where the size and complexity of DNA segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules. Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for

T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a “polynucleotide of interest” (a polynucleotide engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired). Also present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker sequence and the sequence of interest are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from *Agrobacterium* to plant cells. This plasmid often contains the virulence functions (Vir genes) that allow infection of plant cells by *Agrobacterium*, and transfer of DNA by cleavage at border sequences and vir-mediated DNA transfer, as is understood in the art (Hellens and Mullineaux (2000) *Trends in Plant Science*, 5:446-451). Several types of *Agrobacterium* strains (e.g., LBA4404, GV3101, EHA101, EHA105, etc.) can be used for plant transformation. The second plasmid vector is not necessary for introduction of polynucleotides into plants by other methods such as microprojection, microinjection, electroporation, polyethylene glycol, etc.

F. Plant Transformation

Methods of the invention involve introducing a nucleotide construct into a plant. By “introducing” is intended to present to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not require that a particular method for introducing a nucleotide construct to a plant is used, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods. See, for example, the methods for transforming plant cells and regenerating plants described in: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159 A1, EP 604 662 A1, EP 672 752 A1, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174 A1, EP 486 233 A1, EP 486 234 A1, EP 539 563 A1, EP 674 725 A1, WO 91/02071,

WO 95/06128, and WO2011/095460, each of which is herein incorporated by reference, particularly with respect to the transformation methods described therein.

In general, plant transformation methods involve transferring heterologous DNA into target plant cells (e.g. immature or mature embryos, suspension cultures, undifferentiated callus, protoplasts, etc.), followed by applying a maximum threshold level of appropriate selection (depending on the selectable marker gene) to recover the transformed plant cells from a group of untransformed cell mass. Explants are typically transferred to a fresh supply of the same medium and cultured routinely. Subsequently, the transformed cells are differentiated into shoots after placing on regeneration medium supplemented with a maximum threshold level of selecting agent. The shoots are then transferred to a selective rooting medium for recovering rooted shoot or plantlet. The transgenic plantlet then grow into mature plants and produce fertile seeds (e.g. Hiei *et al.* (1994) *The Plant Journal* 6:271-282; Ishida *et al.* (1996) *Nature Biotechnology* 14:745-750). Explants are typically transferred to a fresh supply of the same medium and cultured routinely. A general description of the techniques and methods for generating transgenic plants are found in Ayres and Park (1994) *Critical Reviews in Plant Science* 13:219-239 and Bommineni and Jauhar (1997) *Maydica* 42:107-120. Since the transformed material contains many cells; both transformed and non-transformed cells are present in any piece of subjected target callus or tissue or group of cells. The ability to kill non-transformed cells and allow transformed cells to proliferate results in transformed plant cultures. Often, the ability to remove non-transformed cells is a limitation to rapid recovery of transformed plant cells and successful generation of transgenic plants. Molecular and biochemical methods can be used to confirm the presence of the integrated heterologous gene of interest in the genome of transgenic plant.

Generation of transgenic plants may be performed by one of several methods, including, but not limited to, introduction of heterologous DNA by *Agrobacterium* into plant cells (*Agrobacterium*-mediated transformation), bombardment of plant cells with heterologous foreign DNA adhered to particles, and various other non-particle direct-mediated methods (e.g. Hiei *et al.* (1994) *The Plant Journal* 6:271-282; Ishida *et al.* (1996) *Nature Biotechnology* 14:745-750; Ayres and Park (1994) *Critical Reviews in Plant Science* 13:219-239; Bommineni and Jauhar (1997) *Maydica* 42:107-120) to transfer DNA.

Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917; Svab and Maliga (1993) *EMBO J.* 12:601-606. The method relies on particle gun delivery of DNA containing
5 a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA*
10 91:7301-7305.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having
15 constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as “transgenic seed”)
20 having a nucleotide construct of the invention, for example, an expression cassette of the invention, stably incorporated into their genome. In various embodiments, the seed can be coated with at least one fungicide and/or at least one insecticide, at least one herbicide, and/or at least one safener, or any combination thereof.

25 G Evaluation of Plant Transformation

Following introduction of heterologous foreign DNA into plant cells, the transformation or integration of the heterologous gene in the plant genome is confirmed by various methods such as analysis of nucleic acids, proteins and metabolites associated with the integrated gene.

30 PCR analysis is a rapid method to screen transformed cells, tissue or shoots for the presence of incorporated gene at the earlier stage before transplanting into the soil (Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)). PCR is carried out using

oligonucleotide primers specific to the gene of interest or *Agrobacterium* vector background, etc.

Plant transformation may be confirmed by Southern blot analysis of genomic DNA (Sambrook and Russell (2001) *supra*). In general, total DNA is extracted from
5 the transformant, digested with appropriate restriction enzymes, fractionated in an agarose gel and transferred to a nitrocellulose or nylon membrane. The membrane or “blot” can then be probed with, for example, radiolabeled ³²P target DNA fragment to confirm the integration of the introduced gene in the plant genome according to standard techniques (Sambrook and Russell, 2001, *supra*).

10 In Northern analysis, RNA is isolated from specific tissues of transformant, fractionated in a formaldehyde agarose gel, and blotted onto a nylon filter according to standard procedures that are routinely used in the art (Sambrook and Russell (2001) *supra*). Expression of RNA encoded by nucleotide sequences of the invention is then tested by hybridizing the filter to a radioactive probe derived from a GDC by methods
15 known in the art (Sambrook and Russell (2001) *supra*)

Western blot, ELISA, lateral flow testing, and biochemical assays and the like may be carried out on the transgenic plants to determine the presence of protein encoded by the herbicide tolerance gene by standard procedures (Sambrook and Russell (2001) *supra*) using antibodies that bind to one or more epitopes present on
20 the herbicide tolerance protein.

In one aspect of the invention, the HPPD genes described herein are useful as markers to assess transformation of bacterial or plant cells.

H. Use as a marker for transformation

25 The invention also relates to the use, in a method for transforming plants, of a nucleic acid which encodes an HPPD according to the invention as a marker gene or as a coding sequence which makes it possible to confer to the plant tolerance to herbicides which are HPPD inhibitors, and the use of one or more HPPD inhibitor(s) on plants comprising a nucleic acid sequence encoding a HPPD according to the
30 invention. See, for example, U.S. Patent 6,791,014, which is herein incorporated by reference in its entirety.

In this embodiment, an HPPD inhibitor can be introduced into the culture medium of the competent plant cells so as to bleach said cells before the transformation step. The bleached competent cells are then transformed with the gene

for tolerance to HPPD inhibitors, as a selection marker, and the transformed cells which have integrated said selection marker into their genome become green, enabling them to be selected. Such a process makes it possible to decrease the time required for selecting the transformed cells.

5 Thus, one embodiment of the present invention consists of a method for transforming plant cells by introducing a heterologous gene into said plant cells with a gene for tolerance to HPPD inhibitors as selection markers, wherein the method comprises preparing and culturing competent plant cells capable of receiving the heterologous gene in a suitable medium and introducing a suitable amount of HPPD
10 inhibitor into the suitable culture medium of the competent plant cells. The competent cells are then transformed with the heterologous gene and the selection marker, and the transformed cells comprising the heterologous gene are grown in a suitable medium and transformants selected therefrom. The transformed cells can then be regenerated into a fertile transformed plant.

15

I. Plants and Plant Parts

By “plant” is intended whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, propagules, embryos and progeny of the same. Plant cells can be differentiated or undifferentiated (e.g., callus, suspension culture cells, protoplasts,
20 leaf cells, root cells, phloem cells, pollen). The present invention may be used for introduction of polynucleotides into any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (maize), sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape, *Brassica* sp., alfalfa,
25 rye, millet, safflower, peanuts, sweet potato, cassava, coffee, coconut, pineapple, citrus trees, cocoa, tea, banana, avocado, fig, guava, mango, olive, papaya, cashew, macadamia, almond, oats, vegetables, ornamentals, and conifers.

Vegetables include, but are not limited to, tomatoes, lettuce, green beans, lima beans, peas, and members of the genus *Curcumis* such as cucumber, cantaloupe, and
30 musk melon. Ornamentals include, but are not limited to, azalea, hydrangea, hibiscus, roses, tulips, daffodils, petunias, carnation, poinsettia, and chrysanthemum. Crop plants are also of interest, including, for example, maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, oilseed rape, etc.

This invention is suitable for any member of the monocot plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, and dates.

5 *J. Methods for increasing plant yield*

Methods for increasing plant yield are provided. The methods comprise providing a plant comprising, or introducing into a plant or plant cell, a polynucleotide comprising a nucleotide sequence encoding an HPPD of the invention, growing the plant or a seed thereof in a field, and producing a harvest from said plants
10 or seeds. As defined herein, the “yield” of the plant refers to the quality and/or quantity of biomass produced by the plant. By “biomass” is intended any measured plant product. An increase in biomass production is any improvement in the yield of the measured plant product. Increasing plant yield has several commercial applications. For example, increasing plant leaf biomass may increase the yield of
15 leafy vegetables for human or animal consumption. Additionally, increasing leaf biomass can be used to increase production of plant-derived pharmaceutical or industrial products. An increase in yield can comprise any statistically significant increase including, but not limited to, at least a 1% increase, at least a 3% increase, at least a 5% increase, at least a 10% increase, at least a 20% increase, at least a 30%, at
20 least a 50%, at least a 70%, at least a 100% or a greater increase.

In specific methods, the plant comprising an HPPD sequence of the invention is treated with an effective concentration of an HPPD herbicide, such as one or more HPPD inhibitor herbicide(s) selected from the group consisting of HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or
25 N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolinates, such as pyrasulfotole and topramezone, particularly
30 selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione, where the herbicide application results in enhanced plant yield.

Methods for conferring herbicide tolerance in a plant or plant part are also provided. In such methods, a nucleotide sequence encoding an HPPD of the

invention is introduced into the plant, wherein expression of the polynucleotide results in HPPD inhibitor herbicide tolerance. Plants produced via this method can be treated with an effective concentration of an herbicide (such as one or more HPPD inhibitor herbicide(s) selected from the group consisting of HPPD inhibitor herbicides of the

5 class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of

10 pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione) and display an increased tolerance to the herbicide. An “effective concentration” of an herbicide in this application is an amount sufficient to slow or stop the growth of plants or plant parts that are not naturally tolerant or rendered

15 tolerant to the herbicide.

K. Methods of controlling weeds in a field

The present invention therefore also relates to a method of controlling undesired plants or for regulating the growth of plants in crops of plants comprising a

20 nucleotide sequence encoding an HPPD according to the invention, where one or more HPPD inhibitor herbicides, for example, one or more HPPD inhibitor herbicides selected from the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-

25 (methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, or mesotrione, are applied to the plants (for example harmful plants such

30 as monocotyledonous or dicotyledonous weeds or undesired crop plants), to the seeds (for example grains, seeds or vegetative propagules such as tubers or shoot parts with buds) or to the area on which the plants grow (for example the area under cultivation). In this context, an effective concentration of one or more HPPD inhibitor herbicide(s), for example, one or more HPPD inhibitor herbicides selected from the group

consisting of HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione, can be applied for example pre-planting (if appropriate also by incorporation into the soil), pre-emergence or post-emergence, and may be combined with the application of other herbicides to which the crop is naturally tolerant, or to which it is resistant via expression of one or more other herbicide resistance transgenes. See, e.g., U.S. App. Pub. No. 2004/0058427 and PCT App. Pub. No. WO 98/20144. By "effective concentration" is intended the concentration which controls the growth or spread of weeds or other untransformed plants without significantly affecting the HPPD inhibitor-tolerant plant or plant seed. Those of skill in the art understand that application of herbicides can take many different forms and can take place at many different times prior to and/or throughout the seed planting and growth process. "Pre-emergent" application refers to a herbicide which is applied to an area of interest (e.g., a field or area of cultivation) before a plant emerges visibly from the soil. "Post-emergent" application refers to a herbicide which is applied to an area after a plant emerges visibly from the soil. In some instances, the terms "pre-emergent" and "post-emergent" are used with reference to a weed in an area of interest, and in some instances these terms are used with reference to a crop plant in an area of interest. When used with reference to a weed, these terms may apply to a particular type of weed or species of weed that is present or believed to be present in the area of interest. "Pre-plant incorporation" of a herbicide involves the incorporation of compounds into the soil prior to planting.

Thus, the present invention comprises a method of controlling weeds in a field comprising planting in a field a plant or a seed thereof comprising an HPPD of the invention and applying to said plant or area surrounding said plant an effective concentration of one or more HPPD inhibitor herbicides.

In one embodiment of this invention, a field to be planted with plants (such as soybean, cotton, corn, or wheat plants, e.g.) containing an HPPD nucleotide sequence

of the invention, can be treated with an HPPD inhibitor herbicide, such as isoxaflutole (IFT), before the plants are planted or the seeds are sown, which cleans the field of weeds that are killed by the HPPD inhibitor, allowing for no-till practices, followed by planting or sowing of the plants in that same pre-treated field later on (burndown application using an HPPD inhibitor herbicide). The residual activity of IFT will also protect the emerging and growing plants from competition by weeds in the early growth stages. Once the plants have a certain size, and weeds tend to re-appear, glufosinate or glyphosate, or an HPPD inhibitor or a mixture of an HPPD inhibitor with another herbicide such as glyphosate, can be applied as post-emergent herbicide over the top of the plants, when such plants are tolerant to said herbicides.

In another embodiment of this invention, a field in which seeds containing an HPPD nucleotide sequence of the invention were sown, can be treated with an HPPD inhibitor herbicide, such as IFT, before the plants emerge but after the seeds are sown (the field can be made weed-free before sowing using other means, typically conventional tillage practices such as ploughing, chisel ploughing, or seed bed preparation), where residual activity will keep the field free of weeds killed by the herbicide so that the emerging and growing plants have no competition by weeds (pre-emergence application of an HPPD inhibitor herbicide). Once the plants have a certain size, and weeds tend to re-appear, glufosinate or glyphosate, or an HPPD inhibitor or a mixture of an HPPD inhibitor with another herbicide such as glyphosate, can be applied as post-emergent herbicide over the top of the plants, when such plants are tolerant to said herbicides.

In another embodiment of this invention, plants containing an HPPD nucleotide sequence of the invention, can be treated with an HPPD inhibitor herbicide, over the top of the plants that have emerged from the seeds that were sown, which cleans the field of weeds killed by the HPPD inhibitor, which application can be together with (e.g., in a spray tank mix), followed by or preceded by a treatment with glyphosate or glufosinate as post-emergent herbicide over the top of the plants (post-emergence application of an HPPD inhibitor herbicide (with or without glyphosate)), when such plants are tolerant to such herbicides.

Examples of individual representatives of the monocotyledonous and dicotyledonous weeds which can be controlled with an HPPD inhibitor herbicide include:

Monocotyledonous harmful plants of the genera: Aegilops, Agropyron, Agrostis, Alopecurus, Apera, Avena, Brachiaria, Bromus, Cenchrus, Commelina, Cynodon, Cyperus, Dactyloctenium, Digitaria, Echinochloa, Eleocharis, Eleusine, Eragrostis, Eriochloa, Festuca, Fimbristylis, Heteranthera, Imperata, Ischaemum, Leptochloa, Lolium, Monochoria, Panicum, Paspalum, Phalaris, Phleum, Poa, Rottboellia, Sagittaria, Scirpus, Setaria, Sorghum.

Dicotyledonous weeds of the genera: Abutilon, Amaranthus, Ambrosia, Anoda, Anthemis, Aphanes, Artemisia, Atriplex, Bellis, Bidens, Capsella, Carduus, Cassia, Centaurea, Chenopodium, Cirsium, Convolvulus, Datura, Desmodium, Emex, Erysimum, Euphorbia, Galeopsis, Galinsoga, Galium, Hibiscus, Ipomoea, Kochia, Lamium, Lepidium, Lindernia, Matricaria, Mentha, Mercurialis, Mullugo, Myosotis, Papaver, Pharbitis, Plantago, Polygonum, Portulaca, Ranunculus, Raphanus, Rorippa, Rotala, Rumex, Salsola, Senecio, Sesbania, Sida, Sinapis, Solanum, Sonchus, Sphenoclea, Stellaria, Taraxacum, Thlaspi, Trifolium, Urtica, Veronica, Viola, Xanthium.

HPPD inhibitor herbicides useful in the present invention, including but not limited to HPPD inhibitor herbicides of the class of N-(1,2,5-oxadiazol-3-yl)benzamides, N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, triketones, such as tembotrione, sulcotrione and mesotrione, the class of isoxazoles such as isoxaflutole, or of the class of pyrazolines, such as pyrasulfotole and topramezone, particularly selected from tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, and mesotrione, can be formulated in various ways, depending on the prevailing biological and/or physico-chemical parameters. Examples of possible formulations are: wettable powders (WP), water-soluble powders (SP), water-soluble concentrates, emulsifiable concentrates (EC), emulsions (EW), such as oil-in-water and water-in-oil emulsions, sprayable solutions, suspension concentrates (SC), oil- or water-based dispersions, oil-miscible solutions, capsule suspensions (CS), dusts (DP), seed-dressing products, granules for application by broadcasting and on the soil, granules (GR) in the form of microgranules, spray granules, coated granules and adsorption granules, water-

dispersible granules (WG), water-soluble granules (SG), ULV formulations, microcapsules and waxes.

These individual types of formulation are known in principle and are described, for example, in: Winnacker-Küchler, "Chemische Technologie" [Chemical
5 technology], volume 7, C. Hanser Verlag Munich, 4th Ed. 1986; Wade van Valkenburg, "Pesticide Formulations", Marcel Dekker, N.Y., 1973; K. Martens, "Spray Drying" Handbook, 3rd Ed. 1979, G. Goodwin Ltd. London.

The formulation auxiliaries required, such as inert materials, surfactants, solvents and further additives, are also known and are described, for example, in:
10 Watkins, "Handbook of Insecticide Dust Diluents and Carriers", 2nd Ed., Darland Books, Caldwell N.J., H.v. Olphen, "Introduction to Clay Colloid Chemistry"; 2nd Ed., J. Wiley & Sons, N.Y.; C. Marsden, "Solvents Guide"; 2nd Ed., Interscience, N.Y. 1963; McCutcheon's "Detergents and Emulsifiers Annual", MC Publ. Corp., Ridgewood N.J.; Sisley and Wood, "Encyclopedia of Surface Active Agents", Chem.
15 Publ. Co. Inc., N.Y. 1964; Schönfeldt, "Grenzflächenaktive Äthylenoxidaddukte" [Interface-active ethylene oxide adducts], Wiss. Verlagsgesell., Stuttgart 1976; Winnacker-Küchler, "Chemische Technologie" [Chemical technology], volume 7, C. Hanser Verlag Munich, 4th Ed. 1986.

Based on these formulations, it is also possible to prepare combinations with
20 other pesticidally active substances such as, for example, insecticides, acaricides, herbicides, fungicides, and with safeners, fertilizers and/or growth regulators, for example in the form of a ready mix or a tank mix.

L. Methods of introducing gene of the invention into another plant

25 Also provided herein are methods of introducing the HPPD nucleotide sequence of the invention into another plant. The HPPD nucleotide sequence of the invention, or a fragment thereof, can be introduced into second plant by recurrent selection, backcrossing, pedigree breeding, line selection, mass selection, mutation breeding and/or genetic marker enhanced selection.

30 Thus, in one embodiment, the methods of the invention comprise crossing a first plant comprising an HPPD nucleotide sequence of the invention with a second plant to produce F1 progeny plants and selecting F1 progeny plants that are tolerant to an HPPD inhibitor herbicide or that comprise the HPPD nucleotide sequence of the invention. The methods may further comprise crossing the selected progeny plants

with the first plant comprising the HPPD nucleotide sequence of the invention to produce backcross progeny plants and selecting backcross progeny plants that are tolerant to an HPPD inhibitor herbicide or that comprise the HPPD nucleotide sequence of the invention. Methods for evaluating HPPD inhibitor herbicide tolerance are provided elsewhere herein. The methods may further comprise repeating these steps one or more times in succession to produce selected second or higher backcross progeny plants that are tolerant to an HPPD inhibitor herbicide or that comprise the HPPD nucleotide sequence of the invention.

Any breeding method involving selection of plants for the desired phenotype can be used in the method of the present invention. In some embodiments, The F1 plants may be self-pollinated to produce a segregating F2 generation. Individual plants may then be selected which represent the desired phenotype (e.g., HPPD inhibitor herbicide tolerance) in each generation (F3, F4, F5, etc.) until the traits are homozygous or fixed within a breeding population.

The second plant can be a plant having a desired trait, such as herbicide tolerance, insect tolerance, drought tolerance, nematode control, water use efficiency, nitrogen use efficiency, improved nutritional value, disease resistance, improved photosynthesis, improved fiber quality, stress tolerance, improved reproduction, and the like. The second plant may be an elite event as described elsewhere herein

In various embodiments, plant parts (whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, propagules, embryos, and the like) can be harvested from the resulting cross and either propagated or collected for downstream use (such as food, feed, biofuel, oil, flour, meal, etc).

M. Methods of obtaining a plant product

The present invention also relates to a process for obtaining a commodity product, comprising harvesting and/or milling the grains from a crop comprising an HPPD sequence of the invention to obtain the commodity product. Agronomically and commercially important products and/or compositions of matter including but not limited to animal feed, commodities, and plant products and by-products that are intended for use as food for human consumption or for use in compositions and commodities that are intended for human consumption, particularly devitalized seed/grain products, including a (semi-)processed products produced from such grain/seeds, wherein said product is or comprises whole or processed seeds or grain,

animal feed, corn or soy meal, corn or soy flour, corn, corn starch, soybean meal, soy flour, flakes, soy protein concentrate, soy protein isolates, texturized soy protein concentrate, cosmetics, hair care products, soy nut butter, natto, tempeh, hydrolyzed soy protein, whipped topping, shortening, lecithin, edible whole soybeans (raw, 5 roasted, or as edamame), soy yogurt, soy cheese, tofu, yuba, as well as cooked, polished, steamed, baked or parboiled grain, and the like are intended to be within the scope of the present invention if these products and compositions of matter contain detectable amounts of the nucleotide and/or amino acid sequences set forth herein as being diagnostic for any plant containing such nucleotide sequences.

10 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

15 Example 1. Preparation of *Pseudomonas fluorescens* HPPD mutant G336W (PfG336W) and kinetic characterization of the HPPD enzymes.

The native *Pseudomonas fluorescens* HPPD nucleotide sequence (PfHPPD, 1077 bp, as described in WO2009144079), which encodes the amino acid sequence listed herein as SEQ ID NO:1, and as described in WO2009144079, WO 96/38567, 20 and in Rüetschi et al. (*Eur. J. Biochem.*, 205, 459-466, 1992), was initially cloned into the unique NcoI site of the expression vector pKK233-2 (Pharmacia) that provides a start codon.

At the 5' end, directly downstream to the ATG, a nucleic acid sequence encoding an alanine amino acid and a nucleic acid sequence encoding a N-terminal 25 HIS6-Tag (6x HIS, encoded by: cat cac cat cac cat cac (SEQ ID NO:77) was inserted. Upstream to the ATG, two additional cysteine base pairs were added in order to obtain a sequence corresponding to the recognition site of the restriction enzyme NcoI and downstream to the stop codon the sequences corresponding to the recognition site of the restriction enzyme XbaI were added. The DNA sequence corresponding to the 30 gene, including the sequence encoding the HIS-TAG, was cut with the restriction enzymes NcoI and XbaI, and then cloned into the modified expression vector pSE420(RI)NX (5261 bp).

The cloning and expression vector pSE420(RI)NX (5261 bp) is based on the plasmid pSE420 by Invitrogen (Karlsruhe, Germany). Modifications of this vector

include the addition of a nptII gene (neomycin phosphotransferase; Sambrook and Russell, 2001, Molecular Cloning: a laboratory manual (Third edition)) conferring tolerance to the antibiotic kanamycin and which is missing the majority of the superlinker region (multiple cloning site).

5 The plasmid possesses the trp-lac (trc) promoter and the lacI^q gene that provides the lac repressor in every *E. coli* host strain. The lac repressor binds to the lac operator (lacO) and restricts expression of the target gene; this inhibition can be alleviated by induction with Isopropyl β -D-1-thiogalactopyranoside (IPTG).

10 The resulting vector was called pSE420(RI)NX-PfHPPD and it was used to transform *Escherichia coli* BL21 cells (Merck, Darmstadt, Germany).

15 The plasmid pSE420(RI)NX-PfHPPD was subjected to PCR-mediated site-directed mutagenesis to alter a defined codon at corresponding sites of the PfHPPD gene. The codon encoding Glycine (G) at position 336 was replaced by a codon encoding tryptophan (W). The resulting mutant was called PfG336W, and the resulting vector pSE420(RI)NX-PfG336W.

20 Expression of HPPD was carried out in *E. coli* K-12 BL21 containing pSE420(RI)NX-PfHPPD or pSE420(RI)NX-PfG336W. Cells were allowed to grow until OD reached 0.5, then expression was initiated from the trp-lac (trc) promoter by induction with 1 mM IPTG which binds to the lac repressor and causes its dissociation from the lac operon. Expression was carried out over 15 h at 28 °C.

25 To prepare the pre-starter culture, 2 mL of TB medium (100 $\mu\text{g}\cdot\text{mL}^{-1}$ carbenicillin) were inoculated with 50 μL of an *E. coli* K-12 BL21 glycerol stock. The pre-starter culture was incubated at 37 °C with shaking at 140 rpm for 15 h. 200 μL of the pre-starter culture was used to initiate the starter culture (5mL TB supplement with 100 $\mu\text{g}\cdot\text{L}^{-1}$), which was incubated 3 h at 37°C.

30 To prepare the main culture, 400 mL of TB medium (100 $\mu\text{g}\cdot\text{mL}^{-1}$ carbenicillin) were inoculated with 4 mL of the starter culture. This starter culture was incubated at 37 °C with shaking at 140 rpm until OD₆₀₀ 0.5 was reached. Then recombinant protein expression was induced with 400 μL of 1M IPTG solution. The cells were allowed to grow for an additional hour under these conditions, then the temperature was lowered to 28°C and the culture was shaken at 140 rpm for 15 h. Cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C. Then cell pellets were stored at -80 °C.

Isolation and purification of His₆-PfHPPD and His₆-PfG336W in native form*Lysis of cells*

- Cells were lysed using Lysozyme, an enzyme that cleaves the 1,4- β -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in
- 5 peptidoglycan which forms the bacterial cell wall. Cell membranes were then disrupted by the internal pressure of the bacterial cell. In addition, the lysis buffer contained BENZONASE[®] Nuclease, an endonuclease that hydrolyzes all forms of DNA and RNA without damaging proteins and thereby largely reduces viscosity of the cell lysate. Lysis under native conditions was carried out on ice.
- 10 For purification of His₆-tagged proteins the QIAEXPRESS[®] Ni-NTA Fast Start Kit was used following the user manual instruction.

*Purification of His₆-tagged proteins by immobilized metal ion affinity*15 *chromatography (IMAC)*

- The cleared cell lysate (10 mL) obtained after centrifugation of the lysis reaction was loaded onto a Ni-NTA Fast Start Column from the QIAEXPRESS[®] Ni-NTA Fast Start Kit (Qiagen, Hilden, Germany) and purification was carried out according to the instruction manual. The His₆-tagged protein was eluted with 2.5 mL
- 20 of elution buffer.

Desalting of HPPD solutions by gel filtration

- HPPD solutions eluted from a Ni-NTA Fast Start Column with 2.5 mL of elution buffer were applied to a Sephadex G-25 PD-10 column (GE Healthcare, Freiburg, Germany) following the user manual instruction. After the whole sample
- 25 had entered the gel bed, elution was performed with 3.5 mL of storage buffer.

The HPPD solutions eluted from the desalting column were frozen at -80 °C in 1 mL aliquots.

30 *Determination of HPPD protein concentration using the Bradford protein assay*

Protein concentration was determined using the standard Bradford assay (Bradford, (1976), Anal Biochem 72: 248-254).

Determination of purity of HPPD solutions using SDS-PAGE

The integrity of the eluted protein was checked by SDS-PAGE protein gel electrophoresis using the gel NUPAGE® Novex 4-12 % Bis-Tris Gels (Invitrogen, Karlsruhe, Germany), and approximately 10 µg of protein were loaded. Ten µL of Laemmli Sample Buffer were added to 1-10 µL of protein solution and the mixture was incubated at 90 °C for 10 min. After a short centrifugation step, the whole mixture was loaded into a slot of an SDS gel previously fixed in an XCELL SURELOCK™ Novex Mini-Cell gel chamber filled with NUPAGE® MOPS SDS Running Buffer (diluted from the 20 x-solution with ddH₂O). A voltage of 150 was then applied to the gel chamber for 1 h. For staining of protein bands, the gel was immersed in Coomassie Brilliant Blue R-250 Staining Solution. For destaining of the polyacrylamide gel, it was immersed in Coomassie Brilliant Blue R-250 Destaining Solution until protein bands appeared blue on a white gel.

15 Example 2. Kinetic characterization and evaluation of tolerance to HPPD inhibitors of HPPD enzymes PfHPPD and PfG336W.

The HPPD activity was checked by a standard spectrophotometric assay (which is described WO 2009/144079, herein incorporated by reference in its entirety).

20

Determination of HPPD in vitro kinetic properties

K_m , V_{max} , and k_{cat} values for different HPPD enzyme preparations and K_i , $K_I=K_{on}$, and $K_{-I}=K_{off}$ for different HPPD inhibitors were determined or can be determined using an HPLC assay for measurements of HPPD activity. The assay mixtures contained in a volume of 1 ml 150 mM Tris-HCl buffer at pH 7.8, 10 mM sodium ascorbate, 650 units of bovine catalase (Sigma C30 (Sigma-Aldrich, Munich, Germany), 34 mg protein/ml, 23,000 units/mg), and appropriate amounts of HPP, purified HPPD enzyme and HPPD inhibitors. For K_m , V_{max} , and k_{cat} value determination, HPP concentrations in the assay mixture varied between 10 and 400 µM. For K_i , $K_I=K_{on}$, and $K_{-I}=K_{off}$ value determination, 2 mM HPP was used or can be used. All assays were started by the addition of HPPD enzyme to the assay mixture and stopped at a series of times between 0 and 240 s by addition of 200 µl of the reaction mixture to reaction assay tubes containing 20 µl 10% perchloric acid. Precipitated protein was pelleted by a 5 minute centrifugation at 10,000 g. One

hundred μ l of the supernatant were loaded onto a 250 x 4mm Knauer (Berlin, Germany) Eurospher 100-5 C18-column equilibrated with 10% methanol, 0.1% trifluoroacetic acid (buffer A). The column was eluted, also at 1.5 ml/min, using a 4 minute wash with buffer A, followed by a 3 min wash with 95% methanol and by a further 2 minute wash with buffer A. The elution of HGA (homogentisic acid) and HPP (hydroxyphenylpyruvate) was monitored at 292 nm. HGA elutes at around 5 minutes and HPP elutes later. A standard set of concentrations of HGA were used to provide a standard curve in order to calibrate the 292 nm absorbance of the HGA peak versus HGA concentration.

For K_m and V_{max} value determinations the initial rates of the HPPD reaction at different substrate concentrations were determined from plots of HGA formed versus time and fitted to the Michaelis-Menten equation for unireactant enzymes using the ID Business Solutions Ltd. (www.idbs.com) XLfit software suite. For the determination of K_i , $K_1=K_{on}$, and $K_{-1}=K_{off}$ values, the time-courses of the HPPD reaction at different inhibitor concentrations were fitted to the equations for Mechanism A, competitive inhibition, for tight-binding inhibitors (Cha, S. (1975) Tight-binding inhibitors – I. Kinetic behaviour. *Biochemical Pharmacology* 24, 2177-2185) using the ID Business Solutions Ltd. XLfit software suite

Table 1: Kinetic characterization of HPPD enzymes (Pf HPPD and PfG336W).

In below given Table 1, “ K_m ” (Michaelis-Menten constant) means the kinetic parameter that is used to characterize an enzyme, and it is defined as the concentration of substrate that permits half maximal rate of the reaction. K_m is further defined as the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$) where V_{max} has the meaning of being the maximum velocity of the reaction.

$K_{on}=K_1$ equals the association rate constant of the enzyme-substrate binding and $K_{off}=K_{-1}$ equals the rate constant of the enzyme-inhibitor complex dissociation. K_i defines the inhibition constant. For the determination of the specific activity of the enzymes, samples were incubated, and the reaction was stopped after 24 min. The specific activity was estimated by μ g of protein.

Table 1

	HPP	HPP	Specific Activity
	K_m (μM)	K_{cat} (s^{-1})	$\Delta E/24min$ $\mu gProtein$
PfHPPD	187	4.4	0.76
PfG336W	141	5.4	0.40

Table 1 demonstrates that the kinetic parameters K_m and K_{cat} of the wild type bacterial HPPD (PfHPPD) and of the mutant HPPD (PfG336W) do not show significant differences. However, the specific activity is significantly reduced in the mutant compared to the wild type protein.

10 *Determination of HPPD activity in presence of several HPPD inhibitors*

In this content, pI_{50} -value means the log value of the concentration of inhibitor necessary to inhibit 50% of the enzyme activity in molar concentration.

pI_{50} -values for HPPD inhibitors were determined from dose-response plots of HPPD activity versus inhibitor concentration using the assay extensively described in WO 2009/144079 at 2 mM fixed HPP concentration and 3 minutes fixed incubation time using the ID Business Solutions Ltd. XLfit software suite.

The pI_{50} of the PfHPPD and PfG336W enzymes was measured using a spectrophotometric based method. Tolerance to the several listed below HPPD inhibitors tembotrione, diketonitrile, mesotrione was measured. The symbol ">" means that the value was far higher than the one indicated but could not be precisely calculated within in the range of concentration of inhibitor tested (5.0×10^{-6} , 1.0×10^{-5} , 2.5×10^{-5} , 4.0×10^{-5} , 7.0×10^{-5} , 1.0×10^{-4} , 2.0×10^{-4} and $5.0 \times 10^{-4} M$). In this experiment, the read out is made 24 min after the beginning of the experiment. The results are shown in Table 2.

Table 2

	Tembotrione	Diketoneitrile	Mesotrione
PfHPPD	>5.6	>5.6	>5.6
PfG336W	>5.6	4.9	5.3

The pI50 of the HPPD enzymes was also measured using an HPLC based method. Tolerance to the HPPD inhibitors tembotrione, diketoneitrile, and mesotrione was measured. In this experiment, the measurement is taken 3 min after the beginning of the experiment. The results are shown in Table 3.

Table 3

	Tembotrione	Diketoneitrile	Mesotrione
PfHPPD	4.9	4.7	4.7
PfG336W	4.6	4.2	4.3

10

In an alternative method to measure HPPD activity and HPPD tolerance to HPPD inhibitors, HPPD activity was measured at room temperature by adding appropriate amounts of HPPD to a solution of 200 mM Tris-HCl pH 7.6, 10 mM ascorbate, 20 μ M FeSO₄, 650 units of catalase, 8 μ g HGA dioxygenase (HGA: Homogentisate) and 10 – 100 μ M HPP in a total volume of 1 ml. Initial reaction rates were determined from the increase in absorbance at 318 nm due to the formation of maleylacetoacetate ($\epsilon_{318} = 11,900 \text{ M}^{-1} \text{ cm}^{-1}$). K_m and V_{max} values were determined by fitting initial velocities of HPP turnover determined at different HPP concentrations to the Michaelis-Menten equation using Model 350 of the ID Business Solutions Ltd Xlfit version 5.1.0.0 software suite. This method is called the HGD assay.

20

The pI50 of the HPPD enzymes was also measured using an HPPD coupling assay (HGD; PfHPPD and PfG336W) and their respective tolerance to the HPPD inhibitors tembotrione, diketoneitrile, and mesotrione. The results are shown in Table 4.

25

Table 4

	Tembotrione	Diketoneitrile	Mesotrione
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PfHPPD	6.4	5.8	5.8
PfG336W	6.0	5.3	5.3

On Tables 2, 3 and 4, it can be clearly seen that the mutation at the position 336 of the HPPD from *Pseudomonas fluorescens* increased significantly the tolerance of the HPPD to several HPPD inhibitors.

5

Example 3. First generation point mutant library.

The PfG336W mutant was further mutagenized at 16 positions. Randomization of these positions was carried out using the QUIKCHANGE® lightning kit. The theoretical diversity of the library was about 300. Mutants were pooled and transformed into DH5alpha *E. coli* cells. Six hundred individual clones were screened for tolerance to the HPPD inhibitor tembotrione (TBT). The clones were grown in LB media plus kanamycin at 37 degrees C in a shaker until an OD₆₀₀ nm of 0.3 was reached. Cultures were then switched to 30 degrees C and incubated for an additional 17 hours. Cultures were spun down and cell pellets resuspended in 10 mM Hepes/KOH pH 7.6, 4 mM MgCl₂, 1 mM DTT. The cells were lysed by bead beating and soluble cell extracts were obtained after centrifugation.

The mutants were analyzed using a brown color assay. Specifically, the HPPD extracts were assayed in 96 well format for HPPD inhibitor tolerance by spotting on solid media containing LB-agar, kanamycin, 5 mM tyrosine, 42 mM succinate and an HPPD inhibitor. In the primary screen, 20 ul extract was spotted in triplicate on plates containing 250 uM tembotrione. Plates were covered with airpore tape and incubated at 37 degrees C. After 24 hours, brown pigment formation was visually compared to a sample containing PfG336W. Variants showing increased pigment formation in the presence of TBT were re-assayed on 250 uM TBT and 250 uM diketonitrile (DKN) active compound of isoxaflutole (IFT). Those variants that again showed improved inhibitor tolerance were again expressed, and extract was titrated on 250 uM TBT and 250 uM DKN to determine the extent of improvement. Extract samples were also analyzed by SDS-PAGE and the extracts were found to contain equal amounts of HPPD protein.

Titration showed that variant PfHPPDEvo33 (SEQ ID NO:6) has 4x improved tolerance to TBT and DKN compared to PfG336W. This variant has a substitution of

proline for glutamic acid at position 335 relative to PfG336W. This mutation is located on a c-terminal alpha helix that acts as a gate to the active site.

Example 4. Second generation permutational library screening

5 The sequences of the top performing first-generation variants were analyzed and a second generation permutational library in the region combining positions 335, 336, 339, 340 was generated. The theoretical diversity of this library was 640. Screening was carried out as described in Example 3. Another second generation permutational library was generated targeting positions 188, 189 and 190 and
10 measured using the brown color assay.

 Titration data showed that variant PfHPPDEvo36 (SEQ ID NO:7) had 16x improved tolerance to TBT and DKN compared to PfG336W. It has 4x improved tolerance compared to PfHPPDEvo33. Again protein expression was analyzed by SDS-PAGE and variants were found to express equal amounts of HPPD protein.
15 PfHPPDEvo36 has a substitution of serine for glutamic acid at position 335, serine for tryptophan at position 336, threonine for lysine at position 339, and glutamine for alanine at position 340 relative to PfG336W.

 Titration data showed that variant PfHPPDEvo37 (SEQ ID NO:3) had improved tolerance to TBT and DKN compared to PfG336W. PfHPPDEvo37 has a
20 substitution of tryptophan for alanine at position 188 relative to PfG336W. SDS-PAGE analysis was carried out and showed no differences in HPPD expression levels between variants.

 Titration data below shows variant PfHPPDEvo40 (SEQ ID NO:8) had improved tolerance to TBT and DKN compared to PfG336W. SDS-PAGE analysis
25 was carried out and showed no differences in HPPD expression levels between variants.

 Variants were also tested by plating whole *E. coli* cells expressing HPPDs on media containing various HPPD inhibitors. For these experiments, DH5alpha cells containing HPPD expressing plasmids were grown in LB media + kanamycin until an
30 OD_{600nm}=0.5 was reached. Serial dilutions of cells were prepared in LB media + kanamycin corresponding to OD₆₀₀ values of 0.016, 0.008, 0.004, and 0.002. Ten microliters of each dilution were plated in triplicate on plates containing no HPPD inhibitor, 250 uM TBT, 250 uM DKN and 250 uM mesotrione (MST). Plates were

incubated for 18 hours at 37 degrees C. SDS-PAGE analysis was carried out and showed no differences in HPPD expression levels between variants.

PfHPPDEvo40 and PfHPPDEvo41 (SEQ ID NO:16) showed improved tolerance to TBT and MST in this assay compared to PfG336W.

5

Example 5. Enzymatic analysis of permutational mutants

The kinetic activity of the (PfHPPD enzyme was compared to several of the permutational mutants and the results are set forth in Table 5 below. “Km” (Michaelis-Menten constant) means the kinetic parameter that is used to characterize an enzyme, and it is defined as the concentration of substrate that permits half maximal rate of the reaction. Km is further defined as the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$) where Vmax has the meaning of being the maximum velocity of the reaction.

For the determination of the specific activity of the enzymes, samples were incubated, and the reaction was stopped after 24 min. The specific activity was estimated by μg of protein.

Table 5

	HPP	HPP	SEQ ID NO:
	K_m (μM)	Kcat (s^{-1})	
PfHPPD	187	4.4	1
PfG336W	141	5.4	2
PfHPPDEvo37	220	3.0	3
C023E6	424	6.9	4
C024H11	188	0.8	5
PfHPPDEvo33	581	2.3	6
PfHPPDEvo36	602	3.3	7
PfHPPDEvo40	509	3.2	8
CO210d10	1181	1.4	9

CO212f3	297	0.9	10
C644	232	0.5	11
C645	156	0.8	12
c0216C6	1541	1.2	14
c0213H10	489	2.6	15
PfHPPDEvo41	336	1.1	16

The specific activity of the HPPD from *Pseudomonas fluorescens* HPPD (PfHPPD) and mutants thereof was obtained using the spectrophotometric method. Samples were incubated, and the reaction was stopped after 24 min. The specific

5 activity was estimated by μg of protein.

Table 6

HPPD	Specific activity (Delta Absorbance / 24min / μg protein)	SEQ ID NO:
PfHPPD	0.76	1
PfG336W	0.4	2
PfHPPDEvo37	0.36	3
C023E6	0.38	4
C024H11	0.07	5
PfHPPDEvo33	0.25	6
PfHPPDEvo36	0.82	7
PfHPPDEvo40	0.42	8
CO210d10	0.43	9
CO212f3	0.42	10
C644	0.38	11
C645	0.36	12
c0218A5	0.13	13
c0216C6	0.50	14
c0213H10	0.51	15
PfHPPDEvo41	0.44	16

C0228G9	0.17	17
C0232D2	0.06	18
C0234A4	0.33	19
C0235F6	0.53	20
C0235E2	0.34	21
C0236H7	0.36	22
C0236F8	0.47	23
C0240D2	0.32	24
C0240D12	0.29	25
C0242D4	0.21	26
C0244A2	0.25	27
C0244F5	0.22	28
C0247B6	0.37	29
C0247H7	0.28	30
C0252F11	0.26	31
C0255B12	0.21	32
C0255C1	0.17	33
C0255C3	0.19	34
C0255E6	0.15	35
C0255E10	0.24	36
C0256B1	0.31	37
C0256G11	0.33	38
C0256H4	0.19	39
C0257C5	0.3	40
C0260E11	0.79	41
C0260C6	0.51	42
C0262C4	0.81	43
C0262F11	0.76	44
C0263B7	0.43	45
C0263G12	0.77	46
C0261H2	0.42	47
C0264G5	0.71	48

C0264G7	0.35	49
C0266A11	0.33	50

As shown in Table 6, there was no significant difference in activity between the wild type and most of the mutants of the enzyme. Therefore, it is likely that the observed tolerance is due to intrinsic properties of the enzyme and not to a dysfunction or a slower activity into converting 4-hydroxyphenyl pyruvate into homogentisate.

The tolerance to diketonitrile (active form of isoxaflutole) and mesotrione was also measured using the spectrophotometric assay. Values represent pI₅₀. “>” means that the value was out of the range of measurement of this assay (e.g., the enzyme is more sensitive than the value indicated thereafter).

Table 7

HPPD	pI ₅₀ Diketoneitrile	pI ₅₀ Mesotrione
	OD	OD
PfHPPD	>5.6	>5.6
PfG336W	4.9	5.3
PfHPPDEvo37	4.7	5.3
C023E6	4.4	5.5
C024H11	4.1	4.6
PfHPPDEvo33	4.3	5.1
PfHPPDEvo36	4.5	n.d.
PfHPPDEvo40	3.5	4.9
CO210d10	4.3	5.7
CO212f3	4.7	5.4
C644	5.3	>5.6
C645	>5.6	>5.6
c0218A5	4.4	5.1
c0216C6	4.0	4.9
c0213H10	4.9	5.2

PfHPPDEvo41	3.4	4.7
C0228G9	5.4	5.7
C0232D2	5.1	5.5
C0234A4	4.9	>5.6
C0235F6	4.7	5.5
C0235E2	4.7	5.4
C0236H7	4.7	5.3
C0236F8	4.9	>5.6
C0240D2	4.9	5.4
C0240D12	4.5	5.0
C0242D4	3.6	4.5
C0244A2	4.7	5.6
C0244F5	4.3	5.3
C0247B6	5.1	>5.6
C0247H7	4.4	5.2
C0252F11	3.7	4.6
C0255B12	4.0	5.0
C0255C1	3.6	4.7
C0255C3	4.8	5.5
C0255E6	3.7	4.4
C0255E10	4.8	>5.6
C0256B1	5.0	>5.6
C0256G11	4.9	5.5
C0256H4	3.6	4.8
C0257C5	3.8	4.6
C0260E11	4.7	5.4
C0260C6	4.6	5.3
C0262C4	4.8	5.3
C0262F11	5.0	5.5
C0263B7	4.1	4.6
C0263G12	4.9	5.6
C0261H2	4.5	5.3

C0264G5	4.9	5.6
C0264G7	4.1	4.8
C0266A11	4.0	4.9

The tolerance to tembotrione, diketonitrile (active form of isoxaflutole) and mesotrione was also measured using the HPLC based assay. Values represent pI₅₀.

- 5 “>” means that the value was out of the range of measurement of this assay (e.g., the enzyme is more than sensitive than the value indicated thereafter).

Table 8

HPPD	pI ₅₀ Tembotrione	pI ₅₀ Diketonnitril	pI ₅₀ Mesotrione
	HPLC	HPLC	HPLC
PfHPPD	4.9	4.7	4.7
PfG336W	4.6	4.2	4.3
PfHPPDEvo37	4.1	3.6	n.d.
C023E6	4.3	3.5	n.d.
C024H11	4.8	4.0	n.d.
PfHPPDEvo33	5.2	3.6	n.d.
PfHPPDEvo36	5.2	4.2	n.d.
PfHPPDEvo40	5.0	3.2	n.d.
CO210d10	5.9	3.9	n.d.
CO212f3	4.8	3.8	n.d.
C644	5.5	4.5	5.1
C645	5.2	4.7	5.1
c0218A5	5.0	3.7	4.6
c0216C6	4.8	3.1	4.2
c0213H10	4.5	3.4	4.3
PfHPPDEvo41	4.9	3.4	n.d.
C0228G9	4.6	4.2	4.2
C0232D2	n.d.	n.d.	n.d.
C0234A4	5.0	n.d.	n.d.

C0235F6	5.0	n.d.	n.d.
C0235E2	4.9	n.d.	n.d.
C0236H7	5.1	n.d.	n.d.
C0236F8	4.9	n.d.	n.d.
C0240D2	4.8	n.d.	n.d.
C0240D12	4.9	n.d.	n.d.

As demonstrated in Table 8, several HPPD mutants displayed improved tolerance to each of tembotrione, diketonitrile (Isoxaflutole), and mesotrione.

- 5 The tolerance to tembotrione, diketonitrile and mesotrione of selected HPPD mutants was also measured using the HPPD coupling assay (described in Example 2). Values represent pI50.

Table 9

HPPD	pI ₅₀ Tembotrione	pI ₅₀ Diketoneitrile	pI ₅₀ Mesotrione	Km (μM)	SEQ ID NO:
	HGD	HGD	HGD		
PfHPPD	6.4	5.8	5.8	187	1
PfG336W	6.0	5.3	5.5	141	2
PfHPPDEvo33	6.2	4.6	5.5	581	6
PfHPPDEvo40	6.2	4.2	4.8	509	8
PfHPPDEvo41	5.6	4.2	4.3	336	16

10

As shown in Table 9, the HPPD mutants are more tolerant to any HPPD inhibitor tested compared to the wild type HPPD enzyme. Thus, the present invention includes mutant HPPD enzymes that show significantly improved tolerance to several HPPD inhibitors at the same time.

15

Tolerance to other HPPD inhibitors from the class described in WO2012/028579 (which is herein incorporated by reference in its entirety, but particularly with respect to the compounds described in Table 10 herein) was also

measured for the HPPD enzymes listed in Table 10. Tolerance was estimated *in vitro* using the HPPD coupling assay (HGD) described above. Values are pI50.

Table 10

	pI50 (HGD assay)	
	Compound No.:4-253 as defined in: WO 2012/028579	Compound No.:4-278 as defined in: WO 2012/028579
PfG336W	6.2	6.6
PfHPPD	6.4	5.9
PfHPPDEvo40	5.5	6.1
PfHPPDEvo41	5.4	5.6

5

Thus, the selected mutants displayed tolerance to a broad range of HPPD inhibitor herbicides.

Example 6. Analysis of HPPD inhibitor tolerance *in planta*

10 Soybean plants expressing an HPPD inhibitor tolerant enzyme of the present invention, along with a gene conferring tolerance to glyphosate and a gene conferring tolerance to glufosinate, were tested for tolerance to tembotrione. A DeVries Tracker Sprayer was calibrated prior to each spraying. The chemical formulation used for tembotrione testing was LAUDIS® SC formulation. Spray tests were conducted

15 using 184 grams/ hectare; 34.5% TBT. Tolerance was evaluated one week after spraying. A rating of “+” was assigned to plants that were completely bleached in the actively growing tissue. A rating of “++” was assigned to plants having slight tolerance, i.e., the newest plant tissues had some green and are not bleached completely. A rating of “+++” was assigned to plants showing very little effect from

20 spray, i.e., some chlorosis or very slight bleaching was present. The results of these tests are shown in Table 11.

Table 11

HPPD	Number of plants treated	+	++ or +++	Percent ++ or +++
PfG336W	2248	811	160	7.12
PfHPPDEvo37	35	17	3	8.57

PfHPPDEvo33	151	50	54	35.76
PfHPPDEvo40	207	60	65	31.4
PfHPPDEvo41	852	412	279	32.75
PfHPPDEvo36	88	47	18	20.45

T1 events expressing PfG336W and PfHPPDEvo41 (each also expressing a gene conferring tolerance to glyphosate and a gene conferring tolerance to glufosinate) were evaluated in field trials. The plants were sprayed at the v2-v3 stage with 1x glufosinate. Five days after treatment with glufosinate, the surviving plants were sprayed with 2x tembotrione, 2x mesotrione, or 2x isoxaflutole and evaluated for phytotoxicity after 7 days. . Nine out of 18 events expressing PfHPPDEvo41 and 0 out of 18 events expressing PfG336W exhibited less than or equal to 20% average phytotoxicity from a post-emergent application of tembotrione at 200 g ai/ha.

In a field trial in Argentina, 5 out of 10 T2 PfHPPDEvo41, and 1 out of 3 PfGW336 events exhibited less than or equal to 18% maximum phytotoxicity from a post-emergent application of isoxaflutole at 210 g ai/ha.

In a field trial conducted in the United States (Minnesota), 7 out of 7 T2 PfHPPDEvo41, and 1 out of 11 PfGW336 events exhibited less than or equal to 25% maximum phytotoxicity from a post-emergent application of tembotrione at 200 g ai/ha.

Example 7: Generation of mutants of other HPPD enzymes

Strains ATX22717 and ATX1974 were identified by using an assay that couples HPPD activity to pink/orange color production that can be visually detected. Incorporation of quinone quenching agents was used to improve the sensitivity of the *in vitro* assay. The quinone quench used was MBTH, or 3-methyl-2-benzothiazolinone hydrazine. Both phenol oxidases and HPPDs are dioxygenase enzymes that produce a quinone compound as an intermediate product; these quinones then rapidly and spontaneously go through electron rearrangements that lead to the formation of a downstream product that is more stable, such as melanin (in the case of phenol oxidases) or homogentisate (in the case of HPPDs) and insoluble. When complexed with a quinone, the MBTH-quinone product produces a pink/orange

color. The addition of the MBTH quenching agent increases the sensitivity of the assay by approximately 5-fold.

The strains were grown in LB agar for approximately 24 hours. Cells were pelleted by centrifugation, resuspended in ½ volume of 20mM HEPES buffer, pH 7.1, and lysed by beadbeating. 50ul of strain extract was added to 150ul MBTH assay mix in a conical bottom 96 well block. The final assay mixture contained 10mM MBTH, 0 or 1mM hydroxyphenyl pyruvate (HPP), 20mM HEPES buffer, pH 7.1, and either 0, 10µM or 1mM tembotrione (TBT). The 96 well assay blocks were then shaken at 200rpm in a floor shaker, 30 degrees C for approximately 22 hours prior to scoring. Strains ATX22717 and ATX1974 were shown to be tolerant to TBT.

Strain ATX22717 is a *Pseudomonas aeruginosa* strain isolated from a soil sample collected in North Carolina, United States.

Strain ATX1974 is a *Pseudomonas agarici* strain isolated from a soil sample collected in North Carolina, United States.

15

The HPPD genes were identified from the strains using the following steps:

- Preparation of total DNA from the strain. Total DNA contains both genomic DNA and extrachromosomal DNA. Extrachromosomal DNA contains a mixture of some or all of the following: plasmids of various size; phage chromosomes; other uncharacterized extrachromosomal molecules.
- Sequencing of the DNA. Total DNA is sequenced via Next-Generation Sequencing methods.
- Assembly of the DNA sequence by various software programs, including Newbler, phredPhrapm and CLC.
- Identification of HPPD genes by DNA and protein homology algorithms.

25

Axmi305H was identified from strain ATX22717. The nucleotide sequence encoding Axmi305H is set forth in SEQ ID NO:60 and the amino acid sequence is set forth in SEQ ID NO:57. Axmi305H shares 99.7% identity to SEQ ID NO:29696 in United States Patent Application Publication No. 20070020624.

30

Axmi309H was identified from strain ATX1974. The nucleotide sequence encoding Axmi309H is set forth in SEQ ID NO:61 and the amino acid sequence is set

forth in SEQ ID NO:58. Axmi309H shares 99.7% identity to GENBANK®
Accession No. YP_348648.

The strain *Comamonas testosteroni* was selected as a potential source for an
enzymatically favorable HPPD because it was reported to produce pyomelanin, a
5 derivative of homogentisic acid which is the product of HPPD activity (Turick et al.,
2005, Microbial Metabolite Field Deployment Report. WSRC-TR-2005-00455).

Searches of the BLAST public databases indicated that there are two distinct
HPPDs present in the bacterial species *Comamonas testosteroni*, one is 362 amino
acids in length and the other 373 amino acids in length. *Comamonas testosteroni*
10 genomic DNA (ATCC® catalog number 700441) was PCR amplified using primers
designed for each of the two HPPD genes based on the reported nucleonucleotide
sequence of *Comamonas testosteroni* strains CNB-2, KF-1, and S44 as found in the
BLAST database.

The PCR products were digested with BspHI and Xba I and cloned into pSE420 cut
15 with NcoI and XbaI.

The nucleotide and derived amino acid sequence of the 373 amino acid length
HPPD is not identical to any reported sequence of *Commamonas testosteroni* HPPD
as indicated in BLAST searches. It is 99% similar to *Comamonas testosteroni* S44.
This novel sequence is now referred to as Axmi428H. The nucleotide sequence
20 encoding Axmi428H is set forth in SEQ ID NO:62 and the amino acid sequence is set
forth in SEQ ID NO:59.

Active HPPD enzyme will produce a brown pigment, pyomelanin, as it
converts hydroxyphenylpyruvate to homogentisic acid. The production of this
pigment can be visualized. DH5alpha cells expressing Axmi428H protein were
25 grown to saturation in LB media and then 10µl of saturated culture was spotted onto
100µl LB agar plates containing 0, 0.5, 1.0, and 2.0mM tembotrione. Cells were
photographed after approximately 16 hours of additional growth at 37 degrees C.

Axmi428H was also characterized kinetically using an *in vitro* kinetic assay
30 that couples the production of homogentisic acid with the enzyme homogentisate 1,2-
dioxygenase (HGO). HGO converts homogentisic acid to maleoacetoacetate which is
monitored as it absorbs strongly at 321nm. The real-time production of product is
monitored continuously in a 96-well spectrophotometer in the presence of varying

concentrations of substrate, from limiting to saturating, makes it possible to determine the K_m of the enzyme using standard Michaelis-Menten kinetics. A K_i can be determined by graphing the change of this K_m in the presence of varying amounts of the inhibitor tembotrione.

- 5 For this assay, Axmi428H enzyme was prepared by growing transformed DH5alpha cells with shaking at 250 rpm at 37 °C until cultures reached an OD600 of 0.6 – 0.7. The temperature was then reduced to 30 °C and cultures continued to shake for approximately 20 hours. Cell cultures were pelleted by centrifugation and resuspended in 1/20th volume 20mM HEPES, pH 7.0, 50mM NaCl buffer. Cells
- 10 were lysed by the addition of LYSONASE™ (Novagen) for 45 min at room temperature and frozen at -20 degrees C for at least 1 hour. Cell extracts were thawed just prior to assay, clarified through centrifugation and assayed for activity in the presence of varying substrate and inhibitor levels with excess HGO enzyme. Analysis of the kinetic data yields the kinetic constants indicated in the table below.
- 15 Axmi428H has a similar level of tolerance to tembotrione as the mutated PfG336W gene and both show higher tolerance than the native soybean HPPD.

20 Table 12

Gene	K_m HPP (μ M)	V_{max}	K_i TBT (μ M)
Soy HPPD	32.5	79	0.04
W336	152	35	2.49
Axmi428H	52	22.6	1.37

- The mutations present in some of the mutant HPPD enzymes described herein were introduced in the corresponding positions of other native HPPD enzymes, including Axmi305H, Axmi309H, and Axmi428H in an attempt to improve the
- 25 tolerance of these enzymes

Table 13 shows the sequence identity between different HPPD proteins. Alignment was performed with AlignX.

Table 13

	PfG336W	Axmi309H	Axmi428H	Axmi305H
PfG336W	100	94	56	53
Axmi309H	94	100	56	54
Axmi428H	56	56	100	54
Axmi305H	53	54	54	100

A QUIKCHANGE® Lightning site-directed mutagenesis kit was used for site-directed mutagenesis of the genes in vector pSE420. The mutants generated by this approach were transformed into BI21-DE3* cells and grown to saturation in LB media. Aliquots were then spotted onto LB agar plates containing various amounts of tembotrione. By this method, active HPPD enzyme will produce a brown pigment after approximately 24 hours of growth on plates. Using this assay, all of the mutants generated were shown to have HPPD activity that was highly resistant to inhibition by the tembotrione.

The tolerance of the mutants was also measured against tembotrione, diketone nitrile (isoxaflutole) and mesotrione using the HPPD coupling method. The results are shown in Table 14. “>.” means that is out of range of measurement but highly superior than the number listed thereafter.

Table 14

	pI ₅₀ Tembotrione	pI ₅₀ Diketone nitrile	pI ₅₀ Mesotrione	K _m (μM)
PfG336W	6.4	5.8	5.8	187
PfHPPDEvo40	6.2	4.2	4.8	509
PfHPPDEvo41	5.6	4.2	4.3	336
Axmi305H	6.7	6.5	6.7	116
Axmi305H-Evo40	5.8	5.5	5.3	721
Axmi305H-Evo41	6.8	5.0	5.2	816
Axmi309H	6.4	6.4	6.1	318
Axmi309H-Evo40	5.9	4.4	5.2	290
Axmi309H-Evo41	6.2	4.5	4.7	807

Axmi428H	7.2	7.0	6.7	47
Axmi428H-Evo40	6.1	5.2	5.5	1490
Axmi428H-Evo41	5.6	4.3	5	>>1000

The data of Table 14 shows that the mutations or combinations of mutations identified herein are effectively responsible for the improved tolerance to the HPPD inhibitors, whatever the nature of the HPPD protein in which they are introduced.

5

Example 8. Cloning of HPPD genes into a plant expression cassette.

For each of the HPPD genes described herein, the open reading frame (ORF) may be amplified by PCR from a full-length DNA template. *Hind* III restriction sites may be added to each end of the ORFs during PCR. Additionally, the nucleotide sequence ACC may be added immediately 5' to the start codon of the gene to increase translational efficiency (Kozak (1987) *Nucleic Acids Research* 15:8125-8148; Joshi (1987) *Nucleic Acids Research* 15:6643-6653). The PCR product may be cloned and sequenced using techniques well known in the art to ensure that no mutations are introduced during PCR.

15 The plasmid containing the PCR product may be digested with *Hind* III and the fragment containing the intact ORF may be isolated. This fragment may be cloned into the *Hind* III site of a plasmid such as pAX200, a plant expression vector containing the rice actin promoter (McElroy *et al.* (1991) *Molec. Gen. Genet.* 231:150-160) and the PinII terminator (An *et al.* (1989) *The Plant Cell* 1:115-122).
20 The promoter – gene – terminator fragment from this intermediate plasmid may be then subcloned into plasmid pSB11 (Japan Tobacco, Inc.) to form a final pSB11-based plasmid. These pSB11-based plasmids are typically organized such that the DNA fragment containing the promoter – *gene*– terminator construct may be excised by double digestion by restriction enzymes, such as *Kpn* I and *Pme* I, and used for
25 transformation into plants by aerosol beam injection. The structure of the resulting pSB11-based clones may be verified by restriction digest and gel electrophoresis, and by sequencing across the various cloning junctions.

The plasmid may be mobilized into *Agrobacterium tumefaciens* strain LBA4404 which also harbors the plasmid pSB1 (Japan Tobacco, Inc.), using
30 triparental mating procedures well known in the art, and plating on media containing

spectinomycin. The pSB11-based plasmid clone carries spectinomycin resistance but is a narrow host range plasmid and cannot replicate in *Agrobacterium*.

Spectinomycin resistant colonies arise when pSB11-based plasmids integrate into the broad host range plasmid pSB1 through homologous recombination. The cointegrate
5 product of pSB1 and the pSB11-based plasmid may be verified by Southern hybridization. The *Agrobacterium* strain harboring the cointegrate may be used to transform maize by methods known in the art, such as, for example, the PureIntro method (Japan Tobacco).

10 Example 9. Soybean transformation

Soybean transformation is achieved using methods well known in the art, such as the one described using the *Agrobacterium tumefaciens* mediated transformation soybean half-seed explants using essentially the method described by Paz et al. (2006), Plant cell Rep. 25:206. Transformants were identified using isoxaflutole or
15 tembotrione as selection marker. The appearance of green shoots was observed, and documented as an indicator of tolerance to the herbicide isoxaflutole or tembotrione. The tolerant transgenic shoots will show normal greening comparable to wild-type soybean shoots not treated with isoxaflutole or tembotrione, whereas wild-type soybean shoots treated with the same amount of isoxaflutole or tembotrione will be
20 entirely bleached. This indicates that the presence of the HPPD protein enables the tolerance to HPPD inhibitor herbicides, like isoxaflutole or tembotrione.

Tolerant green shoots are transferred to rooting media or grafted. Rooted plantlets will be transferred to the greenhouse after an acclimation period. Plants containing the transgene are then sprayed with HPPD inhibitor herbicides, as for
25 example with tembotrione at a rate of 100g AI/ha. Ten days after the application the symptoms due to the application of the herbicide are evaluated and compared to the symptoms observed on wild type plants under the same conditions.

Example 10: Cotton T0 plant establishment and selection.

30 Cotton transformation is achieved using methods well known in the art, especially preferred method in the one described in the PCT patent publication WO 00/71733. Regenerated plants are transferred to the greenhouse. Following an acclimation period, sufficiently grown plants are sprayed with HPPD inhibitor herbicides as for example tembotrione equivalent to 100 gAI/ha supplemented with

ammonium sulfate and methyl ester rapeseed oil. Seven days after the spray application, the symptoms due to the treatment with the herbicide are evaluated and compared to the symptoms observed on wild type cotton plants subjected to the same treatment under the same conditions.

5

Example 11. Transformation of Maize Plant Cells by *Agrobacterium*-Mediated Transformation

Ears are best collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are preferred for use in transformation.

10 Embryos are plated scutellum side-up on a suitable incubation media, and incubated overnight at 25°C in the dark.

However, it is not necessary *per se* to incubate the embryos overnight.

Embryos are contacted with an *Agrobacterium* strain containing the appropriate vectors having a nucleotide sequence of the present invention for Ti plasmid mediated

15 transfer for about 5-10 min, and then plated onto co-cultivation media for about 3 days (25°C in the dark). After co-cultivation, explants are transferred to recovery period media for about five days (at 25°C in the dark). Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is
20 transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated as known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants.

25 All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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

THAT WHICH IS CLAIMED

1. A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, wherein said nucleic acid molecule encodes an amino acid sequence having at least 55% sequence identity to SEQ ID NO:1, or a fragment thereof, and has one or more amino acid substitutions selected from the group consisting of:
- (a) a tryptophan, glycine, or serine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1;
 - (b) a serine, cysteine, or arginine at the amino acid position corresponding to amino acid position 189 of SEQ ID NO:1;
 - (c) a proline, serine, histidine, alanine, glycine, or glutamine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1;
 - (d) a serine or tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
 - (e) a threonine, alanine, or serine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1;
 - (f) a glutamine, alanine, valine, or glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
 - (g) a leucine at the amino acid position corresponding to amino acid position 215 of SEQ ID NO:1;
- wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide.

2. A recombinant nucleic acid molecule of claim 1, wherein said amino acid sequence has:
- (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;
 - (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide.

3. A recombinant nucleic acid molecule of claim 1, wherein said nucleic acid sequence encodes an amino acid sequence having at least 90% sequence identity to the amino acid sequence set forth in any of SEQ ID NO:3-59 or fragments thereof.

4. A recombinant nucleic acid molecule encoding an HPPD enzyme, wherein said nucleic acid molecule encodes an amino acid sequence having at least 80% sequence identity to SEQ ID NO:1 or a fragment thereof and wherein said HPPD comprise amino acid substitution(s) of:

(a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1;

(d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to

amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide.

5. A recombinant nucleic acid molecule encoding an HPPD enzyme, wherein said nucleic acid molecule encodes an amino acid sequence having at least 80% sequence identity to SEQ ID NO:1 or a fragment thereof and wherein said HPPD comprise amino acid substitution(s) of:

- (a) a tryptophan at amino acid position 188 and a tryptophan at amino acid position 336; or
(b) a proline at amino acid position 335;
wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide.

6. The recombinant nucleic acid molecule of any of claims 1-5, wherein said nucleotide sequence is a synthetic sequence that has been designed for expression in a plant.

7. The recombinant nucleic acid molecule of any of claims 1-5, wherein said nucleotide sequence is operably linked to a promoter capable of directing expression of the nucleotide sequence in a plant cell.

8. The recombinant nucleic acid molecule of claim 1, wherein said HPPD inhibitor herbicide is selected from the group consisting of an N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamide, an N-(1,2,5-oxadiazol-3-yl)benzamide, tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, pyrasulfotole, and mesotrione.

9. A vector comprising the nucleic acid molecule of claim 1.

10. The vector of claim 9, further comprising a nucleic acid molecule encoding a heterologous polypeptide.

11. A host cell that contains the recombinant nucleic acid molecule of claim 1.

12. The host cell of claim 11 that is a bacterial host cell.

13. The host cell of claim 11 that is a plant cell.

14. A transgenic plant comprising the recombinant nucleic acid molecule of claim 1.

15. The plant of claim 14, wherein said plant is selected from the group consisting of maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape.

16. A transgenic seed comprising the recombinant nucleic acid molecule of claim 1.

17. A recombinant polypeptide comprising an HPPD enzyme, wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide, and wherein said polypeptide has at least 55% sequence identity to SEQ ID NO:1 or a fragment thereof and comprises amino acid substitution(s) of:

(a) a tryptophan, glycine, or serine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1;

(b) a serine, cysteine, or arginine at the amino acid position corresponding to amino acid position 189 of SEQ ID NO:1;

(c) a proline, serine, histidine, alanine, glycine, or glutamine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1;

(d) a serine or tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(e) a threonine, alanine, or serine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1;

(f) a glutamine, alanine, valine, or glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(g) a leucine at the amino acid position corresponding to amino acid position 215 of SEQ ID NO:1.

5

18. The recombinant polypeptide of claim 17, wherein said amino acid sequence comprises amino acid substitution(s) of:

(a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a tryptophan at the amino acid position
10 corresponding to amino acid position 336 of SEQ ID NO:1;

(b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the
15 amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1 and a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1;

(d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or

(e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tryptophan at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the
25 amino acid position corresponding to amino acid position 340 of SEQ ID NO:1.

19. The recombinant polypeptide of claim 18, wherein said amino acid
30 sequence has at least 90% sequence identity to the amino acid sequence set forth in any of SEQ ID NO:3-59.

20. A recombinant polypeptide comprising an HPPD enzyme, wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide, and wherein said

polypeptide comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO:1, or a fragment thereof, and wherein said amino acid sequence comprises amino acid substitution(s) of:

- 5 (a) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1;
- (b) a serine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a threonine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the
10 amino acid position corresponding to amino acid position 340 of SEQ ID NO:1;
- (c) a tryptophan at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1;
- (d) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a serine at the amino acid position corresponding to
15 amino acid position 336 of SEQ ID NO:1, and a glutamic acid at the amino acid position corresponding to amino acid position 340 of SEQ ID NO:1; or
- (e) a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 339 of SEQ ID NO:1, and a glutamine at the amino acid position
20 corresponding to amino acid position 340 of SEQ ID NO:1.

21. A recombinant polypeptide comprising an HPPD enzyme, wherein said HPPD enzyme is tolerant to an HPPD inhibitor herbicide, and wherein said polypeptide comprises an amino acid sequence having at least 80% sequence identity
25 to SEQ ID NO:1, or a fragment thereof, and wherein said HPPD comprises amino acid substitution(s) of:

- (a) a tryptophan at amino acid position 188 and a tryptophan at amino acid position 336; or
- (b) a proline at amino acid position 335.

30

22. The recombinant polypeptide of claims 17-21, wherein said HPPD inhibitor herbicide is selected from the group consisting of an N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamide, an N-(1,2,5-oxadiazol-3-yl)benzamide, tembotrione,

sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, pyrasulfotole, and mesotrione.

23. The recombinant polypeptide of claims 17-21 further comprising a
5 heterologous amino acid sequence.

24. A method for producing a polypeptide with HPPD inhibitor herbicide tolerance activity, comprising culturing the host cell of claim 11 under conditions in which a nucleic acid molecule encoding the polypeptide is expressed.
10

25. A method for conferring tolerance to an HPPD inhibitor herbicide in a plant, said method comprising transforming said plant with a DNA construct, said construct comprising a promoter that drives expression in a plant cell operably linked with the nucleotide sequence of any of claims 1-8.
15

26. A plant having stably incorporated into its genome a DNA construct, said construct comprising a promoter operably linked with the nucleotide sequence of any of claims 1-8.

27. The plant of claim 26, wherein said plant is selected from the group consisting of a plant cell, a plant tissue, and a plant seed.
20

28. The plant of claim 26, wherein said plant is selected from the group consisting of maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape.
25

29. A commodity product comprising a detectable amount of the nucleic acid molecule of any of claims 1-5, or the amino acid sequence of any of claims 17-21.
30

30. Transgenic seed of the plant of claim 26.

31. A method of controlling weeds in a field comprising planting the plant of claim 26 or a seed thereof in a field and applying to said field an effective concentration of an HPPD inhibitor herbicide.

5 32. The method of claim 31, wherein said HPPD inhibitor herbicide is selected from the group consisting of an N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamide, an N-(1,2,5-oxadiazol-3-yl)benzamide, tembotrione, sulcotrione, topramezone, bicyclopyrone, tefuryltrione, isoxaflutole, pyrasulfotole, and mesotrione.

10

33. The transgenic plant of claim 26-28, wherein said plant further comprises a nucleotide sequence that confers tolerance to glyphosate and a nucleotide sequence that confers tolerance to glufosinate.

15 34. Use of the nucleotide sequence of any of claims 1-8 for rendering a plant tolerant to one or more HPPD inhibitor herbicide(s).

FIG. 1A

2/3

Pseudomonas_fluorescens	S--SIYDIDFVYLEG---VERNPGAGLKVIDHLTHNVYRGRMVYWANFY	179
Avena_sativa	D--LPFLPGFERVS-----SPGAVDYG LTRFDHV VGN--VPEMAPVIDYM	231
Avena_sativa	D--LPFLPGFERVS-----SPGAVDYG LTRFDHV VGN--VPEMAPVIDYM	232
Zea_mays	AG-EPFLPGFEGVA-----SPGAADYG LSRFDHIVGN--VPELAPAAAYF	233
Streptomyces_avermitilis	G---PYLPGYVAAAPIVEPPAHR---TFQAIDHCVGNVELGRMNEWVG FY	203
Arabidopsis_thaliana	TEKSEFLPGFERVEDA--SSFP-LDYGIRRLDHAVGN--VPELGPALTYV	241
Hordeum_vulgare	D--VPFLPGFEGVT-----NPDAVDYG LTRFDHV VGN--VPELAPAAAYI	226
Daucus_carota	---GLFLPGFEAVEGT--ASFPDL DYGIRRLDHAVGN--VTELG PVVEYI	234
Mycosphaerella_graminicola	G---PFLPGYRSC TT VDSANKFLPPVNLEAIDHCVGNQDWD EMSDACDFY	238
Coccicoides_immitis	G---GFMPGYRMESNADATSKFLPKVVLERIDHCVGNQDWD EMERVC DYY	217
	: .:	: **: . *
Pseudomonas_fluorescens	EKLNFNFREARYF---DIKGEYTG LTSKAMSAPDGMIRIPLNE--ESSKGA	224
Avena_sativa	KGFLGFHEFAEFTAEDVGTTESGLNSVVLANNSEAVLLPLN EPVHG TKRR	281
Avena_sativa	KGFLGFHEFAEFTAEDVGTTESGLNSVVLANNSEAVLLPLN EPVHG TKRR	282
Zea_mays	AGFTGFHEFAEFTTEDVGTAE SGLNSMVLANNSENVLLPLN EPVHG TKRR	283
Streptomyces_avermitilis	NKVMGFTNMKEFVGDDIATEYSALMSKV VADGTLKV KFPIN EPALAKK-K	252
Arabidopsis_thaliana	AGFTGFHQFAEFTADDVGTAE SGLNSAVLASNDEM VLLPIN EPVHG TKRK	291
Hordeum_vulgare	AGFTGFHEFAEFTAEDVGTTESGLNSVVLANNSEGVLLPLN EPVHG TKRR	276
Daucus_carota	KGFTGFHEFAEFTAEDVGTLESGLNSVVLANNSEMVLLPLN EPVYGT KRK	284
Mycosphaerella_graminicola	ERCLGFHFRFWSVDDKDICTEFSALKSIVMSSPNQVVKMPIN EPAGKK-K	287
Coccicoides_immitis	EKILGFHFRFWSVDDKDICTEFSALKSIVMASPNDIVKMPIN EPAGKK-Q	266
	. * . . *	: .: * * .: : : : * * . *
Pseudomonas_fluorescens	GQIEEF LMQFN GEGIQHVAFLTDDL VKTWDALKKIG---MRFMTAPPDT	270
Avena_sativa	SQIQTYLEYHGGPGVQHIALASNDVLR TLREMRARTPMGGFEFMAPPQAK	331
Avena_sativa	SQIQTYLEYHGGPGVQHIALASNDVLR TLREMRARTPMGGFEFMAPPQAK	332
Zea_mays	SQIQTF LDH HGGPGVQH MALASDDVLR TLREMQARSAMGGFEFMAPP TSD	333
Streptomyces_avermitilis	SQIDEYLEFYGGAGVQHIALNTGDIVETVRTMRAA---GVQFLDTP-DS	297
Arabidopsis_thaliana	SQIQTYLEHN EGAGLQHLALMSEDI FRTLREMRKRSSIGGFDFMPSPPPT	341
Hordeum_vulgare	SQIQTFLEHHGGPGVQHIAVASSDVLRTL RKMARSAMGGFDFLPPPLPK	326
Daucus_carota	SQIQTYLEHN EGAGVQH LALVSEDI FRTLREMRKRSC LGGFDFMPSPPPT	334
Mycosphaerella_graminicola	SQIEEYVDFYNGPGVQHIALRTPNII EAVSNLRSR---GVEFISVP-DT	332
Coccicoides_immitis	SQIEEYVDFYNGAGVQHIALRTNNIIDAITNLKAR---GTEFIKVP-ET	311
	. **: : :	* *: **: * . : : : : : : : *
Pseudomonas_fluorescens	YYEMLEGRLPDHG----EPVDQLQARGILLD GSSVEGDKRLL LQIFSETL	316
Avena_sativa	YYEGVRRRIAGDVLS--EEQIKECQELGVLVD----RDDQGVLLQIFTKPV	375
Avena_sativa	YYEGVRRRIAGDVLS--EEQIKECQELGVLVD----RDDQGVLLQIFTKPV	376
Zea_mays	YYDGVRRRAGDVLT--EAQIKECQELGVLVD----RDDQGVLLQIFTKPV	377
Streptomyces_avermitilis	YYDTLG EWVGD T----RVPVDTLRELKILAD----RDEGDYLLQIFTKPV	339
Arabidopsis_thaliana	YYQNLKKRVGDVLS--DDQIKECEELGILVD----RDDQG TLLQIFTKPL	385
Hordeum_vulgare	YYEGVRRRIAGDVLS--EAQIKECQELGVLVD----RDDQGVLLQIFTKPV	370
Daucus_carota	YYKNLKNRVGDVLS--DEQIKECEDLGILVD----RDDQG TLLQIFTKPL	378
Mycosphaerella_graminicola	YYENMRRLKAAGMKLEESFDIIQKLNILID----FDEGGYLLQLFTKPL	378
Coccicoides_immitis	YYEDMKIRLKRQGLVLDEDFETLKS LDILID----FDENGYLLQLFTKHL	357
	** . :	. . . : * * .: * *: * : : :

FIG. 1B

3/3

Pseudomonas_fluorescens	MG--PVFFFEFIQRK-----GDDGFGEENFKALFESIERDQ	349
Avena_sativa	GDRPTFFLEMIQRIGCMKDEVGQEYQKGGCGGFGKGNFSELFKSIEDYE	425
Avena_sativa	GDRPTFFLEMIQRIGCMKDEVGQEYQKGGCGGFGKGNFSELFKSIEDYE	426
Zea_mays	GDRPTLFLEIIQRIGCMKDEKQEYQKGGCGGFGKGNFSOLFKSIEDYE	427
Streptomyces_avermitilis	QDRPTVFFFEIIERH-----GSMGFGKGNFKALFEAIEREQ	374
Arabidopsis_thaliana	GDRPTIFIEIIQRVGCMMKDEEGKAYQSGGCGGFGKGNFSELFKSIEEYE	435
Hordeum_vulgare	GDRPTLFLEMIQRIGCMKDERGEEYQKGGCGGFGKGNFSELFKSIEDYE	420
Daucus_carota	GDRPTLFIEIIQRVGCMLKDDAGQMYQKGGCGGFGKGNFSELFKSIEEYE	428
Mycosphaerella_graminicola	MDRPTVFIEIIQRN-----NFDGFGAGNFKSLFEAIEREQ	413
Coccicoides_immitis	MDRPTVFIEIIQRN-----NFSGFGAGNFRALFEAIEREQ	392
	. . .*:~*:~* . *** **~**~*:~*~* :	
Pseudomonas_fluorescens	VRRGVLTAD-----	358
Avena_sativa	KSLEVKQSVVAQKS---	439
Avena_sativa	KSLEVKQSVVAQKS---	440
Zea_mays	KSLEAKQAAAAAAQGS	444
Streptomyces_avermitilis	EKRGNL-----	380
Arabidopsis_thaliana	KTLEAKQLVG-----	445
Hordeum_vulgare	KSLEAKQSAAVQGS---	434
Daucus_carota	KTLEAKQITGSAAA---	442
Mycosphaerella_graminicola	DLRGNL-----	419
Coccicoides_immitis	ALRGTLI-----	399

FIG. 1C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/059598

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 453 012 A1 (BAYER CROPSCIENCE AG [DE]) 16 May 2012 (2012-05-16) abstract paragraph [0055] paragraph [0066] paragraph [0072] paragraph [0073] paragraph [0083] paragraph [0089] - paragraph [0090] paragraph [0091] -----	1-34
A	WO 2009/144079 A1 (BAYER BIOSCIENCE NV [BE]; BAYER CROPSCIENCE AG [DE]; BUSCH MARCO [DE];) 3 December 2009 (2009-12-03) abstract page 5, line 8 - line 33 page 13, line 9 - line 30 ----- -/-	1-34



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 November 2013

Date of mailing of the international search report

11/02/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Mundel, Christophe

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/059598

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/053557 A1 (MS TECHNOLOGIES LLC [US]; SEKAR VAITHILINGAM [US]; HELD BRUCE [US]; CH) 5 May 2011 (2011-05-05) the whole document -----	1-34

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/059598

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☒

in the international application as filed

☐

together with the international application in electronic form

☐

subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/059598

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-34(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-34(partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for tryptophan, glycine or serine at a position corresponding to amino acid 188 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for tryptophan, glycine or serine at a position corresponding to position 188 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

2. claims: 1, 3, 6-17, 22-34(all partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for serine, cysteine or arginine at a position corresponding to amino acid 189 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for serine, cysteine or arginine at a position corresponding to position 189 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

3. claims: 1-34(partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for proline, serine, histidine, alanine, glycine or glutamine at a position corresponding to amino acid 335 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for proline,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

serine, histidine, alanine, glycine or glutamine at a position corresponding to position 335 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

4. claims: 1-34(partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for serine or tryptophan at a position corresponding to amino acid 336 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for serine or tryptophan at a position corresponding to position 336 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

5. claims: 1-4, 6-20, 22-34(all partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for threonine, alanine or serine at a position corresponding to amino acid 339 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for threonine, alanine or serine at a position corresponding to position 339 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

6. claims: 1-4, 6-20, 22-34(all partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for glutamine, alanine,

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

valine or glutamic acid at a position corresponding to amino acid 340 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for glutamine, alanine, valine or glutamic acid at a position corresponding to position 340 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

7. claims: 1, 3, 6-17, 22-34(all partially)

A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme having at least 55% sequence identity to SEQ ID NO: 1 and having at least an amino acid substitution for leucine at a position corresponding to amino acid 215 of SEQ ID NO: 1. A vector, host cell, transgenic plant or transgenic seed comprising said nucleic acid. A recombinant polypeptide comprising an HPPD enzyme having at least 55% sequence identity to SEQ ID NO: 1 and comprising an amino acid substitution for leucine at a position corresponding to position 215 of SEQ ID NO: 1. Use of the polypeptide/polynucleotide for the generation of transgenic plants tolerant to an HPPD inhibitor herbicide. A commodity product comprising a detectable amount of the nucleic acid or the polypeptide. A method of controlling weeds in a field comprising the transgenic plant.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2013/059598

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EP 2453012	A1	16-05-2012	EP 2453012 A1 16-05-2012
			EP 2669369 A1 04-12-2013
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			EP 2669373 A1 04-12-2013

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