HPPD VARIANTS AND METHODS OF USE

Abstract: In the present invention, HPPD enzymes and plants containing them showing a full tolerance against several classes of HPPD-inhibitors are described. A set of HPPD enzymes have been designed which have either no or only a significantly reduced affinity to HPPD inhibitors and, at the same time, the rate of dissociation of the HPPD inhibitors of the enzyme is increased to such an extent that the HPPD inhibitors no longer act as slow-binding or slow, tight-binding inhibitors but, instead of this, have become fully reversible inhibitors. 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 APPLICATIONS

This application is a PCT Application of US Provisional Application No. 61/951,455, filed March 11, 2014, the contents of which are incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file name of "APA146008_ST25", created on March 9, 2015, and having a size of 138 kilobytes. This sequence listing is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

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

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. 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.

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 (Ruetschi et al, Eur. J. Biochem., 205, 459-466, 1992, WO96/38567), Kordia (WO201 1/076889) Synchococcus (WO201 1/076877), and Rhodococcus (WO201 1/076892), of protists such as Blepharisma (WO201 1/076882), of

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-[(2,2,2,2,-tri-
fluoroethoxy)methyl] benzoyl]-1,3-cyclo-hexanedione]; tefuryltrione [i.e. 2-[2-chloro-4-(methylsulfonyl)-3-[[tetrahydro-2-furanyl]methoxy]methyl]benzoyl]- 1,3-cyclohexanedione];
bicyclopypyrone [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];
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;

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

4) the pyrazolinates, 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-trifluoromethyl)phenyl)methanone]; pyrazofen [i.e. 2-[4-(2,4-dichlorobenzoyl)-1,3-dimethylpyrazol-5-yl]oxy]acetophenone];

5) N-(1,2,5-oxadiazol-3-yl)benzamides (WO2011/035874) and N-(1,3,4-oxadiazol-2-yl)benzamides (WO2012/126932), eg. 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (hereinafter also named "Cmpd. 1");

6) N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides (WO2012/028579), eg. 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (hereinafter also named "Cmpd.2"); 4-(difuoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (hereinafter also named "Cmpd. 3"); 2-chloro-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (hereinafter also named "Cmpd. 4"); 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (hereinafter also named "Cmpd. 5");

7) Pyridazinone derivatives as described in WO2013/050421 and WO2013/083774;

8) Substituted 1,2,5-oxadiazoles as described in WO2013/072300 and WO2013/072402; and

9) Oxoprazin derivatives as described in WO2013/054495.
These HPPD inhibitor 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, 7501-7507; Pallett et al. (2001), Pest Management Science, Vol 57, 133-142). In order to extend the scope of use of these HPPD inhibitor 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 (W096/38567). Overexpression of HPPD resulted in better pre-emergence tolerance to the diketonitrile derivative (DKN) of isoxaflutole (IFT), but the tolerance level 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 inhibitor herbicides belonging to the diketonitriles family (W099/24585). Pro215Leu, Gly336Glu, Gly336Ile, and more particularly Gly336Trp (positions of the mutated amino acid are indicated with reference to the Pseudomonas fluorescens HPPD) were identified as mutations which are responsible for an increased tolerance to treatment with these diketonitrile herbicides.

More recently, introduction of a Pseudomonas fluorescens 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(l): 118-33).
In WO2004/024928, the inventors sought to increase the prenylquinone biosynthesis (e.g., synthesis of plastoquinones, tocoherols) 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 prephenate dehydrogenase (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 WO2009/144079, nucleic acid sequences encoding an hydroxyphenylpyruvate dioxygenase (HPPD) with specific mutations at position 336 of the Pseudomonas fluorescens HPPD protein and their use for obtaining plants which are tolerant to HPPD inhibitor herbicides was disclosed.

In WO2002/046387, 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 WO2008/150473, 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 US201/0173718, 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 WO201/094199 and US201/0185444, 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 2453012, several
mutants of HPPD have been described; however, the improved tolerance of the described
mutants was not demonstrated in planta against several HPPD inhibitor herbicides.

The currently described and partly commercialized HPPD inhibitor herbicides act as
slow-binding or slow, tight-binding inhibitors (see Morrison (1982) Trends Biochem. Sci. 7,
102-105). These inhibitors bind slowly (i.e., they have slow rates of association, kon) but
noncovalently to the HPPD enzyme (i.e., they produce time-dependent inhibition), and are
released very slowly (i.e., they have exceptionally slow rates of dissociation, koff) due to their
exceedingly tight interaction with the enzyme.

These inhibitors bind so tightly that stoichiometric titrations with the enzyme are possible.

It has become increasingly recognized that the slow-binding or slow, tight-binding
inhibitors are not only extraordinary potent HPPD-inhibitors, but, in addition, have features that
make them attractive agrochemicals for weed control. The slow rate of dissociation enhances an
inhibitor effectiveness to such an extent that ideally only one inhibitor molecule per enzyme
active site is sufficient to fully inhibit the enzyme’s activity and to maintain this level of
inhibition for a long time period even in the absence of free inhibitor molecules in the plant cell.
This translates into low application rates of these inhibitors to control undesired weeds in crop
growing areas.

The properties of slow-binding or slow, tight-binding inhibitors are advantageous when
achieving HPPD inhibition and herbicidal activity is the goal. However, these properties are a
major disadvantage when HPPD enzymes tolerant to these inhibitors are to be designed.
Mutations in the HPPD enzyme that solely reduce the affinity of the inhibitor to the enzyme (pI50) do not fully overcome HPPD inhibition since binding of the inhibitor and inhibition of the HPPD enzyme can still take place and, therefore, the achieved level of inhibition will be maintained for a long time period even in the absence of free inhibitor in the plant cell.

Due to the above described kinetic properties of all the currently described and partly commerczialized HPPD inhibitor herbicides, up to now, no HPPD-inhibitor tolerant plants with full tolerance against HPPD-inhibitor herbicides have been achieved, despite the many efforts to generate them.

SUMMARY OF INVENTION

In the present invention, HPPD enzymes and plants containing them showing a full tolerance against several classes of HPPD-inhibitors are described. To obtain a high level of inhibitor tolerance, mutants were generated which show an increase in the rate of dissociation (koff) of a slow-binding or slow, tight-binding inhibitor. In some embodiments, reduction of the affinity of an inhibitor to the HPPD enzyme (pI50) and an increase of the rate of dissociation of the inhibitor off the HPPD enzyme (koff) was also achieved simultaneously in the mutant enzyme. Thus, in the present invention, a set of HPPD enzymes was designed which have either no or only a significantly reduced affinity to HPPD inhibitors and, at the same time, the rate of dissociation of the HPPD inhibitors of the enzyme is increased to such an extent that the HPPD inhibitors no longer act as slow-binding or slow, tight-binding inhibitors but, instead of this, have become fully reversible inhibitors.

In the present invention compositions and methods for obtaining HPPD enzymes having the aforementioned characteristics (i.e no or only a significantly reduced affinity to HPPD inhibitors, increased rate of dissociation of the HPPD inhibitors of the enzyme; HPPD inhibitors no longer act as slow-binding or slow, tight-binding inhibitors but have become fully reversible inhibitors) are provided. Compositions include HPPD 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 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
topolypeptides, including nucleic acid molecules encoding a Pseudomonas fluorescens HPPD
protein having a proline at the amino acid position corresponding to amino acid position 335 of
SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position corresponding to amino acid
position 336 of SEQ ID NO: 1 and, optionally, one or more amino amino acid substitutions at the
positions corresponding to amino acid positions 188, 200, 226, 339, and 340 of SEQ ID NO:1,
including the HPPD protein set forth in any of SEQ ID NO: 11-21 as well as fragments thereof.

Also encompassed are the nucleic acid molecules encoding the HPPD protein of the present
invention, including SEQ ID NO: 40, 41, 42, 43, or 44.

Compositions also comprise transformed plants, plant cells, tissues, 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 plants, plant cells, tissues, and seeds. 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
additional 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 encoding an HPPD according to the invention can be grown in a field and
harvested to obtain a plant product. The compositions of the invention are also useful 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 (Pf, SEQ ID NO:1), Avena sativa (SEQ ID NO:23),
a variant of the HPPD from Avena sativa (SEQ ID NO:24), Zea mays (SEQ ID NO:25),
Streptomyces avermitilis (SEQ ID NO:29), Arabidopsis thaliana (SEQ ID NO:26), Hordeum
vulgare (SEQ ID NO:27), Daucus carota (SEQ ID NO:28), Mycosphaerella graminicola (SEQ
ID NO:30), and Coccicoides immitis (SEQ ID NO:31), Axmi428H (SEQ ID NO:6) and
Axmi309H (SEQ ID NO:5).

Figure 2A shows an example of time dependent inhibition of a time dependent HPPD
mutant enzyme, in presence of 1 µM HPPD inhibitor. Figure 2B shows an example of reversible
inhibition of an reversible HPPD mutant enzyme in presence of 10 µM inhibitor.

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.

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 (W096/38567), 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.

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 pyrazolinites (e.g., topramezone and pyrasulfotole), N-(1,2,5-Oxadiazol-3-yl)benzamides (WO2011/035874), N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides (WO2012/028579), pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495).

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-(l-methyl-lH-tetrazol-5-yl)benzamide and 2-Chloro-3-(methoxymethyl)-4-(methylsulfonyl)-N-(l-methyl-lH-tetrazol-5-yl)benzamide; N-(1,3,4-oxadiazol-2-yl)benzamides, such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(l-methyl-lH-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(l-methyl-lH-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfonyl)-N-(l-methyl-lH-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), 2-(methoxymethyl)-3-(methylsulfinyl)-N-(l-methyl-lH-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles such as isoxaflutole; or of the class of pyrazolinites, such as pyrasulfotole and topramezone, have an outstanding herbicidal activity against a broad spectrum of economically important monocotyledonous and dicotyledonous annual harmful 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, bactericides, nematicides, 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-y1)- or N-(triazol-3-yl)arylcarboxamides, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfanyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd.2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), and 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolines, preferably such as pyrasulfotole and topramezone. The HPPD inhibitor herbicide tolerance gene of the invention may also show
tolerance towards the "coumarone-derivative herbicides" (described in WO2009/090401, WO2009/090402, WO2008/009908). 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 WO2011/100302, which is herein incorporated by reference in its entirety.

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. In various embodiments, the HPPD gene of the invention is selected from SEQ ID NO:40, 41, 42, 43, or 44. 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 (HPP) is transformed into homogentisate. The catalytic activity of an HPPD enzyme may be defined by various methods well-known in the art. WO2009/144079 describes various suitable screening methods.

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 \( \text{C}_0 \) substrates, or to measure the accumulation of any of the products derived from the enzymatic reaction, i.e., homogentisate or \( \text{C}_0 \). In particular, the HPPD activity can be 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 HPPD nucleic acid of the invention is being compared. For the purposes of describing the HPPD proteins of the present invention, the terms "protein" and "polypeptide" are used interchangeably. 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 as the PfP215L and PfG336F mutants described in International Patent Publication WO2009/144079 and set forth herein as SEQ ID NO:20 and 2, respectively, or can be either of the PfHPPDevo33, PfHPPDevo36, PfHPPDevo37, PfHPPDevo40, or PfHPPDevo41, Axmi309H, Axmi428H, Axmi309H-Evo4 1, or Axmi428H-Evo4 1 proteins set forth herein as SEQ ID NO:22, 37, 38, 4 3, 5, 6, 10, and 8, respectively, which are also described in International Patent Application No, PCT/US20 13/59598, filed September 13, 2013, and which is herein incorporated by reference. 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 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 protein encoded by a 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 protein encoded by 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)arylcaboxamides, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcaboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), and 2-(methoxymethyl)-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolines, preferably such as pyrasulfotole and topramezone) has a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 336 of SEQ ID NO:1 and, optionally, one or more amino acid substitutions at the positions corresponding to amino acid positions 172, 188, 200, 226, 339, and 340 of SEQ ID NO:1, including the HPPD proteins set forth in any of SEQ ID NO:11-1. 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, 215, 335, 336, 339, and 340 of SEQ ID NO:1 correspond to amino acid positions 241, 271, 412, 413, 416, and 417, respectively, of the HPPD from Avena sativa (SEQ ID NO:23); to amino acid positions 235, 265, 406, 407, 410, and 411, respectively, of the HPPD from Hordeum vulgare (SEQ ID NO:27) to amino acid positions 242, 272, 413, 414, 417, and 418, respectively, of the HPPD from Zea mays (SEQ ID NO:25), to amino acid positions 209, 236, 351, 352, 355, and 356 of Axmi428H (SEQ ID NO:6). An alignment of numerous HPPD
amino acid sequences from various species is also found in Tables 2a and 2b of European Patent Publication No. EP2453012, which is herein incorporated by reference. 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 172, 188, 200, 226, 335, 336, 339, and 340 in such concerned HPPD protein.

In one embodiment, the HPPD of the present invention (including the nucleotide sequence encoding it and recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleotide sequence encoding the HPPD of the invention) consists of an amino acid sequence comprising a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 336 of SEQ ID NO: 1.

In another embodiment, the HPPD of the present invention (including the nucleotide sequence encoding it and recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleotide sequence encoding the HPPD of the invention) consists of an amino acid sequence comprising a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 336 of SEQ ID NO: 1 and

(a) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO: 1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO: 1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO: 1;

(b) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO: 1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO: 1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO: 1;

(c) a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO: 1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO: 1;
(d) a glycine at the amino acid position corresponding to amino acid position 172 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1 and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1, an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1.

In another embodiment, the HPPD of the present invention (including the nucleotide sequence encoding it and recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleotide sequence encoding the HPPD of the invention) consists of an amino acid sequence comprising

(a) a tyrosine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, a glycine at the position corresponding to amino acid position 339 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(b) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1, a serine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, an alanine at the amino acid position corresponding to amino acid position 335 of SEQ ID NO:1, a tyrosine at the amino acid position corresponding to amino acid position 336 of SEQ ID NO:1, and an alanine at the position corresponding to amino acid position 340 of SEQ ID NO:1; and

(c) 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 a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1.
In another embodiment, the HPPD of the present invention (including the nucleotide sequence encoding it and recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts, and seeds comprising the nucleotide sequence encoding the HPPD of the invention) consists of an amino acid sequence comprising a proline at the amino acid position corresponding to amino acid position 351 of SEQ ID NO:6 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 352 of SEQ ID NO:6 and

(a) an alanine at the amino acid position corresponding to amino acid position 209 of SEQ ID NO:6, a histidine at the amino acid position corresponding to amino acid position 247 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6;

(b) an isoleucine at the amino acid position corresponding to amino acid position 221 of SEQ ID NO:6, a histidine at the amino acid position corresponding to amino acid position 247 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6;

(c) a histidine at the amino acid position corresponding to amino acid position 247 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6;

(d) a glycine at the amino acid position corresponding to amino acid position 193 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6;

(e) an isoleucine at the amino acid position corresponding to amino acid position 221 of SEQ ID NO:6 and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6; or

(f) an alanine at the amino acid position corresponding to amino acid position 209 of SEQ ID NO:6, an isoleucine at the amino acid position corresponding to amino acid position 221 of SEQ ID NO:6, a histidine at the amino acid position corresponding to amino acid position 247 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6.

In another embodiment, the HPPD of the present invention (including the nucleotide sequence encoding it and recombinant and chimeric genes thereof, vectors, host cells, plants, plant parts,
and seeds comprising the nucleotide sequence encoding the HPPD of the invention) consists of an amino acid sequence comprising

(a) a tyrosine at the amino acid position corresponding to amino acid position 352 of SEQ ID NO:6, a glycine at the position corresponding to amino acid position 355 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6;

(b) an alanine at the amino acid position corresponding to amino acid position 209 of SEQ ID NO:6, a serine at the amino acid position corresponding to amino acid position 221 of SEQ ID NO:6, a histidine at the amino acid position corresponding to amino acid position 247 of SEQ ID NO:6, an alanine at the amino acid position corresponding to amino acid position 351 of SEQ ID NO:6, a tyrosine at the amino acid position corresponding to amino acid position 352 of SEQ ID NO:6, and an alanine at the position corresponding to amino acid position 356 of SEQ ID NO:6; and

(c) a proline at the amino acid position corresponding to amino acid position 351 of SEQ ID NO:6, a tryptophan at the amino acid position corresponding to amino acid position 352 of SEQ ID NO:6, and a glycine at the position corresponding to amino acid position 356 of SEQ ID NO:6.

The relevant amino acid position of the reference HPPD proteins and of the HPPD proteins according to the invention comprising one or more amino acid substitutions are summarized in Table 1.

Table 1. Amino acid substitutions of the reference HPPD proteins and the HPPD proteins according to the invention relative to SEQ ID NO:1, also containing the respective SEQ ID NO. In case of open boxes, the wild-type amino acid sequence (PfHPPD) is present at this position.
In another embodiment, HPPD proteins according to the invention has at least 53%, at least 60%, at least 65%, at least 70%, 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, 5, or 6.

Exemplary HPPD sequences that can be modified according to the present invention include those from bacteria, for example, of the Pseudomonas sp. type, or otherwise cyanobacteria, for example of the Synechocystis genus. The sequence can also be of plant origin, in particular derived from dicotyledonous plants or monocotyledonous plants. Advantageous examples which may be cited are plants such as tobacco, Arabidopsis thaliana (WO96/38567), Daucus carota (WO96/38567), Zea mays (corn, WO2012/021785), wheat (Triticum aestivum, WO2002/046387), barley (EP2453012), Avena sativa (WO2002/046387/WO201 1/068567), Brachiaria platyphylla (WO2002/046387), Cenchrus echinatus (WO2002/046387), Lolium rigidum (WO2002/046387), Festuca arundinacea (WO2002/046387), Setaria faberi (WO2002/046387), Eleusine indica (WO2002/046387), or Sorghum (WO2002/046387, WO2012/021785). In a particular embodiment of the invention, the HPPD that can be modified according to the present invention is from a bacterial or protist origin, particularly from Pseudomonas sp., more particularly from Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas testosteroni (Comamonas testosteroni), Rhodococcus

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<th>Amino acid positions in Axmi309H</th>
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<td>Axmi309H-Evo41</td>
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sp. (WO201 1/076892), Blepharisma japonicum (WO201 1/076882), Synechococcus sp. (WO201 1/076877), Kordia algicida (WO201 1/076889), from the euryarchaeote Picrophilus torridus (WO201 1/076885), or from a plant origin, including from 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 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 any of SEQ ID NO:2-10, 22, 37 or 28, the variant HPPD from Avena sativa set forth herein as SEQ ID NO:24, 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 WO201 1/068567, which is herein incorporated by reference in its entirety; 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 WO2009/144079 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 WO201 1/068567.

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, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triadiazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4); 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO20 13/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolinates, preferably such as pyrasulfotole and topramezone) encodes the amino acid sequence set forth in any one of SEQ ID NO: 11-21, and fragments and variants thereof that encode a HPPD inhibitor herbicide tolerance polypeptide.

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, for 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, corn, 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 inhibitor herbicides (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, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), 2-(methoxymethyl)-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolines, preferably such as pyrasulfotole and topramezone) 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, a *Pseudomonas fluorescens* HPPD-encoding DNA, under control of the same promoter as the nucleic acid encoding the HPPD protein of the invention. Accordingly, the term "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 1x, 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 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 plso (plso-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 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, 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 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.

**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 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. 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 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™ lightening mutagenesis kit from Qiagen or Stratagene). 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 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.

C. Isolated polynucleotides, and variants and fragments thereof

In some embodiments, the present invention comprises isolated or recombinant, polynucleotides. A "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, a recombinant 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, a "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. In some embodiments, the nucleic acid molecule of the invention is operably linked to a promoter capable of directing expression of the nucleic acid molecule in a host cell (e.g., a plant host cell or a bacterial host cell).

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: 11-21. 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: 11-21. 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 lx, 2x, 3x, 4x, or higher dose of an
HPPD inhibitor herbicide, or the fragments will be as or more tolerant based on pI50 or Ki
between the fragment and SEQ ID NO: 11-21.

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 HPPD protein having a proline at the amino acid position corresponding to
amino acid position 335 of SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position
corresponding to amino acid position 336 of SEQ ID NO: 1 and, optionally, one or more amino
acid substitutions at the positions corresponding to amino acid positions 172, 188, 200, 226, 339, and 340 of SEQ ID NO: 1., including the HPPD protein set forth in any of SEQ ID
NO: 1 1-21.

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 53%, 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.

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
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 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 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, 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.

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 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 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 $K_m$, $K_i$, $k_{cat}$), protein stability, protein thermostability, or protein temperature and pH optimum.

D. **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 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.

"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 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 53%, 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:1 1-21, wherein said variant has HPPD enzyme activity and HPPD inhibitor 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:11-21 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:11-21, or a fragment thereof. In some embodiments, the antibody specifically binds to the region of the protein corresponding to amino acid position 178 of SEQ ID NO:1, or the region of the protein corresponding to amino acid position 188 of SEQ ID NO:1, or the region of the protein corresponding to amino acid position 200 of SEQ ID NO:1, or the region of the protein corresponding to amino acid position 226 of SEQ ID NO:1, or the region of the protein corresponding to amino acid positions 335-340 of SEQ ID NO:1. In other embodiments, the antibody specifically binds to the region of the protein corresponding to amino acid position 193 of SEQ ID NO:6, or the region of the protein corresponding to amino acid
position 209 of SEQ ID NO:6, or the region of the protein corresponding to amino acid position 221 of SEQ ID NO:6, or the region of the protein corresponding to amino acid position 247 of SEQ ID NO:6, or the region of the protein corresponding to amino acid positions 351-356 of SEQ ID NO:6.

5 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.

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E. 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 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 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 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 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 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 confer tolerance to certain diseases, DNAs that encodes RNAs that provide nematode or insect control, and the like.


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 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 (WO2004/074443), and which is described in Patent Application US 6566587, hereinafter named double mutant maize EPSPS or 2mEPSPS, or the gene which
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 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) inhibitor (WO2007/024782); 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 metabolization (U.S. Patent 6,153,401); and, genes encoding Dicamba monooxygenases conferring tolerance to dicamba (3,6-dichloro-2-methoxybenzoic acid) by metabolization (US 2008/01 19361 and US 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 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 (WO97/17432 & WO98/08932).

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 CryIF protein or hybrids derived from a CryIF protein (e.g., the hybrid CryIA-CryIF proteins
described in US 6,326,169; US 6,281,016; US 6,218,188, or toxic fragments thereof), the Cryl A-type proteins or toxic fragments thereof, preferably the Cry1Ac protein or hybrids derived from the Cryl Ac protein (e.g., the hybrid Cryl Ab-Cryl Ac protein described in US 5,880,275) or the Cryl Ab or Bt2 protein or insecticidal fragments thereof as described in EP45 1878, the Cry2Ae, Cry2Af or Cry2Ag proteins as described in WO2002/057664 or toxic fragments thereof, the Cry1 A.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 WO 2007/142840), the VIP3A proteins produced in the COT202 or COT203 cotton events (WO2005/054479 and WO2005/054480, respectively), the Cry proteins as described in WO2001/47952, the VIP3Aa protein or a toxic fragment thereof as described in Estruch et al. (1996), Proc Natl Acad Sci U S A. 28;93(1 1):5389-94 and US 6,291,156, the insecticidal proteins from Xenorhabdus (as described in 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(1 1):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 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 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 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-GHK04 (cotton, insect control, described in WO2002/040677), Event 1143-14A (cotton, insect control, not deposited, described in WO2006/128569); Event 1143-5 IB (cotton, insect control, not deposited, described in WO2006/128570); Event 1445 (cotton, herbicide tolerance, not deposited, described in US-A 2002-120964 or WO2002/034946 Event 17053 (rice, herbicide
tolerance, deposited as PTA-9843, described in WO2010/17737; Event 17314 (rice, herbicide tolerance, deposited as PTA-9844, described in WO2010/17735); Event 281-24-236 (cotton, insect control - herbicide tolerance, deposited as PTA-6233, described in WO2005/103266 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 WO2005/03266); Event 3272 (corn, quality trait, deposited as PTA-9972, described in WO2006/098952 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-1 1508, described in WO 11/075593); Event 43A47 (corn, insect control - herbicide tolerance, deposited as ATCC PTA-1 1509, described in WO2011/075595); Event 5307 (corn, insect control, deposited as ATCC PTA-9561, described in WO2010/077816); Event ASR-368 (bent grass, herbicide tolerance, deposited as ATCC PTA-4816, described in US-A 2006-162007 or WO2004/053062); 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 WO2010/080829); 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 WO2006/128573); Event CE44-69D (cotton, insect control, not deposited, described in US-A 2010-0024077); Event CE44-69D (cotton, insect control, not deposited, described in WO2006/128571); Event CE46-02A (cotton, insect control, not deposited, described in WO2006/128572); Event COT102 (cotton, insect control, not deposited, described in US-A 2006-130175 or WO2004/039986); Event COT202 (cotton, insect control, not deposited, described in US-A 2007-067868 or WO2005/054479); Event COT203 (cotton, insect control, not deposited, described in WO2005/054480); Event DAS2 1606-3/1606 (soybean, herbicide tolerance, deposited as PTA-1 1028, described in WO2012/033794), Event DAS40278 (corn, herbicide tolerance, deposited as ATCC PTA-10244, described in WO2011/022469); Event DAS-44406-6/pDAB8264.44.06.1 (soybean, herbicide tolerance, deposited as PTA-1 1336, described in WO2012/075426), Event DAS-14536-7/pDAB829 1.45.36.2 (soybean, herbicide tolerance, deposited as PTA-1 1335, 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
tolerance, not deposited, described in WO2002/036831 or US-A 2008-070260); Event
SYHT0H2 / SYN-000H2-5 (soybean, herbicide tolerance, deposited as PTA-1 1226, described in
WO2012/082548), Event T227-1 (sugar beet, herbicide tolerance, not deposited, described in
WO2002/44407 or US-A 2009-265817); Event T25 (corn, herbicide tolerance, not deposited,
described in US-A 2001-029014 or WO2001/05 1654); Event T304-40 (cotton, insect control -
herbicide tolerance, deposited as ATCC PTA-8171, described in US-A 2010-077501 or
WO2008/122406); Event T342-142 (cotton, insect control, not deposited, described in
WO2006/128568); Event TC1507 (corn, insect control - herbicide tolerance, not deposited,
described in US-A 2005-039226 or WO2004/099447); Event VIP1034 (corn, insect control -
herbicide tolerance, deposited as ATCC PTA-3925., described in WO2003/052073), Event
32316 (corn, insect control-herbicide tolerance, deposited as PTA-1 1507, described in
WO2011/084632), Event 4114 (corn, insect control-herbicide tolerance, deposited as PTA-
11506, described in WO2011/084621), event EE-GM3 / FG72 (soybean, herbicide tolerance,
ATCC Accession N° PTA-1 1041) optionally stacked with event EE-GM1/LL27 or event EE-
GM2/LL55 (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-1 1508, WO2011/075593A1), event DP-043A47-3
(corn, insect control, ATCC Accession N° PTA-1 1509, WO2011/075595A1), event DP-
004114-3 (corn, insect control, ATCC Accession N° PTA-1 1506, WO2011/084621A1),
event DP-032316-8 (corn, insect control, ATCC Accession N° PTA-1 1507,
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-1 1028, WO2012/033794A2), event MON-87712-4 (soybean, quality trait,
ATCC Accession No. PTA-1 10296, WO2012/05 1199A2), event DAS-44406-6 (soybean, stacked
herbicide tolerance, ATCC Accession N°. PTA-1 1136, WO2012/075426A1), event DAS-14536-
7 (soybean, stacked herbicide tolerance, ATCC Accession N°. PTA-1 1135,
PTA-1 1226, WO2012/082548A2), event DP-061061-7 (oilseed rape, herbicide tolerance, no
deposit N° available, WO2012/071039A1), event DP-073496-4 (oilseed rape, herbicide tolerance,
no deposit N° available, US2012/131692), event 8264.44.06.1 (soybean, stacked herbicide

F. Polynucleotide Constructs

The polynucleotides encoding the HPPD polypeptides of the present invention 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' 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 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 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 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 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 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. 15:2343-2361. 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 (PC1SV) 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 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) EMBOJ. 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); Brassica napus ALS3 (PCT application WO97/41228); a plant ribulose-bisphosphate/oxigenase (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).

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 Al), the phaseolin promoter, the glutenin promoter, the helianthinin promoter (WO92/17580), the albumin promoter (WO98/45460), the oleosin promoter (W098/45461), 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 (W098/45445). 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 PC1SV 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 WO87/07644, or of the tobacco etch virus (TEV) described by Carrington & Freed 1990, J. Virol. 64: 1590-1597, for example, or introns such as the adhl intron of maize or intron 1 of rice actin. See also PCT WO96/23898, WO20 12/02 1794, WO20 12/02 1797, WO201 1/084370, and WO201 1/028914.

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.


In one aspect of the invention, synthetic DNA sequences are designed for a 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 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, creation of a DNA sequence that in 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 host-preferred codon usage frequency. See, for example, Campbell and Gowri (1990) \textit{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 \textit{et al.} (1989) \textit{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.
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-rnQdiated 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.

G. Plant Transformation


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.

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 91:7301-7305.

The plant 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 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") 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.

**H 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.

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 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 $^{32}$P target DNA fragment to confirm the integration of the introduced gene in the plant genome according to standard techniques (Sambrook and Russell, 2001, supra).
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 known in the art (Sambrook and Russell (2001) supra). RNA can also be detected and/or quantified using reverse transcriptase PCR as known in the art (e.g., Green and Sambrook (2012) Molecular Cloning: A Laboratory Manual, 4th Edition, Cold Spring Harbor Laboratory Press, Woodbury, NY).

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 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.

I. Use as a marker for transformation

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 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.

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 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.

J. 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, 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, 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 *Curcumin* such as cucumber, cantaloupe, and 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.

K. 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 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 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 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 inhibitor 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 N-(triazol-3-yl)arylcaboxamides, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcaboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-1-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), and 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyrazolinates, preferably such as pyrasulfotole and topramezone, 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 class of N (1,2,5-oxadiazol-3-yl)benzamides; N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide and 2-Chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolinates, preferably such as pyrasulfotole and topramezone) 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 tolerant to the herbicide.

L. 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 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, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), and 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolinates, preferably such as pyrasulfotole and topramezone, are applied to the plants (for example harmful plants such 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, preferably 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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), and 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione
and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolinates, preferably such as pyrasulfotole and topramezone, the class of isoxazoles preferably such as isoxaflutole, or of the class of pyrazolinates, preferably 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. WO98/20144. 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.


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; N-(1,3,4-oxadiazol-2-yl)benzamides, preferably such as 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide (Cmpd. 1); N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, preferably such as 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 2), 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide (Cmpd. 3), 2-chloro-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 4), 2-(methoxymethyl)-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide (Cmpd. 5); pyridazinone derivatives (WO2013/050421 and WO2013/083774); substituted 1,2,5-oxadiazoles (WO2013/072300 and WO2013/072402); and oxoprazin derivatives (WO2013/054495); triketones, preferably such as tembotrione, sulcotrione and mesotrione; the class of isoxazoles preferably such as isoxaflutole; or of the class of pyrazolinates, preferably such as pyrasulfotole and topramezone, 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.


Based on these formulations, it is also possible to prepare combinations with 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.

**M. Methods of introducing gene of the invention into another plant**

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.
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).

N. 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, 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.

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

EXPERIMENTAL

Example 1. Mutagenesis of Axmi428H

Axmi428H (described in International Patent Application No. PCT/US2013/59598, filed September 13, 2013 and set forth herein as SEQ ID NO:6) was chosen as a template for mutagenesis based on its high resistance to tembotrione (TBT), and homology to a Pseudomonas fluorescens enzyme (W09638567A3) that was previously shown to possess TBT tolerance (PfG336W; see WO1999024585 and WO2009144079). The PfG336W substitution was engineered into the native Pseudomonas HPPD to improve tolerance to TBT; the tolerance is proposed to be mediated by the larger tyrosine side chain crowding out the bulky TBT inhibitor in the active site of the enzyme.

Amino acids near the active site of HPPD Axmi428H were selected for mutagenesis based on structural modeling. Individual amino acids were then fully randomized, and the randomized proteins were screened for activity in the presence of TBT using a colorimetric assay in E. coli (based on production of pyomelanin by Axmi428H mutants), and were further screened
by an enzymatic assay that quantifies the product formation by HPPD enzymes. Enzymes with improved tolerance to TBT were identified by this approach. The three-dimensional structure of Pseudomanas HPPD was studied to identify residues in the active site that were likely to be involved in substrate binding.

**Generation of Axmi428H genes that encode randomized amino acids**

A nucleotide sequence encoding the HPPD enzyme Axmi428H (SEQ ID NO: 6) was cloned into an E. coli expression vector (based on pRSFl-b) that guides overexpression of Axmi428H in E. coli cell lines (such as BL21*DE3) following induction with IPTG. A QUIKCHANGE® Lightning Site directed mutagenesis kit (Stratagene cat # 210519) was used to guide site directed mutagenesis of Axmi428H in plasmid pSE420 using primers that were designed for the mutagenesis.

**Primary screening of randomized Axmi428H enzymes using pyomelanin assay**

The Axmi428H proteins containing randomized amino acid positions were evaluated for their HPPD activity using a solid-phase, growth-based assay. Briefly, overexpression of HPPD enzymes (in E. coli) on rich growth medium leads to the production of the compound pyomelanin. Pyomelanin is deep brown in color, therefore, inspection of HPPD enzymes grown on LB agar in a multi-well plate allows visual comparison of the enzymatic activity of HPPD enzymes. This experimental approach also allows varying concentrations of HPPD inhibitors (such as tembotrione) to be added to individual sample wells, which allows visual assessment of the tolerance of HPPD inhibitors by the randomized HPPD enzymes.

**Secondary screening of randomized Axmi428H enzymes using in vitro kinetic assay**

The Axmi428H mutants selected during primary screening (pyomelanin assay) were promoted to a secondary screen that utilized an HPPD in vitro kinetic assay. The in vitro kinetic assay couples the production of Homogentisic acid with the enzyme Homogentisate 1,2-dioxygenase (HGO). HGO converts homogentisic acid to maleoacetoacetate, which is easily quantified by UV absorption at 321nm. The assay is carried out in real-time, and product can be quantified continuously in a 96-well spectrophotometer.
The kinetic assay was performed in two stages. In the first stage, enzymes were evaluated under 3 conditions: 1) HPPD activity was quantified under conditions of high substrate concentration (500 µM HPPD) and the rate obtained under this condition was a measure of catalytic activity (Vmax) of the enzyme; 2) HPPD activity was quantified under high substrate, with addition of a single concentration of the tembotrione inhibitor (500 µM HPP + 5 µM TBT). Comparison of the enzymatic rate with tembotrione relative to the rate without tembotrione (500 µM HPP only) was used to measure the resistance to the inhibitor tembotrione, which is an indication of the inhibitor binding constant (Ki); 3) HPPD activity was quantified under limiting substrate conditions (125 µM HPP). Comparison of the enzymatic rate at limiting substrate concentration, relative to the rate at high substrate concentration (500 µM HPP), provides a measure of the affinity of the enzyme for the substrate (Km).

Experimentally, the first stage enzymatic assays were performed as follows. Clones for the top Axmi428H mutants identified in the primary screen (pyomelanin assay) were transformed into DH5a, and a loopful of colonies was used to inoculate 25 ml of LB + Kanamycin in a 125 ml flask. The cultures were incubated at 37 degrees C. When the OD reached 0.6 - 0.7, the temperature was decreased to 30 degrees C and the cultures were incubated overnight. Cultures were sampled the next afternoon for the pyomelanin assay and the remaining culture was pelleted at 6500 rpm in an SA600 rotor, the supernatant was removed, and the pellets were frozen overnight at -20 degrees Celsius or until assay.

Just before assay, the pellets were resuspended in 1 ml of buffer (20 mM Hepes pH 7.0, 50 mM NaCl). Two ul of LYSONASE™ (Novagen) was added to each resuspended pellet, and then mixed. Two ml of buffer and 4 ul of LYSONASE™ were then added to the HGO pellet. The suspensions were incubated for 45 minutes at room temperature, and then frozen at -20 degrees for at least 1 hour. Then, the solutions were thawed at room temperature with occasional brief immersion in a 37 degree C water bath and a little agitation. The cultures were spun down for 15 min at 14000 rpm in a Sorvall SA 600 rotor. The supernatants were carefully pipetted off the pellets (with care not to disturb the pellets), and transferred into 1 column of a 96 well plate, and 0.5 ml of buffer was added to each of the samples and pipetted up and down to mix. The HGO supernatant was collected and the reactions were performed in the following order. The HGO was added to the 500 µM HPP solution and the 500 µM solution + 5 µM TBT. Immediately 100 ul of the 500 µM HPP solution was pipetted into even-numbered rows (5 reps).
and 100 µl of the 500 µM HPP + 5 µM TBT solution was pipetted into odd-numbered rows (5 reps). The reactions were divided between two 96-well plates to allow a sufficient number of samples to be analyzed (4 assays total in one 96-well plate, and 6 in another). Then, 100 µl of the enzyme solutions or buffer was pipetted first into a row of wells containing 500 µM HPP+ 5 µM TBT followed by gentle mixing by stirring with the pipette tips, and next into a row of wells containing 500 µM HPP followed by gentle mixing by stirring with the pipette tips. Product formation was measured by absorbance at 321 nm at 5 sec intervals. The reduced values (equivalent to the absorbance change over 50 sec, and calculated as OD/min) were analyzed. Five sets of data were collected. This was then repeated after addition of the HGO to the 125 µM HPP, using the same steps as above.

Individual Axmi428H mutants that performed well in the stage 1 kinetic assays were promoted to deeper characterization using a stage 2 format. As with stage 1, the stage 2 assays utilized an in vitro kinetic assay that coupled the production of Homogentisic acid with the enzyme Homogentisate 1,2-dioxygenase (HGO). HGO converts homogentisic acid to maleoacetoacetate which was easily monitored as it absorbs strongly at 321nm. In stage 2, the real-time production of product was quantified across a broader range of substrate concentrations, and also inhibitor concentrations, to allow a more accurate determination of the Michaelis-Menten binding constant (Km) and the inhibitor binding constant (Ki) to be calculated. The Ki can be determined by graphing the change of this Km in the presence of varying amounts of the inhibitor tembotrione. Alternately, the Ki can be calculated from the IC50 value of the enzyme when adjusted for Km and substrate concentration. This latter technique for calculating Ki was used for the majority of the Axmi428H mutants.

Experimentally, the second stage enzymatic assays were performed as follows. Individual Axmi428H mutant clones were grown as described above, and E. coli extracts were prepared in the same manner. Final assay concentrations were as follows: 20 mM HEPES, pH 7.0, 50 mM NaCl, 0-500 µM HPP, 0-50 µM TBT, and a saturating amount of freshly prepared HGO enzyme (final concentration was approximately 50 ug/ml). Analysis of the kinetic data yielded the kinetic constants. The Km and Ki of the enzymes was calculated using standard Michaelis-Menten kinetic equations. IC50 was calculated at the HPP concentration of 500 µM, and the measured Km was also used to derive Ki using the following formula:
\[ K_i = \frac{IC50}{1 + (K_m/\left[S\right])} \]

For some Axmi428H mutants, the expressed proteins were examined by SDS-PAGE to determine if expression level differences were present. Briefly, 60 ul of each culture was spun down at 9000 for 2 minutes, and the supernatant was removed and the pellet was resuspended in 100 ul 2x NuPage sample buffer (1:1 diln. NuPage LDS 4x sample buffer (NP0007) with water. The samples were heated at 90 degrees Celsius for 10 minutes. A 4-12% Bis-Tris NuPage gel 10 or 12 well (NP0322) in NuPage 1x Mops buffer (NP0002-02) was loaded with 10 ul of Fermentas PAGERULER™ Protein Ladder (Fermentas #SM0661 from VWR) and 20 ul of each sample. The gels were stained with a Coomassie protein stain.

**Results**

The primary and secondary screening approaches (described above) identified several Axmi428H proteins with improved properties. Table 2 summarizes the kinetic assay data (secondary screen) for several of the top Axmi428H enzymes. The amino acid positions in Table 2 and described in this example correspond to the amino acid positions of SEQ ID NO:6. Each mutant shown was a "stack", in which the indicated substitution was added to a parental Axmi428H enzyme containing substitutions at two positions (G352Y, A356G) (SEQ ID NO:1). The enzymatic assay for each protein at 500 µM HPP is shown as milliOD units/minute, while the relative activity at 500 µM HPP + 5 µM TBT, and also 125 µM HPP, are shown as a percentage of the activity at 500 µM HPP.
Table 2. Kinetic assay data

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<th>500 μM HPP + 5 μM TBT (as % of 500 μM HPP, dataset 2)</th>
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<td>42,39</td>
<td>56</td>
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</tr>
<tr>
<td>L357M</td>
<td>11,24</td>
<td>54,45</td>
<td>73,80</td>
<td>57,76</td>
<td>123,42</td>
<td>70,84</td>
<td></td>
</tr>
</tbody>
</table>
Additional stacking of top mutants

Next, the best amino acids identified above were stacked on 2 mutants with particularly strong activity (M226V and Q247H, each combined with "YG" (G352Y, A356G) as separate mutant pools). The substitution E351P was also included in these stacks, as the substitution appeared to improve the Vmax of Axmi428H. The stacked mutants were constructed as previously described, and were screened by the primary (pyomyelin in vivo assay) and secondary (in vitro quick kinetic assay) as described above. The primary and secondary screening data are shown for two of these groups of stacked mutants in Table 3.

<table>
<thead>
<tr>
<th>Table 3. Kinetic data for Axmi428H-YG stacks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Axmi428HEvo40</td>
</tr>
<tr>
<td>Axmi428HEvo41</td>
</tr>
<tr>
<td>PfHPPD Evo40</td>
</tr>
<tr>
<td>PfHPPD Evo41</td>
</tr>
<tr>
<td>Axmi428H YG Q247 A3 His (HYG)</td>
</tr>
<tr>
<td>Axmi428H YG Q247 A3 His (HYG)</td>
</tr>
<tr>
<td>Axmi428H YG Q247H, E351P (HPYG)</td>
</tr>
<tr>
<td>Axmi428H YG E8 (YG)</td>
</tr>
<tr>
<td>Axmi428H YG E351P (PYG)</td>
</tr>
</tbody>
</table>

There were two Axmi428H mutants that performed better than the parent (Axmi428H-YG, SEQ ID NO: 11) in both the pyomelanin and the quick kinetic assay:

• 428H V221I, E351P G352Y, A356G (Axmi428H-IPYG, SEQ ID NO:17)

There were 3 additional mutants that performed better than the parent (Axmi428H-HPYG, SEQ ID NO: 16) in both the pyomelanin and the quick kinetic assay:


Example 2. Mutagenesis of Axmi309H

Some of the 428H mutations were introduced into the corresponding positions in the HPPD enzyme Axmi309H (SEQ ID NO:5), including the E351P and the A356G mutations. It was observed that the Axmi309H protein containing E335P, G336W, and A340G (Axmi309H-PWG, SEQ ID NO:21) in the active site seems the most tolerant to tembotrione in the brown color assay.

Example 3. Analysis of Axmi428H variants using the HGD method

Candidate mutants identified using the brown color assays were selected. Proteins were produced and purified. The activity and tolerance of the enzymes to HPPD inhibitors were evaluated using the HGD method. HPPD proteins were produced and purified as described in WO2011/076882. With the HGD assay, 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 μM FeS0₄, 650 units of catalase, 8 μg HGA dioxygenase (HGA: homogentisate) and 600 μM HPP in a total volume of 1 ml. Initial reaction rates in the absence or presence of inhibitors were determined from the increase in absorbance at 318 nm due to the formation of maleylacetoacetate (ε318 = 11,900 M⁻¹ cm⁻¹). pI50-values (the negative log value of the concentration of inhibitor necessary to inhibit 50% of the enzyme activity in molar concentration were determined from dose-response plots of HPPD activity versus inhibitor concentration tested using the 4 Parameter Logistic Model or Sigmoidal Dose-Response Model of the ID Business Solutions Ltd. XLfit software suite. Due to the UV absorption of the HPPD inhibitors tested, inhibitor concentrations >100 μM could not be tested. The result "<4" means that the inhibition
at 100 µM inhibitor concentration was lower than 25% and the pI50-value could therefore not be precisely calculated within the range of concentration of inhibitor tested. "n.d." means not determined.

The HGD assay allowed to continuously follow HPPD-catalyzed HGA formation over time was therefore used to determine the inhibition type of the HPPD inhibitors tested. When in the presence of an inhibitor the HPPD activity was found to decrease in a time-dependent manner characteristic for slow-binding or slow, tight-binding inhibitors (for a definition see Morrison (1982) Trends Biochem. Sci. 7, 102-105), the inhibitor was called time-dependent (abbreviation "td"). When in the presence of an inhibitor the HPPD activity was inhibited but the inhibition was found not to decrease in a time-dependent manner, the inhibitor was called reversible (abbreviation "rev").

The abbreviation "no-in" means that the type of inhibition could not be determined due to the fact that no inhibition of the corresponding HPPD variant was observed at 100 µM inhibitor concentration.

Inhibitors tested were tembotrione, diketonitrile (active compound of isoxaflutole) and mesotrione, and 3 selected inhibitors from the NO-chelator class. The results are shown in Tables 4-7. The symbol "»" means that is out of range of measurement but highly superior than the number listed thereafter.
Table 4. Tolerance of Axmi428H mutants to diketonitrile (DKN) using HGD assay

<table>
<thead>
<tr>
<th>Clones</th>
<th>SEQ ID NO</th>
<th>Amino acid position in Axmi428H</th>
<th>pIC50</th>
<th>diketonitrile</th>
<th>K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axmi428H</td>
<td>6</td>
<td>M I V Q E G K A</td>
<td>7.0</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-AHPYG</td>
<td>13</td>
<td>A H P Y G</td>
<td>3.7</td>
<td>326</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-HPYG</td>
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<td>H P Y G</td>
<td>3.8</td>
<td>541</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-AIHPYG</td>
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<td>A I H P Y G</td>
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<td>188</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-PWAQ</td>
<td>20</td>
<td>P W A Q</td>
<td>4.3</td>
<td>&gt;&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-IHPYG</td>
<td>18</td>
<td>I H P Y G</td>
<td>4.3</td>
<td>379</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-IPYG</td>
<td>17</td>
<td>I P Y G</td>
<td>4.5</td>
<td>680</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-Evo40</td>
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<td>P S E</td>
<td>5.2</td>
<td>1490</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-YGG</td>
<td>12</td>
<td>Y G G</td>
<td>5.4</td>
<td>321</td>
<td></td>
</tr>
<tr>
<td>Axmi428H-GPYG</td>
<td>15</td>
<td>G P Y G</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

For the HPPD Axmi428H-GPYG, no activity was detectable in this assay.
Table 5. Tolerance of Axmi428H mutants to tembotrione (TBT) and mesotrione (MST) using HGD assay

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Amino acid position in Axmi428H</th>
<th>pI50 TMB</th>
<th>pI50 MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axmi428H</td>
<td>193 209 221 247 351 352 355 356</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axmi428H-HPYG</td>
<td>16 - - - H P Y G</td>
<td>5.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Axmi428H-AHPYG</td>
<td>13 - A - H P Y G</td>
<td>5.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Axmi428H-IHPYG</td>
<td>18 - I H P Y G</td>
<td>5.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Axmi428H-AIHPYG</td>
<td>14 - A I H P Y G</td>
<td>5.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Axmi428H-IYPY</td>
<td>17 - I - P Y G</td>
<td>5.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Axmi428H-YGG</td>
<td>12 - - - - Y G G</td>
<td>5.9</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Table 6. Tolerance of Axmi428H mutants to NO chelators Cmpd. 1 (2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide), Cmpd. 2 (2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide), and Cmpd. 4 (2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide), using HGD assay

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Amino acid position in Axmi428H</th>
<th>p150</th>
<th>p150</th>
<th>p150</th>
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<tr>
<td></td>
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<td>Cmpd. 2</td>
<td>Cmpd. 1</td>
<td>Cmpd. 4</td>
</tr>
<tr>
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<td>M</td>
<td>I</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>Q</td>
<td>E</td>
<td>G</td>
</tr>
<tr>
<td>Axmi428H-HPYG</td>
<td>16</td>
<td></td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>Axmi428H-AHPYG</td>
<td>13</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Axmi428H-IHPYG</td>
<td>18</td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Axmi428H-AIHPYG</td>
<td>14</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Axmi428H-IYPY</td>
<td>17</td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Axmi428H-YGG</td>
<td>12</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Table 7. Effect of the mutation on the type of inhibition of the enzyme for each single tested inhibitor (DKN, TBT, and MST)

<table>
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<tr>
<th>SEQ ID NO</th>
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<th>221</th>
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<th>352</th>
<th>355</th>
<th>356</th>
<th>pI50</th>
<th>pI50</th>
<th>pI50</th>
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<tbody>
<tr>
<td>Axmi428H</td>
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<td>I</td>
<td>V</td>
<td>Q</td>
<td>E</td>
<td>G</td>
<td>K</td>
<td>A</td>
<td>td</td>
<td>td</td>
</tr>
<tr>
<td>Axmi428H-IHPYG</td>
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<td>-</td>
<td>-</td>
<td>I</td>
<td>H</td>
<td>P</td>
<td>Y</td>
<td>-</td>
<td>G</td>
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<td>rev</td>
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<td>-</td>
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<td>P</td>
<td>Y</td>
<td>-</td>
<td>G</td>
<td>rev</td>
<td>rev</td>
</tr>
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<td>-</td>
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<td>-</td>
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<td>G</td>
<td>G</td>
<td>rev</td>
<td>rev</td>
<td>rev</td>
</tr>
</tbody>
</table>

n.d.: not determined

rev: reversible inhibition of the HPPD enzyme for each tested inhibitor

Table 8. Effect of the mutation on the type of inhibition of the enzyme for each single tested inhibitor (NO chelators)

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>193</th>
<th>209</th>
<th>221</th>
<th>247</th>
<th>351</th>
<th>352</th>
<th>355</th>
<th>356</th>
<th>Cmpd. 2</th>
<th>Cmpd. 1</th>
<th>Cmpd. 4</th>
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</thead>
<tbody>
<tr>
<td>Axmi428H</td>
<td>6</td>
<td>M</td>
<td>I</td>
<td>V</td>
<td>Q</td>
<td>E</td>
<td>G</td>
<td>K</td>
<td>A</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Axmi428H-IHPYG</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>H</td>
<td>P</td>
<td>Y</td>
<td>-</td>
<td>G</td>
<td>rev</td>
<td>rev</td>
</tr>
<tr>
<td>Axmi428H-AIHPYG</td>
<td>14</td>
<td>-</td>
<td>A</td>
<td>I</td>
<td>H</td>
<td>P</td>
<td>Y</td>
<td>-</td>
<td>G</td>
<td>rev</td>
<td>n.d</td>
</tr>
<tr>
<td>Axmi428H-FebHPYG</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>-</td>
<td>P</td>
<td>Y</td>
<td>-</td>
<td>G</td>
<td>rev</td>
<td>rev</td>
</tr>
<tr>
<td>Axmi428H-YGG</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Y</td>
<td>G</td>
<td>G</td>
<td>rev</td>
<td>rev</td>
<td>rev</td>
</tr>
</tbody>
</table>
Example 4. 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. Transforms are identified using 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 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 are transferred to the greenhouse after an acclimation period. Plants containing the transgene are then sprayed with HPPD inhibitor herbicides, as for example with tembotrione at a rate of 100g AI/ha or with mesotrione at a rate of 300g AI/ha supplemented with ammonium sulfate methyl ester rapeseed oil. 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 5. Tolerance of soybean plants to mesotrione

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 mesotrione. A DeVries Tracker Sprayer was calibrated prior to each spraying. The chemical formulation used for mesotrione (MST) testing was Callisto® 4 SC formulation. Spray tests were conducted using 3X the field rate (equivalent to 9 fluid ounce per acre of the same herbicide formulation that containing 40% the active ingredient (AI), mesotrione), which equals 316 grams AI per hectare. Tolerance was evaluated one week after spraying. A tolerance rating of "0" was assigned to plants that their shoot apexes, newly emerged trifoliates and some axillary buds were completely bleached. A rating of "1" was
assigned to plants having slight tolerance, i.e., the newest plant shoot tissues had some green and are not bleached completely. A rating of "2" was assigned to plants showing moderate tolerance, i.e., more than 50% of the leaf area of the top three trifoliates showing no chlorosis or bleaching damage. A rating of "3" was assigned to plants showing nearly perfect tolerance, i.e., less than 10% of the leaf area showing chlorosis or very slight bleaching. The results are shown in Table 9.

Table 9.

<table>
<thead>
<tr>
<th>Clone</th>
<th>nucleotide SEQ ID NO:</th>
<th>amino acid SEQ ID NO:</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>Total # of Plants</th>
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</thead>
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<td>6</td>
<td>5</td>
<td>18</td>
<td>23</td>
<td>12</td>
<td>58</td>
</tr>
<tr>
<td>Axmi428H-Evo41</td>
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<td>8</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>21</td>
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<td>6</td>
<td>12</td>
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<td>27</td>
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<td>7</td>
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<td>22</td>
<td>69</td>
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<td>23</td>
<td>12</td>
<td>58</td>
</tr>
</tbody>
</table>

**Example 6: Cotton T0 plant establishment and selection.**

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 tembotrine equivalent to 100 or 200 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.
Example 7. Transformation of Maize Plant Cells by *Agrobacterium-MQdiatQd* 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. 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 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 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.

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.

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.
CLAIMS

1. A recombinant nucleic acid molecule encoding a 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein consisting of an amino acid sequence comprising a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 336 of SEQ ID NO:1, and wherein said HPPD protein is tolerant to an HPPD inhibitor herbicide, wherein said encoded HPPD protein consists of an amino acid sequence further comprising:

(a) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(b) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(c) a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(d) a glycine at the amino acid position corresponding to amino acid position 172 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1 and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1, an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1.
2. The recombinant nucleic acid molecule of claim 1, wherein said HPPD protein comprises an amino acid sequence having at least 53% sequence identity to the amino acid sequence set forth in SEQ ID NO 1, 5, or 6.

3. The recombinant nucleic acid molecule of claims 1 or 2, wherein its nucleotide sequence is a synthetic sequence that has been designed for expression in a plant.

4. The recombinant nucleic acid molecule of any of claims 1-3, wherein its nucleotide sequence is operably linked to a promoter capable of directing expression of the nucleotide sequence in a plant cell.

5. The recombinant nucleic acid molecule of claim 1, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO:40, 41, 42, 43, 44, and 45.

6. The recombinant nucleic acid molecule of claim 1, wherein said HPPD inhibitor herbicide is selected from the group consisting of N-(1,2,5-oxadiazol-3-yl)benzamides; N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, N-(1,3,4-oxadiazol-2-yl)benzamides, N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, pyridazine derivatives, substituted 1,2,5-oxadiazoles, oxoprazin derivatives triketones, isoxazoles, and pyrazolinates.

7. The recombinant nucleic acid molecule of claim 6, wherein said HPPD inhibitor herbicide is selected from the group consisting of 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, 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide, 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide, 2-(methoxymethyl)-3-(methylsulfinyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide, tembotrione, sulcotrione, mesotrione, isoxaflutole, pyrasulfotole, and topramezone.
8. A host cell that contains the recombinant nucleic acid molecule of claim 1, 2, or 4.

9. The host cell of claim 8 that is a bacterial host cell.

10. The host cell of claim 8 that is a plant cell.

11. A transgenic plant comprising the recombinant nucleic acid molecule of any of claims 1-5.

12. The plant of claim 11, 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.

13. A transgenic seed comprising the recombinant nucleic acid molecule of any of claims 1-5.

14. A recombinant polypeptide comprising an HPPD protein, wherein said HPPD protein is tolerant to an HPPD inhibitor herbicide, and wherein said HPPD protein comprises a proline at the amino acid position corresponding to amino acid position 335 of SEQ ID NO: 1 and a phenylalanine or a tyrosine at the position corresponding to amino acid position 336 of SEQ ID NO:1, wherein said HPPD protein further comprises
   (a) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO: 1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;
   (b) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO: 1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO: 1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;
(c) a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(d) a glycine at the amino acid position corresponding to amino acid position 172 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1;

(e) an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, and a histidine at the position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1; or

(f) an alanine at the amino acid position corresponding to amino acid position 188 of SEQ ID NO:1, an isoleucine at the amino acid position corresponding to amino acid position 200 of SEQ ID NO:1, a histidine at the amino acid position corresponding to amino acid position 226 of SEQ ID NO:1, and a glycine at the position corresponding to amino acid position 340 of SEQ ID NO:1.

15. The recombinant polypeptide of claim 14, wherein said HPPD protein comprises an amino acid sequence having at least 53% sequence identity to the amino acid sequence set forth in SEQ ID NO:1.

16. The recombinant polypeptide of claim 14 or 15, wherein said HPPD protein comprises the amino acid sequence selected from the group consisting of SEQ ID NO: 11-21

17. The recombinant polypeptide of claims 14, 15, or 16, wherein said HPPD inhibitor herbicide is selected from the group consisting of N (1,2,5-oxadiazol-3-yl)benzamides; N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, N-(1,3,4-oxadiazol-2-yl)benzamides, N-(tetrazol-5-yl)- or N-(triazol-3-yl)arylcarboxamides, pyridazinone derivatives, substituted 1,2,5-oxadiazoles, oxoprazin derivatives triketones, isoxazoles, and pyrazolinates.

18. The recombinant polypeptide of claim 17, wherein said HPPD inhibitor herbicide is selected from the group consisting of 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, 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide, 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide, 2-(methoxymethyl)-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)-4-(trifluoromethyl)benzamide, tembotrione, sulcotrione, mesotrione, isoxaflutole, pyrasulfotole, and topramezone.

19. A method for producing a polypeptide with HPPD inhibitor herbicide tolerance activity, comprising culturing the host cell of claim 8 under conditions in which a nucleic acid molecule encoding the polypeptide is expressed.

20. A plant having stably incorporated into its genome a DNA construct, said construct comprising a promoter operably linked with the nucleic acid of any of claims 1-5.

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

22. The plant of claim 20, 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.

23. Transgenic seed of the plant of claim 20.

24. A method of controlling weeds in a field comprising planting the plant of claim 20 or a seed thereof in a field and applying to said field an effective concentration of an HPPD inhibitor herbicide.

25. The method of claim 24, wherein said HPPD inhibitor herbicide is selected from the group consisting of N-(1,2,5-oxadiazol-3-yl)benzamides; N-(tetrazol-4-yl)- or N-(triazol-3-yl)arylcarboxamides, N-(1,3,4-oxadiazol-2-yl)benzamides, N-(tetrazol-5-yl)- or N-(triazol-3-
yl)arylcarboxamides, pyridazinone derivatives, substituted 1,2,5-oxadiazoles, oxoprazin derivatives triketones, isoxazoles, and pyrazolinates.

26. The method of claim 24, wherein said HPPD inhibitor herbicide is selected from the group consisting of 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, 2-methyl-N-(5-methyl-1,3,4-oxadiazol-2-yl)-3-(methylsulfonyl)-4-(trifluoromethyl)benzamide, 2-chloro-3-ethoxy-4-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 4-(difluoromethyl)-2-methoxy-3-(methylsulfonyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, 2-chloro-3-(methylsulfanyl)-N-(1-methyl-1H-tetrazol-5-yl)benzamide, tembotrione, sulcotrione, mesotrione, isoxaflutole, pyrasulfotole, and topramezone.

27. Use of the nucleic acid of any of claims 1-5 for rendering a plant tolerant to one or more HPPD inhibitor herbicide(s).

28. A commodity product comprising the nucleic acid molecule of any of claims 1-5, or the protein of any of claims 14-16, wherein said product is selected from the group consisting of whole or processed seeds or grain, animal feed, corn or soy meal, corn or soy flour, 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, soy yogurt, soy cheese, tofu, yuba, and cooked, polished, steamed, baked or parboiled grain.
FIG. 1C
FIG. 2a

Time dependent HPPD activity vs. Time

- Control - no preincubation
- 1 μM - 10 min preincubation

inhibition

Time dependence of the inhibition