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61/987,965 2 May 2014 (02.05.2014) US(71) Applicant: PIONEER HI-BRED INTERNATIONAL,
INC. [US/US]; 7100 N.W. 62nd Avenue, P.O. Box 1014,
Johnston, IA 50131 (US).(72) Inventors: SIEHL, Daniel; 310 Durham Street, Menlo
Park, CA 94025 (US). PATTEN, Philip A.; 261 La Cuesta
Drive, Menlo Park, CA 94028 (US). TAO, Yumin; 363
Becado Drive, Fremont, CA 94539 (US). BERMUDEZ,
Ericka; 734 Clubhouse Drive, Aptos, CA 95003 (US).
CASTLE, Linda A.; 784 Hans Avenue, Mountain View,
CA 94040 (US). DONG, Yuxia; 46937 Lundy Terrace,
Fremont, CA 94539 (US). SATTERFIELD, Andrew
Duncan; 1524 Robert Burns Court, Bear, DE 19701 (US).
ALBERT, Henrik; 142 Grand Street, Apt #1, Alameda,
CA 94501 (US).(74) Agents: MERCHANT, Mary Anthony et al.; Ballard
Spahr LLP, 999 Peachtree Street, Suite 1000, Atlanta, GA
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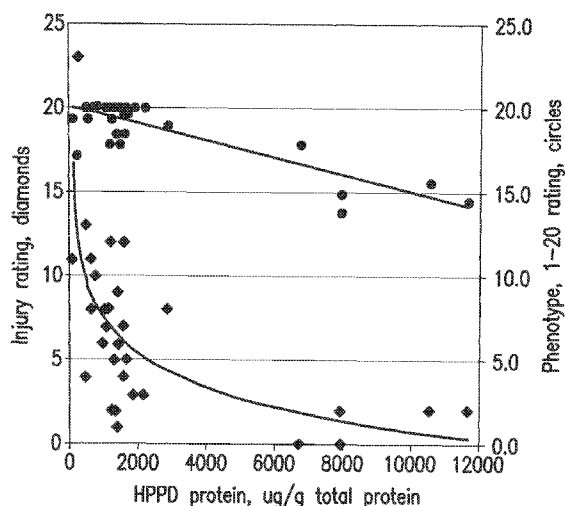
(54) Title: COMPOSITIONS AND METHODS COMPRISING SEQUENCES HAVING HYDROXYPHENYLPYRUVATE DI-
OXYGENASE (HPPD) ACTIVITY

FIG. 14

(57) Abstract: Compositions and methods comprising polynucleotides and polypeptides having 4- hydroxyphenylpyruvate dioxygenase (HPPD) activity and having insensitivity to an HPPD inhibitor are provided. Further provided are nucleic acid constructs, plants, plant cells, explants, seeds and grain having the HPPD sequences. Various methods of employing the HPPD sequences are provided. Such methods include, for example, methods for producing an HPPD inhibitor tolerant plant, plant cell, explant or seed and methods of controlling weeds in a field containing a crop employing the plants and/or seeds disclosed herein. Methods are also provided to identify additional HPPD variants. Further provided are various methods and compositions that allow the various HPPD polypeptides and variant and fragments thereof to be expressed in a chloroplast or transported to a chloroplast.



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**COMPOSITIONS AND METHODS COMPRISING SEQUENCES HAVING
HYDROXYPHENILPYRUVATE DIOXYGENASE (HPPD) ACTIVITY**

CROSS-REFERENCE TO RELATED APPLICATIONS

This Application claims the benefit of U.S. Provisional Application No. 61/987,965, filed on
5 May 2, 2015, which is incorporated herein by reference in its entirety.

**REFERENCE TO A SEQUENCE LISTING SUBMITTED AS
A TEXT FILE VIA EFS-WEB**

The official copy of the sequence listing is submitted electronically via EFS-Web as an
10 ASCII formatted sequence listing with a file named 36446_0199P1_Sequence_Listing.txt , created
on May 2, 2015, and having a size of 356,801 bytes and 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.

15 **BACKGROUND OF THE INVENTION**

In the commercial production of crops, it is desirable to easily and quickly eliminate
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 unharmed. One such treatment system would involve the use of crop plants which
20 are tolerant to a herbicide so that when the herbicide was sprayed on a field of herbicide-tolerant
crop plants, the crop plants would continue to thrive while non-herbicide-tolerant weeds were 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
25 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.

Crop tolerance to specific herbicides can be conferred by engineering genes into crops which
30 encode appropriate herbicide metabolizing enzymes and/or insensitive herbicide targets. In some
cases these enzymes, and the nucleic acids that encode them, originate in a plant. In other cases, they
are derived from other organisms, such as microbes. *See, e.g.*, Padgett *et al.* (1996) “New weed
control opportunities: Development of soybeans with a Roundup Ready[®] gene” and Vasil (1996)
“Phosphinothricin-resistant crops,” both in *Herbicide-Resistant Crops*, ed. Duke (CRC Press, Boca

Raton, Florida) pp. 54-84 and pp. 85-91. Indeed, transgenic plants have been engineered to express a variety of herbicide tolerance genes from a variety of organisms.

For nearly two decades, corn, soybean, and cotton farmers have relied on glyphosate and glyphosate resistant crops for weed control. While favored for its efficacy, economy and convenience, the onset of glyphosate-resistant weeds, now numbering 26 species (Heap, 2014), signals a need for new herbicide and trait systems that meet similar criteria (Green and Castle, 2010; Duke, 2012). The silver bullet solution would be another herbicide and trait combination as effective as glyphosate. Unfortunately, prospects for novel herbicide chemistries are not encouraging (Duke, 2012).

Inhibitors of 4-hydroxyphenylpyruvate dioxygenase (HPPD) disrupt production of tocopherols (antioxidants) and plastoquinone (essential for photosynthetic electron transfer) by blocking conversion of tyrosine, through 4-hydroxyphenyl pyruvate (HPP), to homogentisate (Moran, 2014). The result is that the plant cannot protect itself from the radicals generated by light activation of chlorophyll, causing bleaching, necrosis, and death. Registered HPPD inhibitors include mesotrione, tembotrione, sulcotrione, isoxaflutole, and topramezone. HPPD inhibitors are most effective on broad-leaf weeds but control some grasses as well. Currently, HPPD herbicides are selective for use in corn, while soybeans and other dicot crop species are sensitive. A broad spectrum HPPD tolerance trait in soybeans, used in combination with glyphosate tolerance and other traits or selective herbicides, will prolong the positive impact of the glyphosate systems and slow appearance of resistant weeds. Two such products, one for tolerance to isoxaflutole (APHIS, 2009) and the other to mesotrione and isoxaflutole (APHIS, 2012) are in the USDA regulatory approval process. The former uses a *Pseudomonas fluorescens* HPPD with a single amino acid change (Matringe et al., 2005), while the latter uses HPPD from oat, with a single amino acid change (Hawkes et al., 2010). There are presently no reports of tembotrione or broad spectrum tolerance.

To develop a robust and stable HPPD tolerance trait in soybean, expression patterns of the native gene and localization of native and transgenic protein were studied, and the efficacy of low to moderate expression of a desensitized protein were evaluated. Data regarding the subcellular location of HPPD are ambiguous, perhaps resulting from species diversity. Early work with organelle fractions attributed most HPPD activity to the chloroplast in spinach (Fiedler et al., 1982) or *Lemna gibba* (Loeffelhardt and Kindl, 1979). Organelle targeting can be conjectured from the observation that the N-terminal sequence of mature HPPD isolated from maize leaf begins at either ala-17 (Fritze et al., 2004) or ala-23 (Yang et al., 2004) with respect to the translated full-length gene. Two HPPD genes identified from EST libraries prepared from cotton tissue were 98.6 percent identical, each with a 23-amino acid sequence deemed likely to function in chloroplast targeting by analysis with

the ChloroP prediction program (Moshiri et al., 2007). In the same publication, tomato, but not *Brassica* HPPD was predicted to have a CTP. Subcellular fractionation supported a cytosolic location of the carrot cell enzyme (Garcia et al., 1997). Although the N-terminal sequence of the purified enzyme was truncated with respect to the translated cDNA, this was attributed to proteolysis during purification. Later, the same authors determined that after PSORT analysis failed to identify a targeting signal within the Arabidopsis HPPD amino acid sequence, native Arabidopsis HPPD heterologously expressed in tobacco was located exclusively in the cytosol (Garcia et al., 1999).

While a number of HPPD crop plants are presently commercially available, improvements in every aspect of crop production, weed control options, extension of residual weed control, and improvement in crop yield are continuously in demand. Particularly, due to local and regional variation in dominant weed species as well as preferred crop species, a continuing need exists for customized systems of crop protection and weed management which can be adapted to the needs of a particular region, geography, and/or locality. A continuing need therefore exists for compositions and methods of crop protection and weed management.

BRIEF SUMMARY OF THE INVENTION

Compositions and methods comprising polynucleotides and polypeptides having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity and having an insensitivity to at least one HPPD inhibitor are provided. Further provided are nucleic acid constructs, plants, plant cells, explants, seeds and grain having the HPPD sequences.

Various methods of employing the HPPD sequences are provided. Such methods include methods for producing an HPPD inhibitor tolerant plant, plant cell, explant or seed and methods of controlling weeds in a field containing a crop employing the plants and/or seeds disclosed herein.

Methods are also provided to identify additional HPPD variants.

Further provided are methods and compositions that allow the various HPPD polypeptides and variants and fragments thereof to be expressed in a chloroplast or transported to a chloroplast. Such methods and compositions find use in producing plant cells, plants and explants having tolerance to various HPPD inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Structures of herbicidal HPPD inhibitors and a HPPD substrate, 4-hydroxyphenylpyruvate.

FIG. 2. HPPD inhibitor herbicide site of action and symptoms. FIG. 2A: HPPD reaction and essential downstream products in plants. FIG 2B: Bleaching symptoms of HPPD-inhibiting

herbicides in soybean.

FIGS. 3A – 3M. *Z. mays* HPPD protein characterization. FIG 3A: *Z. mays* HPPD amino acid sequence. FIG. 3C: Fluorescence observed in chloroplasts of maize leaf expressing rubisco activase CTP-Ds-Red2, with the same sample photographed under white light shown in FIG. 3B. FIG. 3E: Fluorescence observed in chloroplasts of maize leaf expressing N-terminal 50 amino acids of maize HPPD-Ds-Red2, with the same sample photographed under white light shown in FIG. 3D. FIG. 3G: Fluorescence observed in cytosol of maize leaf expressing untargeted Ds-Red2, with the same sample photographed under white light shown in FIG. 3F. FIGS. 3H-3J: Maize cells transiently expressing DsRed fused to 30, 40 and 50 amino acid N-terminal region of HPPD protein sequence (FIG. 3H, 3I, and 3J, respectively). FIG. 3K: Expression in both chloroplasts and cytoplasm of soybean leaf epidermal cell transiently expressing AcGFP fused to amino acids 1-50 of maize HPPD. FIG. 3L: Expression in chloroplasts of guard cells in soybean stably expressing AcGFP linked to amino acids 1-50 of maize HPPD. FIG. 3M: alignment of monocot HPPD N-terminal region sequences and consensus monocot functional CTP. Sources of sequences are indicated. Gray shading shows identities in sequences.

FIGS. 4A – 4F. Genomic analysis of soybean HPPD. FIG. 4A: *G. max* HPPD protein sequence with in-frame N-terminal extension (underlined). FIG 4B: Protein gel of *in vitro* transcription-translation products of the two (3' truncated) HPPD transcripts. Predicted protein mass from -237 mRNA: 30.6kD; +1 mRNA: 26.0 kD. FIG 4C: Transient expression of N-terminal fragment 1-44 with DsRed2 in soybean. FIG 4D: Transient expression of N-terminal fragment 42-86 with AcGFP1 in soybean. FIG 4E: Transient expression of N-terminal fragment 1-86 with AcGFP1 in soybean. F, Alignment of *Z. mays* and *G. max* HPPD proteins. An asterisk indicates identity; a colon shows the fusion point of soybean to maize insensitive HPPD proteins.

FIG. 5. Progression of enzyme fitness with rounds of shuffling. Bars indicate ON and OFF rate ratios, the insensitivity parameters that are functions of the rate constants for association and dissociation of enzyme and inhibitor, respectively. “Fitness” is the product of k_{cat}/K_m and insensitivity parameters (ON rate ratio and OFF rate ratio, see text for details), and approximates $k_{cat}/K_m \times K_i$.

FIG. 6. Selection for insensitivity to mesotrione or tembotrione resulted in cross-insensitivity to other herbicidal inhibitors. “Fitness” is the product of k_{cat}/K_m and insensitivity parameters, and approximates $k_{cat}/K_m \times K_i$. The dotted line is drawn at the fitness level that provided tolerance in soybean to twice the labeled field rate (for maize) of tembotrione in 2013 field trials.

FIG. 7. Expression of soybean HPPD ESTs in specific tissues compared to EPSPS and ALS

with ACT2/7 as a moderately expressing control gene. Units of expression are reads/kilobase/million, normalized from raw data (SoyBase.org).

FIGS. 8A and 8B. FIG. 8A: Upstream 1225 bp *G. max* promoter sequence and N-terminal regions of the long and short HPPD proteins. Underlined bold denotes predicted TATA sequences; bold denotes methionine initiation sites for long and short proteins; grey background denotes actual transcription start sites at positions -237 and +1.; Grey box denotes the upORF in the long transcript. FIG. 8B: Schematic of *G. max* HPPD promoter and coding region.

FIGS. 9A-9D. Field trial with transgenic soybean events treated with 2X maize field rate of tembotrione, 14 days after treatment. FIG 9A: Promoter HSP206 and full length maize HPPD variant 6-1. FIG. 9B: 35S enhanced promoter SHP110 with fused Gm:Zm HPPD variant 6-1. FIG. 9C: Non-transgenic unsprayed control. FIG. 9D: Non-transgenic sprayed control. Treatment was as described in Table 6.

FIGS. 10A and 10B. FIG. 10A: Immuno-localization of native HPPD in maize leaf shows label only in chloroplasts. Serum containing anti-maize or anti-soybean HPPD antibodies was raised in rabbits inoculated with recombinant wild-type 6x-his-HPPD produced in E coli and purified by nickel chelate affinity chromatography. The serum was passed through Protein A Ceramin Hyper DF to adsorb the IgG fraction. After washing, IgG was eluted with citrate buffer, pH 2.55, with a yield of 50 mg of IgG per gram of serum protein. Ten mg of IgG protein were subjected to the manufacturer's linkage protocol for Affi-Gel Hz (Bio-Rad, Hercules, CA), which, in the case of the maize enzyme, resulted in the capture of 2 mg of IgG, 20% of which was anti-maize HPPD. Anti-HPPD antibodies were further purified by passage through a column of immobilized Rubisco, to remove a small fraction of antibodies that reacted with both HPPD and Rubisco. Leaf punches from mature line A63 maize plants or native or stably transformed soybean plants expressing a gene encoding the maize HPPD protein driven by an SCP1 synthetic promoter (Cahoon and Coughlan, 2007) were fixed in 2% paraformaldehyde, 0.25% glutaraldehyde in 100mM Na phosphate buffer, pH 7.0, for 3 hours at room temperature, dehydrated by passage through progressively higher concentrations of ethanol, embedded in LR White resin and cured at 55°C for 48 hours. Immuno-localizations were performed with the primary antibody being the double-purified anti-maize HPPD (1:200) and the secondary antibody goat anti-rabbit F(ab') conjugated with ultrasmall gold particles (Aurion, The Netherlands). Gold labeling was followed by silver enhancement (Aurion). Sections were counterstained with 4% uranyl acetate (aqueous) followed by Reynold's lead citrate and examined with a Hitachi S4800 scanning electron microscope. Gold labeling was observed mainly in bundle sheath chloroplasts (range; 5-28 particles per chloroplast, 50 chloroplasts observed. The highest frequency of observations was 6 chloroplasts that contained 8 particles. Particles were also

found in mesophyll chloroplasts, 31 of which had 1 to 5 particles and 19, of which had no particles. A small number of particles were also observed randomly in other locations including cytosol and voids, and were considered to be artifacts. FIG. 10B: shows the inner indicated rectangle at higher digital magnification.

5 **FIGS. 11A-11J.** The N-terminal 50 amino acids of maize HPPD directs fluorescent protein to the chloroplasts of maize leaf guard cells. FIGS. 11A-11D, Co-expression of untargeted C3GFP and HPPD N-terminal 50AA::DsRed2. FIGS. 11E-11G, Co-expression of untargeted C3GFP and Rubisco activase CTP::DsRed. FIGS. 11H-11J, untargeted C3GFP. The red channel (FIGS. 11B, 11F and 11I) shows the pattern of DsRed fluorescence, the green channel (FIGS. 11D, 11G, and 11J) shows untargeted C3GFP fluorescence and the blue channel (FIG. 11C), chlorophyll autofluorescence. Overlays of the red and green channels are shown in FIGS. 11A, 11E and 11H. Plastid targeting by the N-terminal 50 amino acids of maize HPPD is evident from the co-localization of the DsRed with chlorophyll autofluorescence (FIGS. 11B and 11C) and the lack of overlap with the untargeted C3GFP (FIGS. 11B and 11D). Note that exclusion of the C3GFP signal marks the location of the guard cell plastids. Similarly, the positive control vector for chloroplast localization showed a lack of overlap between the cytosolic C3GFP signal and the DsRed signal (FIGS. F and G). Untargeted DsRed shows spatial overlap of the DsRed and C3GFP signals and an absence of DsRed in the regions of C3GFP exclusion that mark the position of the plastids. Maize seedlings were generated in the absence of soil by embedding kernels between two sheets of blotting paper hydrated in tap water amended with 0.1 mg/ml sucrose. Leaf segments were detached from seedlings at 15 days post-planting immediately before biolistic transformation. The lower epidermis of the leaf segments was bombarded and the segments were placed in sterile moist chambers. The tissue was co-bombarded with DNA from DsRed-containing test plasmids and a plasmid encoding untargeted Cycle 3 Green Fluorescence Protein (C3GFP, Life Technologies) using the PDS-1000 He biolistic particle delivery system (Bio-Rad, Hercules CA). Gold particles (0.6 μ m in diameter; Bio-Rad) were coated with plasmid DNA as follows. Fifty μ l of freshly prepared gold particles in water (20 mg/ml), and 20 μ l of DNA mixture (containing 10 μ g of equimolar quantities of the DsRed-containing test plasmid and the untargeted C3GFP plasmid) were combined. While gently vortexing, 50 μ l of a 2.5 M CaCl₂ solution and 20 μ l of freshly prepared 0.1 M spermidine (Sigma-Aldrich, St Louis MO) were slowly added. The mixture was incubated at room temperature for 5 min and pelleted at 13,000g in a micro-centrifuge for 5 sec. The supernatant was carefully removed and the pellet was resuspended in 85 μ l of 100% ethanol. While gently vortexing, a 5 μ l aliquot of suspension was drawn and dispensed onto the center of a macrocarrier membrane. The membrane was allowed to dry completely for 2–5 min and used immediately. Leaf segments were bombarded at

a distance of 9 cm from an 1100-psi rupture disk. Three replicate shots were performed from each coating preparation. Initial examination was conducted at approximately 24 h post-bombardment with a Lumar fluorescence stereomicroscope (Carl Zeiss Inc., Thornwood NY) equipped with both a UV-exciting (Zeiss Set 01) and red-emitting (Zeiss Set 43 HE) filter set to image the C3GFP and the DsRed2, respectively. The leaf segments containing DsRed2-positive cells identified in the stereomicroscope were placed in a 0.01% Tween 20 solution and a vacuum was applied for about 10 min to remove internal air and to wet the leaf surface. The leaves were placed into coverglass chambers in the same solution, sealed with an additional coverglass and examined in the LSM510 (Carl Zeiss). C3GFP fluorescence was captured using a 488 nm argon laser for excitation and a 500-550 nm band pass emission filter. DsRed fluorescence was imaged using a 561 nm diode laser for excitation and a 575-615 nm band pass emission filter. Chlorophyll fluorescence was captured by combining 561 nm excitation and a 650-710 nm band pass emission filter.

FIGS. 12A and 12B. Each of FIG. 12A and 12B show immuno-localization electron microscopy of HPPD in soybean cotyledons shows label in chloroplasts and cytoplasm. Affinity purified rabbit anti-soy HPPD antibody was obtained as described for FIGS. 10A and 10B. Pieces of green soybean cotyledon (6 days after planting) were cut under fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M sucrose in 0.1M Na Phosphate Buffer, pH 7.2) and held in fixative for 20 min under vacuum at room temperature, then overnight at 4°C. Sectioning, labeling, counterstaining and microscopy were as in FIG. 1S with 10 nm gold. Gold particles were observed exclusively in chloroplasts and cytosol. Most of the fields captured show a greater portion of the label in chloroplasts.

FIGS. 13A – 13D. Indirect measurement of rates of association and dissociation of inhibitors and HPPD. Rates of association (k_{ON}) are represented by inactivation during catalytic turnover. At time 0, reactions are started with the simultaneous addition of HPP and inhibitor to an aliquot of enzyme. Reaction rates in the 70 to 90 sec interval are recorded and expressed as the ratio of the rate with inhibitor to that with no inhibitor. FIGS. 13A and 13B: Time course, reaction with wt maize HPPD or a shuffled variant; 100 μ M HPP $\pm$ 4 μ M mesotrione. For wild type maize HPPD, rapidly slowing reaction indicates a relatively high k_{ON} . For the shuffled variant, resistance to inactivation indicates a lower value for k_{ON} . The smaller the actual k_{ON} , the more slowly the HPPD reaction decelerates and the higher the value is for the ON rate ratio. FIGS. 13C and 13D: OFF rate ratio. A quantitative indicator of k_{OFF} was obtained by observing the time course of an HPPD reaction as inhibitor is released from a pre-formed enzyme-inhibitor complex. The ratio of the maximum rate attained in mixtures containing inhibitor to the initial velocity of mixtures lacking inhibitor is termed the “OFF rate ratio”. The plateau of absorbance is due to exhaustion of the substrate. With improved

variants, not only was the steady state attained in a shorter time span, the velocity attained was higher than with wild type, though the enzymes were present at the same concentration (240 nM). This is because a larger fraction of the variant enzyme was free of inhibitor compared to the wild-type enzyme, which can due to a higher KD.

5 **FIG. 14.** The figure shows data indicating the influence of transgene expression level on trait efficacy and plant phenotype of soybean plants treated with tembotrione in the field. Tembotrione was applied at 180 g/ha (twice the labeled rate for maize) at the V4 stage. Phenotype was rated on mature plants in late summer. Filled diamonds: Injury rating, 0, no injury, 100, dead plant; 14 days after treatment. Open circles: Phenotype rating; 20, no symptoms; 1, leaf crinkling, empty seed pods.
10 Correlation (R²), log plot, for injury versus protein = 0.48. Correlation (R²), linear regression, for phenotype versus protein = 0.70.

FIG. 15. Kinectic parameters, insensitivity parameters, trait fitness parameters, and trait fitness (fold vs. wild-type) for the indicated mutants is given. The various data were determined using the methods described herein below. In the figure, the following abbreviations are used:
15 “Tembo” indicates tembotrione; “Meso” indicates mesotrione; “Sulco” indicates sulcotrione; “Isox(DKN)” indicates isoxaflutole (diketonitrile); and “Topra” indicates topramezone.

FIG. 16. The figure shows the chemical structures for various HPPD inhibitors.

FIG. 17. The figure shows the enzymatic conversion of 4-hydroxyphenylpyruvate by HPPD to homogentisate, and the subsequent enzymatic conversion of homogentisate to maleylacetoacetate
20 by HGD. The detection parameters for maleylacetoacetate are shown in the figure.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments or aspects are shown. Indeed, the
25 disclosure 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 disclosure set forth herein will come to mind to one skilled in the art to which this disclosure pertains having the benefit of the teachings
30 presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the disclosure is 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.

I. Compositions

A. Hydroxyphenylpyruvate Dioxygenase (HPPD) Polynucleotides and Polypeptides

Hydroxyphenylpyruvate dioxygenase (HPPD) converts hydroxyphenylpyruvate, derived from the aromatic amino acid biosynthesis pathway, to homogentisate. Homogentisate is a precursor of tocopherols and plastoquinones, an electron carrier essential in the biosynthesis of carotenoids. Consequently, when HPPD is inhibited by herbicide inhibitors, the plant can not protect itself from the radicals generated by light activation of chlorophyll. More specifically, inhibition of HPPD polypeptide leads to the depletion of protective pigments in the plant tissue resulting in bleaching of tissues which leaves the plants vulnerable to damage by light. HPPD inhibitors are an important class of herbicides, and transgenes that confer crop tolerance to HPPD inhibitors would be of significant value, especially for managing weed resistance to glyphosate.

As used herein, "hydroxyphenylpyruvate dioxygenase," "HPPD," "4-hydroxy phenyl pyruvate (or pyruvic acid) dioxygenase (4-HPPD)," and "p-hydroxy phenyl pyruvate (or pyruvic acid) dioxygenase (p-OHPP)" are synonymous and refer to a non-heme iron-dependent oxygenase that catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate (see FIG. 17). In organisms that degrade tyrosine, the reaction catalyzed by HPPD is the second step in the pathway. In plants, formation of homogentisate is necessary for the synthesis of plastoquinone, an essential redox cofactor, and tocopherol (see FIG. 2A). The structures of various HPPD polypeptides are known.

Various methods and compositions are provided which employ polypeptides having HPPD activity and having an increased insensitivity to at least one HPPD inhibitor when compared to an appropriate control, and the polynucleotides encoding these polypeptides. In an aspect, HPPD polypeptides having HPPD activity and having an increased insensitivity to at least one HPPD inhibitor when compared to an appropriate control include those set forth in any one of SEQ ID NO:2-83, and biologically active variants and fragments thereof. In various aspects, HPPD polypeptides having HPPD activity and having an increased insensitivity to at least one HPPD inhibitor when compared to an appropriate control include those set forth in any one of SEQ ID NO:4, 7, 8, 18, and 22, and biologically active variants and fragments thereof. Further provided are the polynucleotides encoding these various polypeptides and active variants and fragments thereof.

As used herein, an "isolated" or "purified" polynucleotide or polypeptide, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide or polypeptide as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide or polypeptide is substantially free of other cellular

material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In various aspects, an "isolated" polynucleotide is free of sequences (optimally protein encoding sequences) that naturally flank the polynucleotide (i.e., sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. For example, in various embodiments, the isolated 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 flank the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. A polypeptide that is substantially free of cellular material includes preparations of polypeptides having less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of contaminating protein. In an aspect, a disclosed polypeptide, or biologically active portion thereof, is recombinantly produced, culture medium represents less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

As used herein, polynucleotide or polypeptide is "recombinant" when it is artificial or engineered, or derived from an artificial or engineered protein or nucleic acid. For example, a polynucleotide that is inserted into a vector or any other heterologous location, e.g., in a genome of a recombinant organism, such that it is not associated with nucleotide sequences that normally flank the polynucleotide as it is found in nature is a recombinant polynucleotide. A polypeptide expressed *in vitro* or *in vivo* from a recombinant polynucleotide is an example of a recombinant polypeptide. Likewise, a polynucleotide sequence that does not appear in nature, for example, a variant of a naturally occurring gene is recombinant.

A "control" or "control plant" or "control plant cell" provides a reference point for measuring changes in phenotype of the subject plant or plant cell, and may be any suitable plant or plant cell. A control plant or plant cell may comprise, for example: (a) a wild-type or native plant or cell, *i.e.*, of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (*i.e.*, with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell which is genetically identical to the subject plant or plant cell but which is not exposed to the same treatment (*e.g.*, herbicide treatment) as the subject plant or plant cell; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

i. Hydroxyphenylpyruvate Dioxygenase Activity

As used herein, "hydroxyphenylpyruvate dioxygenase activity" or "HPPD activity" refers to the conversion of 4-hydroxyphenylpyruvate to homogentisate. As used herein, a polypeptide

having “HPPD activity” comprises an HPPD polypeptide or an active variant or fragment thereof that retains sufficient HPPD activity such that (i) when expressed at sufficient levels in a cell that requires HPPD activity for viability, the HPPD polypeptide or active variant or fragment exhibits sufficient HPPD activity to maintain viability of the cell in which it is expressed; or (ii) when
 5 expressed in a cell that requires HPPD activity for viability, the HPPD polypeptide, or active variant or fragment thereof, when expressed in combination with one or more additional HPPD polypeptides results in the viability of the cell. In one embodiment, the HPPD activity of an HPPD polypeptide, or an active variant or fragment thereof, is such that in the absence of an HPPD inhibitor said polypeptide or active variant or fragment thereof displays at least about 5%, 15%, 20%, 25%, 30%,
 10 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% or greater of the HPPD activity displayed in any one of SEQ ID NO:2-83. Methods to determine such kinetic parameters (i.e., K_m , k_{cat} , k_{cat}/K_m) are known and discussed elsewhere herein.

In still further embodiments, the HPPD polypeptide or active variant or fragment thereof has an activity that is at least equivalent to a native HPPD polypeptide or has an activity that is increased
 15 when compared to a native HPPD polypeptide. An “equivalent” HPPD activity refers to an activity level that is not statistically significantly different from the control as determined through any enzymatic kinetic parameter, including for example, via K_m , k_{cat} , or k_{cat}/K_m . An increased HPPD activity comprises any statistically significant increase in HPPD activity as determined through any enzymatic kinetic parameter, such as, for example, K_m , k_{cat} , or k_{cat}/K_m . In specific embodiments, an
 20 increase in HPPD activity comprises at least a 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 2, 3, 4, 5, 6, 7, 8, 9, or 10 fold or greater improvement in a given kinetic parameter when compared to the maize wild-type HPPD sequence as set forth in SEQ ID NO:1 or when compared to a native HPPD polypeptide. Methods to determine such kinetic parameters are known.

Briefly, HPPD catalyzes the conversion of 4-hydroxyphenylpyruvate (HPP) to homogentisate.
 25 Substrate and product do not differ in absorbance of light at any useful wavelength. However, homogentisate dioxygenase (HGD) catalyzes the conversion of homogentisate into maleylacetoacetate which absorbs strongly at 320 nm. Thus, by combining 4-hydroxyphenylpyruvate with both HPPD and HGD under the appropriate reaction conditions HPPD activity can be assayed.

As used herein, a “native” HPPD polypeptide comprises any wild-type HPPD sequence.
 30 Such sequences are known in the art, and representative native/wild-type HPPD sequences from various monocot and dicot plants are set forth in FIG. 4 and SEQ ID NO:1. In specific embodiments, the biologically active fragments and variants of the HPPD sequences is compared to the maize wild-type HPPD polypeptide (SEQ ID NO:1) or to a native HPPD polypeptide.

As used herein, a “corresponding native” HPPD polypeptide comprises the native or wild type sequence from which the biologically active variant is derived. For example, for a biologically active variant or fragment of a soy HPPD polypeptide, the corresponding native HPPD polypeptide would be the native soy sequence as set forth in SEQ ID NO:100.

5 *ii. Insensitivity to HPPD Inhibitors*

In order to provide plants with tolerance to commercially useful application rates of at least one desired HPPD inhibitor, it is advantageous to use polynucleotides which encode HPPD polypeptides having sufficient HPPD activity and having an insensitivity to inhibition by at least one or more HPPD inhibitor. Thus, in specific embodiments, the HPPD polynucleotides and polypeptides and active variants and fragments thereof provided herein display an increased insensitivity to an HPPD inhibitor when compared to a corresponding native HPPD polypeptide and/or an increased insensitivity when compared to the maize native HPPD polypeptide (SEQ ID NO:1).

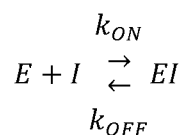
As used herein, an “HPPD inhibitor” comprises any compound or combinations of compounds which decrease the ability of HPPD to catalyze the conversion of 4-hydroxyphenylpyruvate to homogentisate. In specific embodiments, the HPPD inhibitor comprises a herbicidal inhibitor of HPPD. Non-limiting examples of HPPD inhibitors include, triketones (such as, mesotrione, sulcotrione, topramezone, and tembotrione); isoxazoles (such as, pyrasulfotole and isoxaflutole); pyrazoles (such as, benzofenap, pyrazoxyfen, and pyrazolynate); and benzobicyclon. Agriculturally acceptable salts of the various inhibitors include salts, the cations or anions of which are known and accepted in the art for the formation of salts for agricultural or horticultural use. See, for example, International Publication No. WO 2005/053407 herein incorporated by reference.

An “increased” or an “improved” insensitivity are used interchangeably herein. An “increased” or an “improved” insensitivity to an HPPD inhibitor comprises any significant increase in the insensitivity of the HPPD polypeptide to the inhibitor as determined through any enzymatic kinetic parameter, such as, for example, the dissociation constant (K_D) of the enzyme-inhibitor complex, the rates of association (k_{ON}), or dissociation (k_{OFF}) of inhibitor with or from enzyme, or the ratio of k_{OFF}/k_{ON} . This disclosure additionally defines parameters, “ON rate ratio”, “OFF rate ratio”, and “insensitivity parameter” that are not direct measurements of on and off rates but are measurements of the effect of the on and off rates inherent to a particular HPPD enzyme on its catalytic function when the enzyme is exposed to the inhibitor. An improvement in insensitivity need not show an improvement in all kinetic parameters. The improvement of a single kinetic parameter or any combination thereof is sufficient to classify the change as an improvement in insensitivity. In specific embodiments, the increased insensitivity to the HPPD inhibitor is determined by measuring these novel insensitivity parameters of the enzyme. Thus, in specific

embodiments, the increased insensitivity to an HPPD inhibitor comprises at least a 0.2, 0.3, 0.5, 0.7, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70 fold or greater improvement in a given kinetic parameter when compared to a native HPPD polypeptide and/or when compared to the maize wild-type HPPD sequence as set forth in SEQ ID NO:1.

In specific embodiments, an improved insensitivity to an HPPD inhibitor comprises (a) a slower rate of association of enzyme and inhibitor as quantified, for example, by a higher ON rate ratio than native HPPD; (b) a faster rate of dissociation of inhibitor from enzyme as quantified, for example, by a higher OFF rate ratio than native HPPD; and/or (c) both a slower rate of association of inhibitor with enzyme and a faster rate of dissociation of inhibitor from enzyme as quantified, for example, by a higher product of ON rate ratio and OFF rate ratio than native HPPD.

Methods to determine kinetic parameters for measuring the insensitivity of HPPD to an inhibitor are known. See also, Examples 1 and 4. Briefly, herbicidal inhibitors of HPPD form a tight complex with the enzyme by the dual mechanisms of coordination to the active site iron atom through a pair of keto oxygens and a Pi stack of the aromatic ring of the inhibitor between a pair of active site phenylalanines. As a result, conventional I_{50} determinations are not able to distinguish differences in binding affinity among various forms of HPPD and the inhibitor. All values will be the same, namely, 50% of the enzyme concentration. To devise a parameter for detecting changes in inhibitor binding affinity, K_D , one can utilize the relationship between K_D and the rates of binding and release of inhibitor (I) to and from the enzyme (E).



At equilibrium, rates of binding and release are equal. Thus,

$$k_{ON} [E] [I] = k_{OFF} [EI]$$

Written as a dissociation (products over reactants), the equation can be re-arranged to:

$$\frac{[E][I]}{[EI]} = \frac{k_{OFF}}{k_{ON}} = K_D$$

Higher K_D (reduced affinity or increased insensitivity) can be attained with a numerically smaller ON rate, a larger OFF rate or both. To detect changes in ON and OFF rates, one can observe the time course of an HPPD reaction as inhibitor binds to and inactivates the enzyme (ON rate), or is released from a pre-formed enzyme-inhibitor complex (OFF rate). See, for example, Example 1.

A quantitative indicator of ON rate can be obtained by monitoring the time courses of HPPD reactions in the presence and absence of inhibitor (e.g. mesotrione). The ratio of the reaction rate with inhibitor to that without inhibitor during the 70 to 90 second interval of the reaction is the "ON rate ratio".

5 A quantitative indicator of OFF rate can be obtained by observing the time course of an HPPD reaction as the HPPD inhibitor (such as mesotrione) is released from a pre-formed enzyme-inhibitor complex. Reaction velocity accelerates as inhibitor is released from the enzyme until a steady state is reached, during which the reaction velocity is constant. The ratio of the steady state rate in mixtures containing mesotrione (or other herbicidal inhibitor) to the initial velocity of
10 mixtures lacking inhibitor is termed the "OFF rate ratio". Another parameter to record is the time span required for the reactions with inhibitor to reach the steady state. In still further embodiments, to be sure that improvement in ON rates is being taken into account, the ON and OFF rate ratios are multiplied together and the product is termed the "insensitivity parameter."

The increased insensitivity of an HPPD inhibitor can also be determined by assaying the
15 increased insensitivity of a cell, a plant, a plant cell expressing said HPPD polypeptide or active fragment or variant thereof. In such instances, the cell, plant, or plant cell expressing an HPPD sequence having an increased insensitivity to an HPPD inhibitor will display an increased tolerance to the HPPD inhibitor or to a combination of HPPD inhibitors when compared to a control cell, plant or plant cell not expressing the HPPD sequence. "Increased tolerance" to a herbicide is
20 demonstrated when plants which display the increased tolerance to a herbicide are subjected to the HPPD inhibitor and a dose/response curve is shifted to the right when compared with that provided by an appropriate control plant. Such dose/response curves have "dose" plotted on the x-axis and "percentage injury", "herbicidal effect" etc. plotted on the y-axis. Plants which are substantially
25 "resistant" or "tolerant" to the herbicide exhibit few, if any, bleached, necrotic, lytic, chlorotic or other lesions and are not stunted, wilted or deformed when subjected to the herbicide at concentrations and rates which are typically employed by the agricultural community to kill weeds in the field.

For example, a plant expressing an HPPD polypeptide which displays an increased insensitivity to an HPPD inhibitor will tolerate statistically significantly higher levels of the HPPD
30 inhibitor than a control plant not expressing the HPPD polypeptide. In specific embodiments, a plant transfected and expressing one or more HPPD polypeptide sequence disclosed herein, and the active variants and fragments thereof, allow for an increased insensitivity to an HPPD inhibitor comprising, for example, at least a 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 100 fold or greater increase in a given performance parameter when compared to untransformed plants. See,

also, Examples 1 for exemplary assays.

Different HPPD polypeptides can provide different levels of tolerance to different HPPD-inhibitor herbicides. While a given HPPD polypeptide may provide a useful level of tolerance to some HPPD-inhibitor herbicides it may be quite inadequate to provide commercial levels of tolerance to a different HPPD-inhibitor herbicide which, for example, may control a different spectrum of weeds, be cheaper to make or offer environmental benefits. Thus, the various HPPD polypeptides disclosed herein can be used in combination in a single plant, plant explant or plant cell to expand and/or improve the tolerance to a desired HPPD herbicide or combination of HPPD herbicides.

B. Active Fragments and Variants of HPPD Sequences

Methods and compositions are provided which employ polynucleotides and polypeptides having HPPD activity and having an insensitivity to at least one HPPD inhibitor.

i. Polynucleotide and Polypeptide Fragments

Fragments and variants of HPPD polynucleotides and polypeptides are also encompassed by the present disclosure. By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain HPPD activity and HPPD inhibitor insensitivity. Alternatively, fragments of a polynucleotide that is useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, about 150 nucleotides, and up to the full-length polynucleotide encoding the HPPD polypeptides.

A fragment of an HPPD polynucleotide that encodes a biologically active portion of an HPPD protein of the disclosure will encode at least 20, 50, 75, 100, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 410, 415, 420, 425, 430, 435, or 440 contiguous amino acids, or up to the total number of amino acids present in a full-length HPPD polypeptide. Fragments of an HPPD polynucleotide that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of an HPPD protein.

Thus, a fragment of an HPPD polynucleotide may encode a biologically active portion of an HPPD polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an HPPD polypeptide can be prepared by isolating a portion of one of the HPPD polynucleotides, expressing the encoded portion of the HPPD polypeptides (e.g., by recombinant expression *in vitro*), and assessing the activity of the HPPD portion of the HPPD protein. Polynucleotides that are fragments of an HPPD nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650,

700, 800, 900, 1,000, 1,100, 1,200, 1,300, or 1,400 contiguous nucleotides, or up to the number of nucleotides present in a full-length HPPD polynucleotide disclosed herein.

In one embodiment, the HPPD polynucleotides and/or polypeptides comprise or encode an N-terminal truncation of the HPPD polypeptide. Such active HPPD fragments comprise an N-terminal deletion of at least the first 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more amino acids of the HPPD polypeptide of any one of SEQ ID NO:2-83. In specific embodiments, N-terminal deletions are designed to comprise a methionine residue on the N-terminus. In specific embodiments, a fragment of the HPPD polypeptide or polynucleotide comprising or encoding an N-terminal truncated HPPD polypeptide comprises or encodes a polypeptide having a deletion of amino acids 2-23 of any one of SEQ ID NO:2-83.

ii. Polynucleotide and Polypeptide Variants

"Variant" protein is intended to mean a protein derived from a native protein by deletion (i.e., truncation at the 5' and/or 3' end) and/or a deletion, or addition, of one or more amino acids at one or more internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, have HPPD activity and/or display insensitivity to a HPPD inhibitor as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a polynucleotide having a deletion (i.e., truncations) at the 5' and/or 3' end and/or a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the HPPD polypeptides of the disclosure. Naturally occurring variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis or gene synthesis but which still encode an HPPD polypeptide.

Biologically active variants of an HPPD polypeptide (and the polynucleotide encoding the same) will have at least about 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 95.7%, 95.9%, 96%, 96.3%,

96.5%, 96.9%, 97%, 97.3%, 97.5%, 97.9%, 98%, 98.3%, 98.5%, 98.9%, 99%, 99.3%, 99.5%, 99.6% or more sequence identity to the polypeptide of any one of SEQ ID NO:2-83.

In an aspect, biologically active variants of an HPPD polypeptide (and the polynucleotide encoding the same) will have at least about 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 95.7%, 95.9%, 96%, 96.3%, 96.5%, 96.9%, 97%, 97.3%, 97.5%, 97.9%, 98%, 98.3%, 98.5%, 98.9%, 99%, 99.3%, 99.5%, 99.6% or more sequence identity to the polypeptide of any one of SEQ ID NO:4, 7, 8, 18, and 18.

In other embodiments, variants of a HPPD polypeptides (and polynucleotide encoding the same) will have at least about 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 95.7%, 95.9%, 96%, 96.3%, 96.5%, 96.9%, 97%, 97.3%, 97.5%, 97.9%, 98%, 98.3%, 98.5%, 98.9%, 99%, 99.3%, 99.5% or more sequence identity to an HPPD polypeptide having an N-terminal deletion. For example, such polypeptides comprise an N-terminal truncation of any one of SEQ ID NO:2-83, comprising an N-terminal deletion of at least the first 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 amino acids wherein said active variant of the HPPD polypeptide comprises at least 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 95.7%, 95.9%, 96%, 96.3%, 96.5%, 96.9%, 97%, 97.3%, 97.5%, 97.9%, 98%, 98.3%, 98.5%, 98.9%, 99%, 99.3%, 99.5% or more sequence identity to an N-terminal deletion of the HPPD polypeptide. In further embodiments, the N-terminal truncates further comprise a methionine amino acid residue on the N-terminus. In specific embodiments, fragments of the HPPD polypeptide have a deletion of amino acids 2-23 of any one of SEQ ID NO:2-83. Thus, further provided are HPPD polypeptides comprising at least 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 95.7%, 95.9%, 96%, 96.3%, 96.5%, 96.9%, 97%, 97.3%, 97.5%, 97.9%, 98%, 98.3%, 98.5%, 98.9%, 99%, 99.3%, 99.5% or more sequence identity to the amino acid sequence set forth in any one of SEQ ID NO:2-83.

In other embodiments, variants of a particular polypeptide (and polynucleotide encoding the same) will have a bit score of at least 700, 710, 720, 721, 722, 723, 724, 725, 726, 728, 729, 730, 731, 732, 733, 734, 735, 736, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 828, 829, 830, 831, 832, 833, 834, 835, 836, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890 or greater as

determined by parameters described elsewhere herein to a polypeptide of any one of SEQ ID NO:2-83.

The HPPD polypeptide and the active variants and fragments thereof may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of the HPPD proteins can be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal.

Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and optimally will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

Variant polynucleotides and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different HPPD coding sequences can be manipulated to create a new HPPD possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the HPPD sequences disclosed herein and other known HPPD genes to obtain a new gene coding for a protein with an improved property of interest, such as a decreased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Cramer *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Cramer *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

In still further embodiments, the HPPD polypeptide or active variants and fragments thereof have been modified to remove one or more sequences that are present in other proteins which are

allergens. As used herein, a "matching sequence" comprises a region of 8 contiguous identical amino acids present in a known protein allergen. See, for example, Ladics (2008) *Food and Chemical Toxicology* 46: S20-S23, herein incorporated by reference. Such identified 8-amino acid sequences, however, do not necessarily have any allergenic potential or confer allergenicity.

5 Nonetheless, to comply with established criteria governing the potential for allergenic cross-reactivity (FAO/WHO 2001, Codex 2003), the matching sequences can be altered so the resulting polypeptide no longer contains the 8 residue contiguous match. Identification of such matching sequences is done by comparing the predicted amino acid sequence of the transgene product with that of a database of known or putative protein allergens and subsequently altering the identified amino

10 acid sequence to remove the allergenic match. The database utilized for this analysis was the AllergenOnline database of known protein allergens housed at the University of Nebraska (www.allergenonline.org). It is recognized that in specific embodiments, removal of the matching sequence via substitution, deletion and/or addition of amino acids will not impact the HPPD activity or the HPPD inhibitor insensitivity of the protein when compared to an appropriate control.

15 TAAAAGAA (amino acids 6-13 in maize wild-type HPPD SEQ ID NO:1) was changed to TATAAGAA (SEQ ID NO:101) to eliminate the 8 amino acid match to an allergen sequence in the database. Such a change does not alter the activity or insensitivity to herbicides of HPPD enzymes. See, for example, Codex Alimentarius Commission, Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy, June 30

20 – July 5, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and Appendix IV, Annex of the assessment of possible allergenicity, 47-60 and FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. January 22-25, 2001. Rome, Italy.

25 C. Chloroplast Transit Peptides

Further provided are various methods and compositions which comprise HPPD polypeptides and active variants and fragments thereof, and polynucleotides encoding the same, wherein the HPPD sequence comprises a chloroplast transit peptide. As used herein, the term "chloroplast transit peptide" will be abbreviated "CTP" and refers to the N-terminal portion of a chloroplast precursor

30 protein that directs the latter into chloroplasts and is subsequently cleaved off by the chloroplast processing protease. When a CTP is operably linked to the N-terminus of a polypeptide, the polypeptide is translocated into the chloroplast. Removal of the CTP from a native protein reduces or abolishes the ability of the native protein from being transported into the chloroplast. An operably linked chloroplast transit peptide is found at the N-terminus of the protein to be targeted to the

chloroplast and is located upstream and immediately adjacent to the transit peptide cleavage site that separates the transit peptide from the mature protein to be targeted to the chloroplast.

The term "chloroplast transit peptide cleavage site" refers to a site between two amino acids in a chloroplast-targeting sequence at which the chloroplast processing protease acts. Chloroplast transit peptides target the desired protein to the chloroplast and can facilitate the proteins translocation into the organelle. This is accompanied by the cleavage of the transit peptide from the mature polypeptide or protein at the appropriate transit peptide cleavage site by a chloroplast processing protease, native to the chloroplast. Accordingly, a chloroplast transit peptide further comprises a suitable cleavage site for the correct processing of the pre-protein to the mature polypeptide contained within the chloroplast.

Thus, any one of the polypeptides, or the polynucleotide encoding the same, or active variants and fragments thereof set forth in any one of SEQ ID NO:2-83, and biologically active variants and fragments thereof, can comprise a heterologous CTP sequence. Additional CTPs from HPPD polypeptides can be employed (see for example, PCT Publication No. WO 2012/021797, entitled "Methods and Compositions for Targeting Sequences of Interest to a Chloroplast.").

In still further embodiments, an HPPD polypeptide, and a polynucleotide encoding the same, is provided, wherein the HPPD polypeptide comprises a heterologous chloroplast transit peptide that is not from the wild-type maize HPPD polypeptide or an active variant or fragment thereof. Such heterologous chloroplast transit peptides are known, including but not limited to those derived from *Pisum* (JP 1986224990; E00977), carrot (Luo *et al.* (1997) *Plant Mol. Biol.*, 33 (4), 709-722 (Z33383), *Nicotiana* (Bowler *et al.*, EP 0359617; A09029), *Oryza* (de Pater *et al.* (1990) *Plant Mol. Biol.*, 15 (3), 399-406 (X51911), as well as synthetic sequences such as those provided in EP 0189707; U.S. Pat. No. 5,728,925; U.S. Pat. No. 5,717,084 (A10396 and A10398). In one embodiment, the heterologous chloroplast transit peptide is from the ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit precursor protein isolated from any plant. The Rubisco small subunit is well characterized from a variety of plants and the transit peptide from any of them are suitable for use in the compositions and methods of the present disclosure. See for example, *Physcomitrella* (Quatrano *et al.*, AW599738); *Lotus* (Poulsen *et al.*, AW428760); *Citrullus* (J. S. Shin, AI563240); *Nicotiana* (Appleby *et al.* (1997) *Heredity* 79(6), 557-563); alfalfa (Khouidi *et al.* (1997) *Gene*, 197(1/2), 343-351); potato and tomato (Fritz *et al.* (1993) *Gene*, 137(2), 271-4); wheat (Galili *et al.* (1991) *Theor. Appl. Genet.* 81(1), 98-104); and rice (Xie *et al.* (1987) *Sci. Sin.*, Ser. B (Engl. Ed.), 30(7), 706-19). For example, transit peptides may be derived from the Rubisco small subunit isolated from plants including but not limited to, soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, rice, *Arabidopsis*, sugar beet, sugar cane, canola,

millet, beans, peas, rye, flax, and forage grasses. In an aspect, methods and compositions of the present disclosure can utilize the Rubisco small subunit precursor protein from, for example, *Arabidopsis* or tobacco.

In other embodiments, the HPPD polypeptides and active variants and fragments thereof, and polynucleotide encoding the same, do not comprise a CTP. In such instances, the HPPD polypeptide and active variants and fragments thereof, or polynucleotides encoding the same, do not comprise a chloroplast transit peptide. Such polypeptides can be expressed in the cytoplasm of a plant, plant cell or explant and still confer insensitivity of the cell, plant or plant cell to an HPPD inhibitor. In still other embodiments, the HPPD polynucleotides lacking the chloroplast transit peptide are introduced directly into the chloroplast via chloroplast transformation. Such methods of chloroplast transformation are discussed in detail elsewhere herein.

Thus, further provided herein are HPPD polynucleotides and polypeptides and variants and fragments thereof that have HPPD activity and display insensitivity to an HPPD inhibitor and lack a chloroplast transit peptide. Various N-terminal truncations are described elsewhere herein.

In other embodiments, the HPPD polypeptide or polynucleotide encoding the same, lacks the first 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 or more amino acids of any one of SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 383, 384, 385, 386, 387, 388, 389, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 458, or 459 or biologically active variant or fragment thereof. In specific embodiments, the sequence set forth in any one of SEQ ID NO:2-83, and biologically active variants and fragments thereof, or the polynucleotide encoding the same, lack the first the 2-23 amino acid of the corresponding SEQ ID NO.

D. Sequence Comparisons

The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", and, (d) "percent sequence identity."

(a) As used herein, "reference sequence" is a defined sequence used as a basis for

sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence or protein sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polypeptide sequence, wherein the polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two polypeptides. Generally, the comparison window is at least 5, 10, 15, or 20 contiguous amino acid in length, or it can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polypeptide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the

present disclosure. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the present disclosure. BLASTP protein searches can be performed using default parameters. For example, see blast.ncbi.nlm.nih.gov/Blast.cgi.

5 To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, or PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide
10 sequences, BLASTP for proteins) can be used. See www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

In one embodiment, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring
15 matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find
20 the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater
25 than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an
30 integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for

alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored
5 when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same
10 when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity). When sequences differ in conservative substitutions, the percent sequence identity
15 may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percent sequence identity. Thus, for example, where an identical amino acid is given a score of 1
20 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percent sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the
25 polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of
30 positions in the window of comparison, and multiplying the result by 100 to yield the percent sequence identity.

(e) Two sequences are "optimally aligned" when they are aligned for similarity scoring using a defined amino acid substitution matrix (e.g., BLOSUM62), gap existence penalty and gap extension penalty so as to arrive at the highest score possible for that pair of sequences. Amino acids

substitution matrices and their use in quantifying the similarity between two sequences are well-known in the art and described, e.g., in Dayhoff et al. (1978) "A model of evolutionary change in proteins." In "Atlas of Protein Sequence and Structure," Vol. 5, Suppl. 3 (ed. M.O. Dayhoff), pp. 345-352. Natl. Biomed. Res. Found., Washington, DC and Henikoff et al. (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919. The BLOSUM62 matrix (Fig. 10) is often used as a default scoring substitution matrix in sequence alignment protocols such as Gapped BLAST 2.0. The gap existence penalty is imposed for the introduction of a single amino acid gap in one of the aligned sequences, and the gap extension penalty is imposed for each additional empty amino acid position inserted into an already opened gap. The gap existence penalty is imposed for the introduction of a single amino acid gap in one of the aligned sequences, and the gap extension penalty is imposed for each additional empty amino acid position inserted into an already opened gap. The alignment is defined by the amino acids positions of each sequence at which the alignment begins and ends, and optionally by the insertion of a gap or multiple gaps in one or both sequences, so as to arrive at the highest possible score. While optimal alignment and scoring can be accomplished manually, the process is facilitated by the use of a computer-implemented alignment algorithm, e.g., gapped BLAST 2.0, described in Altschul et al, (1997) Nucleic Acids Res. 25:3389-3402, and made available to the public at the National Center for Biotechnology Information Website (<http://www.ncbi.nlm.nih.gov>). Optimal alignments, including multiple alignments, can be prepared using, e.g., PSI-BLAST, available through <http://www.ncbi.nlm.nih.gov> and described by Altschul et al, (1997) Nucleic Acids Res. 25:3389-3402.

As used herein, similarity score and bit score is determined employing the BLAST alignment used the BLOSUM62 substitution matrix, a gap existence penalty of 11, and a gap extension penalty of 1. For the same pair of sequences, if there is a numerical difference between the scores obtained when using one or the other sequence as query sequences, a greater value of similarity score is selected.

E. Plants

Plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequences disclosed herein are provided. In specific embodiments, the plants and/or plant parts have stably incorporated at least one heterologous HPPD polypeptide disclosed herein or an active variant or fragment thereof. Thus, plants, plant cells, plant parts and seed are provided which comprise at least one heterologous HPPD sequence of any one of SEQ ID NO:2-83, and biologically active variants and fragments thereof, or any one of other variants disclosed herein, or a biologically active fragment and/or variant of the HPPD sequence. In specific embodiments, the HPPD sequences are characterized as having HPPD activity and having an insensitivity to an HPPD inhibitor.

Further provided are plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequences having a heterologous CTP as discussed elsewhere herein. In light of employing HPPDs with and without CTP sequences, the term “stably incorporated” in a plant, plant cell, plant part, explant, seed, or grain refers to the integration of the polynucleotide into the genomic DNA or to the integration of the polynucleotide into the genome of a plastid (i.e., the chloroplast, amyloplasts, chromoplasts, statoliths, leucoplasts, elaioplasts, and proteinoplasts).

In specific embodiments, the heterologous polynucleotide in the plant or plant part is operably linked to a constitutive, tissue-preferred, or other promoter for expression in plants.

As used herein, the term “plant” includes immature or mature whole plant, including a plant from which seed, grain, or anthers have been removed, as well as plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like.

As used herein, “grain” is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the disclosure, provided that these parts comprise introduced polynucleotides encoding a disclosed HPPD polypeptide.

As used herein, the term “plant part” or “plant parts” refers to any plant part whether part of an intact living or growing plant or whether isolated or separated from an intact living plant. Plant part includes, but is not limited, to protoplasts, leaves, stems, roots, root tips, anthers, pistils, seed, grain, embryo, pollen, ovules, cotyledon, hypocotyl, pod, flower, shoot, tissue, petiole, cells, meristematic cells, and the like.

As used herein, “explant” refers to plant tissue that is directly excised from an intact plant, such as a leaf, petal, sepal, stamen, filament anther, root, or stem.

The HPPD sequences and active variants and fragments thereof disclosed herein may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut

(*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar
 5 beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus
 10 (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing methods of the present disclosure include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir
 15 (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*), and Poplar and Eucalyptus. In specific embodiments, plants of the present disclosure are
 20 crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean plants are optimal, and in yet other embodiments corn plants are optimal.

Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum,
 25 rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

A “subject plant or plant cell” is one in which genetic alteration, such as transformation, has been affected as to a gene of interest, or is a plant or plant cell which is descended from a plant or
 30 cell so altered and which comprises the alteration. A “control” or “control plant” or “control plant cell” provides a reference point for measuring changes in phenotype of the subject plant or plant cell.

A control plant or plant cell may comprise, for example: (a) a wild-type plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has

been transformed with a null construct (i.e. with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to conditions or stimuli that would induce expression of the gene of interest; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

F. *Polynucleotide Constructs*

The use of the term "polynucleotide" is not intended to limit the present disclosure to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides of the disclosure also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

The HPPD polynucleotides disclosed herein can be provided in expression cassettes for expression in the plant of interest. The cassette can include 5' and 3' regulatory sequences operably linked to an HPPD polynucleotide or active variant or fragment thereof. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of interest and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked is intended that the coding regions are in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the HPPD polynucleotide or active variant or fragment thereof to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette can include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), an HPPD polynucleotide or active variant or fragment thereof, and a transcriptional and translational termination region (i.e., termination region) functional in plants. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the HPPD polynucleotide or active variant or fragment thereof may be native/analogous to the host cell or to each other. Alternatively, the regulatory

regions and/or the HPPD polynucleotide or active variant or fragment thereof may be heterologous to the host cell or to each other. As discussed in further detail elsewhere herein, the expression cassette can comprises a chimeric polynucleotide comprising a heterologous CTP operably linked to an HPPD polynucleotide.

5 As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are
10 substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

While it may be optimal to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs can change expression levels of the HPPD polynucleotide in the plant or plant cell. Thus, the phenotype of the plant or plant cell can be altered.

15 The termination region may be native with the transcriptional initiation region, may be native with the operably linked HPPD polynucleotide or active variant or fragment thereof, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the HPPD polynucleotide or active fragment or variant thereof, the plant host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also
20 Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

25 Where appropriate, the polynucleotides may be optimized for increased expression in the transformed plant. That is, the polynucleotides can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.*
30 (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average

for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene* 165(2):233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385. See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used to express the various HPPD sequence disclosed herein, including the native promoter of the polynucleotide sequence of interest. The promoters can be selected based on the desired outcome. Such promoters include, for example, constitutive, tissue-preferred, or other promoters for expression in plants.

Constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

Tissue-preferred promoters can be utilized to target enhanced HPPD expression within a particular plant tissue. Tissue-preferred promoters include those described in Yamamoto *et al.*

(1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

As discussed in more detail elsewhere herein, promoters that direct expression in a plastid, such as a chloroplast, can also be used to express the HPPD sequences or biologically active variants and fragments thereof.

Synthetic promoters can be used to express HPPD sequences or biologically active variants and fragments thereof. In one non-limiting embodiment, the HPPD sequences are expressed with a synthetic constitutive promoter (see for example U.S. Pat. Nos. 6,072,050 and 6,555,673) or with a promoter disclosed in International Publication No. WO 2012/021794 and US Patent No. 8,993,837, entitled "Chimeric Promoters and Methods of Use."). In still further embodiments, the HPPD variants operably linked to such synthetic promoters further comprise an Arabidopsis ubiquitin3 gene terminator (Callis *et al.* (1995) *Genetics* 139 (2), 921-939; Genbank L05363).

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glyphosate, glufosinate ammonium, bromoxynil, sulfonylureas, dicamba, and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β -galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su *et al.* (2004) *Biotechnol Bioeng* 85:610-9 and Fetter *et al.* (2004) *Plant Cell* 16:215-28), cyan fluorescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science* 117:943-54 and Kato *et al.* (2002) *Plant Physiol* 129:913-42), and yellow fluorescent protein (PhiYFP™ from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science* 117:943-54). For additional selectable markers, see generally,

Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschmidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present disclosure, including for example, DsRed as described in Example 1.

G. Stacking Other Traits of Interest

In some embodiments, the HPPD polynucleotides or active variants and fragments thereof disclosed herein are engineered into a molecular stack. Thus, the various plants, plant cells, plant parts, explants, seeds, and grain disclosed herein can further comprise one or more traits of interest, and in more specific embodiments, the plant, plant part or plant cell is stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired combination of traits. As used herein, the term “stacked” includes having the multiple traits present in the same plant (i.e., both traits are incorporated into the nuclear genome, one trait is incorporated into the nuclear genome and one trait is incorporated into the genome of a plastid, or both traits are incorporated into the genome of a plastid). In one non-limiting example, “stacked traits” comprise a molecular stack where the sequences are physically adjacent to each other. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. In one embodiment, the molecular stack comprises at least one additional polynucleotide that also confers tolerance to at least one HPPD inhibitor and/or at least one additional polynucleotide that confers tolerance to a second herbicide.

Thus, in one embodiment, the plants, plant cells, plant parts, explants, seeds, and grain having the HPPD polynucleotide or active variants or fragments thereof disclosed herein is stacked with at

least one other HPPD sequence, include the HPPD sequence, and variants and fragment thereof disclosed herein, as well as other HPPD sequence, which include but are not limited to the HPPD sequences set forth in U.S. Patent Nos. 6,245,968 B1; 6,268,549; and 6,069,115; and international publication WO 99/23886, each of which is herein incorporated by reference.

5 In still other embodiments, plants, plant cells, plant parts, explants, seeds, and grain comprise expression cassettes comprising the HPPD sequences or active variant and fragment thereof are stacked with a sequence that confers tolerance to HPPD inhibitors through a different mechanism than the HPPD polypeptide. For example, a P450 sequence could be employed which provides tolerance to HPPD-inhibitors by metabolism of the herbicide. Such sequences including, but are not
10 limited to, the NSF1 gene. See, US 2007/0214515 and US 2008/0052797 both of which are herein incorporated by reference in their entirety.

In some embodiments, plants, plant cells, plant parts, explants, seeds, and grain having the HPPD polynucleotides or active variants or fragment thereof may be stacked with other herbicide-tolerance traits to create a transgenic plant of the disclosure with further improved properties. Other
15 herbicide-tolerance polynucleotides that could be used in such embodiments include those conferring tolerance to glyphosate such as, for example, glyphosate N-acetyltransferase. See, for example, WO02/36782, US Publication 2004/0082770 and WO 2005/012515, US Patent No. 7,462,481, US Patent No. 7,405,074, each of which is herein incorporated by reference.

Additional glyphosate-tolerance traits include a sequence that encodes a glyphosate oxido-
20 reductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and 5,463,175. Other traits that could be combined with the HPPD sequence disclosed herein include those derived from polynucleotides that confer on the plant the capacity to produce a higher level or glyphosate insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), for example, as more fully described in U.S. Patent Nos. 6,248,876 B1; 5,627,061; 5,804,425; 5,633,435; 5,145,783; 4,971,908;
25 5,312,910; 5,188,642; 4,940,835; 5,866,775; 6,225,114 B1; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; Re. 36,449; RE 37,287 E; and 5,491,288; and international publications WO 97/04103; WO 00/66746; WO 01/66704; and WO 00/66747. Other traits that could be combined with the HPPD sequences disclosed herein include those conferring tolerance to sulfonylurea and/or imidazolinone, for example, as described more fully in U.S. Patent Nos.
30 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270.

Additional known genes that confer tolerance to herbicides and can be employed in the methods and compositions disclosed herein include, for example e.g., auxin, HPPD, glyphosate, dicamba, glufosinate, sulfonylurea, bromoxynil and norflurazon herbicides can be stacked either as a

molecular stack or a breeding stack with plants expressing the traits disclosed herein. Polynucleotide molecules encoding proteins involved in herbicide tolerance include, but are not limited to, a polynucleotide molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) disclosed in U.S. Pat. Nos. US39,247; 6,566,587 for imparting glyphosate tolerance; polynucleotide molecules
5 encoding a glyphosate oxidoreductase (GOX) disclosed in U.S. Pat. No. 5,463,175 and a glyphosate-N-acetyl transferase (GAT) disclosed in US Pat. Nos. 7,622,641; 7,462,481; 7,531,339; 7,527,955; 7,709,709; 7,714,188 and 7,666,643 also for providing glyphosate tolerance; dicamba monooxygenase disclosed in US Pat. No. 7,022,896 and WO2007146706A2 for providing dicamba tolerance; a polynucleotide molecule encoding AAD12 disclosed in U.S. Pat. App. Pub. No.
10 2005731044 or WO2007053482A2 or encoding AAD1 disclosed in US20110124503A1 or US 7,838,733 for providing tolerance to auxin herbicides (2,4-D); a polynucleotide molecule encoding hydroxyphenylpyruvate dioxygenase (HPPD) for providing tolerance to HPPD inhibitors (e.g., hydroxyphenylpyruvate dioxygenase) disclosed in e.g., US7935869; US20090055976A1; and US20110023180A1; each publication is herein incorporated by reference in its entirety.

15 In other embodiments, plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof is stacked with, for example, a sequence which confers tolerance to an acetolactate synthase (“ALS”) inhibitor. As used herein, an “ALS inhibitor-tolerant polypeptide” comprises any polypeptide which when expressed in a plant confers tolerance to at least one ALS inhibitor. A variety of ALS inhibitors are known and include, for
20 example, sulfonylurea, imidazolinone, triazolopyrimidines, pyrimidinyoxy(thio)benzoates, and/or sulfonylaminocarbonyl triazolinone herbicides. Additional ALS inhibitors are known and are disclosed elsewhere herein. It is known in the art that ALS mutations fall into different classes with regard to tolerance to sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl(thio)benzoates, including mutations having the following characteristics: (1) broad
25 tolerance to all four of these groups; (2) tolerance to imidazolinones and pyrimidinyl(thio)benzoates; (3) tolerance to sulfonylureas and triazolopyrimidines; and (4) tolerance to sulfonylureas and imidazolinones.

Various ALS inhibitor-tolerant polypeptides can be employed. In some embodiments, the ALS inhibitor-tolerant polynucleotides contain at least one nucleotide mutation resulting in one
30 amino acid change in the ALS polypeptide. In specific embodiments, the change occurs in one of seven substantially conserved regions of acetolactate synthase. See, for example, Hattori *et al.* (1995) *Molecular Genetics and Genomes* 246:419-425; Lee *et al.* (1998) *EMBO Journal* 7:1241-1248; Mazur *et al.* (1989) *Ann. Rev. Plant Phys.* 40:441-470; and U.S. Patent No. 5,605,011, each of which is incorporated by reference in their entirety. The ALS inhibitor-tolerant polypeptide can be

encoded by, for example, the SuRA or SuRB locus of ALS. In specific embodiments, the ALS inhibitor-tolerant polypeptide comprises the C3 ALS mutant, the HRA ALS mutant, the S4 mutant or the S4/HRA mutant or any combination thereof. Different mutations in ALS are known to confer tolerance to different herbicides and groups (and/or subgroups) of herbicides; see, e.g., Tranel and Wright (2002) *Weed Science* 50:700-712. See also, U.S. Patent No. 5,605,011, 5,378,824, 5,141,870, and 5,013,659, each of which is herein incorporated by reference in their entirety. The soybean, maize, and Arabidopsis HRA sequences are disclosed, for example, in WO2007/024782, herein incorporated by reference.

In some embodiments, the ALS inhibitor-tolerant polypeptide confers tolerance to sulfonylurea and imidazolinone herbicides. The production of sulfonylurea-tolerant plants and imidazolinone-tolerant plants is described more fully in U.S. Patent Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270, which are incorporated herein by reference in their entireties for all purposes. In specific embodiments, the ALS inhibitor-tolerant polypeptide comprises a sulfonamide-tolerant acetolactate synthase (otherwise known as a sulfonamide-tolerant acetohydroxy acid synthase) or an imidazolinone-tolerant acetolactate synthase (otherwise known as an imidazolinone-tolerant acetohydroxy acid synthase).

In further embodiments, plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof is stacked with, for example, a sequence which confers tolerance to an ALS inhibitor and glyphosate tolerance. In one embodiment, the HPPD sequence or active variant or fragment thereof is stacked with HRA and a glyphosate N-acetyltransferase. See, WO2007/024782, 2008/0051288 and WO 2008/112019, each of which is herein incorporated by reference.

In still other embodiments, plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof may be stacked with, for example, aryloxyalkanoate dioxygenase polynucleotides (which confer tolerance to 2,4-D and other phenoxy auxin herbicides as well as to aryloxyphenoxypropionate herbicides as described, for example, in WO2005/107437) and dicamba-tolerance polynucleotides as described, for example, in Herman *et al.* (2005) *J. Biol. Chem.* 280: 24759-24767, auxin polypeptides and an acetyl coenzyme A carboxylase (ACCase) polypeptides.

Other examples of herbicide-tolerance traits that could be combined with the plant or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof include those conferred by polynucleotides encoding an exogenous phosphinothricin acetyltransferase, as described in U.S. Patent Nos. 5,969,213; 5,489,520; 5,550,318; 5,874,265; 5,919,675; 5,561,236;

5,648,477; 5,646,024; 6,177,616; and 5,879,903. Plants containing an exogenous phosphinothricin acetyltransferase can exhibit improved tolerance to glufosinate herbicides, which inhibit the enzyme glutamine synthase. Other examples of herbicide-tolerance traits that could be combined with the plants or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof include those conferred by polynucleotides conferring altered protoporphyrinogen oxidase (protox) activity, as described in U.S. Patent Nos. 6,288,306 B1; 6,282,837 B1; and 5,767,373; and international publication WO 01/12825. Plants containing such polynucleotides can exhibit improved tolerance to any of a variety of herbicides which target the protox enzyme (also referred to as “protox inhibitors”).

Other examples of herbicide-tolerance traits that could be combined with the plants or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof include those conferring tolerance to at least one herbicide in a plant such as, for example, a maize plant or horseweed. Herbicide-tolerant weeds are known in the art, as are plants that vary in their tolerance to particular herbicides. See, *e.g.*, Green and Williams (2004) “Correlation of Corn (*Zea mays*) Inbred Response to Nicosulfuron and Mesotrione,” poster presented at the WSSA Annual Meeting in Kansas City, Missouri, February 9-12, 2004; Green (1998) *Weed Technology* 12: 474-477; Green and Ulrich (1993) *Weed Science* 41: 508-516. The trait(s) responsible for these tolerances can be combined by breeding or via other methods with the plants or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof to provide a plant of the disclosure as well as methods of use thereof.

In still further embodiments, the HPPD sequences can be stacked with at least one polynucleotide encoding a homogentisate solanesyltransferase (HST). See, for example, WO2010023911 herein incorporated by reference in its entirety. In such embodiments, classes of herbicidal compounds - which act wholly or in part by inhibiting HST can be applied over the plants having the HTS polypeptide.

Plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof can also be combined with at least one other trait to produce plants that further comprise a variety of desired trait combinations including, but not limited to, traits desirable for animal feed such as high oil content (*e.g.*, U.S. Patent No. 6,232,529); balanced amino acid content (*e.g.*, hordothionins (U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,409; U.S. Patent No. 5,850,016); barley high lysine (Williamson *et al.* (1987) *Eur. J. Biochem.* 165: 99-106; and WO 98/20122) and high methionine proteins (Pedersen *et al.* (1986) *J. Biol. Chem.* 261: 6279; Kirihara *et al.* (1988) *Gene* 71: 359; and Musumura *et al.* (1989) *Plant Mol. Biol.* 12:123)); increased digestibility (*e.g.*, modified storage proteins (U.S. Application Serial No. 10/053,410, filed

November 7, 2001); and thioredoxins (U.S. Application Serial No. 10/005,429, filed December 3, 2001)); the disclosures of which are herein incorporated by reference. Desired trait combinations also include LLNC (low linolenic acid content; see, *e.g.*, Dyer *et al.* (2002) *Appl. Microbiol. Biotechnol.* 59: 224-230) and OLCB (high oleic acid content; see, *e.g.*, Fernandez-Moya *et al.* (2005) *J. Agric. Food Chem.* 53: 5326-5330).

Plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof can also be combined with other desirable traits such as, for example, fumonisin detoxification genes (U.S. Patent No. 5,792,931), avirulence and disease resistance genes (Jones *et al.* (1994) *Science* 266: 789; Martin *et al.* (1993) *Science* 262: 1432; Mindrinos *et al.* (1994) *Cell* 78: 1089), and traits desirable for processing or process products such as modified oils (*e.g.*, fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (*e.g.*, ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE)); and polymers or bioplastics (*e.g.*, U.S. Patent No. 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine herbicide-tolerant polynucleotides with polynucleotides providing agronomic traits such as male sterility (*e.g.*, see U.S. Patent No. 5,583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (*e.g.*, WO 99/61619, WO 00/17364, and WO 99/25821); the disclosures of which are herein incorporated by reference.

In other embodiments, plants, plant cells, plant parts, explants, seeds, and grain having the HPPD sequence or an active variant or fragment thereof may be stacked with any other polynucleotides encoding polypeptides having pesticidal and/or insecticidal activity, such as *Bacillus thuringiensis* toxic proteins (described in U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; Geiser *et al.* (1986) *Gene* 48: 109; Lee *et al.* (2003) *Appl. Environ. Microbiol.* 69: 4648-4657 (*Vip3A*); Galitzky *et al.* (2001) *Acta Crystallogr. D. Biol. Crystallogr.* 57: 1101-1109 (*Cry3Bb1*); and Herman *et al.* (2004) *J. Agric. Food Chem.* 52: 2726-2734 (*Cry1F*)), lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.* 24: 825, pentin (described in U.S. Patent No. 5,981,722), and the like. The combinations generated can also include multiple copies of any one of the polynucleotides of interest.

These stacked combinations can be created by any method including, but not limited to, breeding plants by any conventional methodology, or genetic transformation. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. The traits can be introduced simultaneously in a co-

transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different
5 promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example,
10 WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

H. Method of Introducing

Various methods can be used to introduce a sequence of interest into a plant or plant part. "Introducing" is intended to mean presenting to the plant, plant cell or plant part the polynucleotide or polypeptide in such a manner that the sequence gains access to the interior of a cell of the plant.
15 The methods of the disclosure do not depend on a particular method for introducing a sequence into a plant or plant part, only that the polynucleotide or polypeptides gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotide or polypeptides into plants are known in the art including, but not limited to, stable transformation methods, transient
20 transformation methods, and virus-mediated methods.

"Stable transformation" is intended to mean that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. "Transient transformation" is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a plant.

25 Transformation protocols as well as protocols for introducing polypeptides or polynucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing polypeptides and polynucleotides into plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated
30 transformation (U.S. Patent No. 5,563,055 and U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent Nos. 4,945,050; U.S. Patent No. 5,879,918; U.S. Patent No. 5,886,244; and, 5,932,782; Tomes *et al.* (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926);

and Lec1 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Nos. 5,240,855; 5,322,783; and, 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

In specific embodiments, the HPPD sequences or active variant or fragments thereof can be provided to a plant, plant cell, plant part, or host cell using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the HPPD protein or active variants and fragments thereof directly into the plant, plant part, or plant cell. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway *et al.* (1986) *Mol Gen. Genet.* 202:179-185; Nomura *et al.* (1986) *Plant Sci.* 44:53-58; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci.* 91: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science* 107:775-784, all of which are herein incorporated by reference.

In other embodiments, polynucleotides encoding the peptides of the disclosure can be introduced into plants, plant parts, or plant cells by contacting plants, plant parts, or plant cells with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the disclosure within a DNA or RNA molecule. It is recognized that the an HPPD sequence may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that promoters of the disclosure also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing polynucleotides into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos.

5,889,191, 5,889,190, 5,866,785, 5,589,367, 5,316,931, and Porta *et al.* (1996) *Molecular Biotechnology* 5:209-221; herein incorporated by reference.

Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference. Briefly, polynucleotides encoding polypeptides of the disclosure can be contained in transfer cassette flanked by two non-recombinogenic recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its genome a target site which is flanked by two non-recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome. Other methods to target polynucleotides are set forth in WO 2009/114321 (herein incorporated by reference), which describes "custom" meganucleases produced to modify plant genomes, in particular the genome of maize. See, also, Gao *et al.* (2010) *Plant Journal* 1:176-187.

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 progeny 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 disclosure provides transformed seeds (also referred to as "transgenic seed") having a polynucleotide encoding polypeptides of the disclosure, for example, an expression cassette of the disclosure, stably incorporated into their genome.

I. Chloroplast Transformation

In specific embodiments, the HPPD polypeptides and active variants and fragments thereof, and polynucleotide encoding the same, do not comprise or encode a CTP. Such polynucleotides can be expressed from the nuclear genome of plants, plant cells, plant parts, explants, seeds, and grain and the polypeptides acting from the cytoplasm still confer insensitivity of the cell, plant or plant cell to an HPPD inhibitor. In still other embodiments, the HPPD polynucleotides lacking the chloroplast transit peptide are introduced directly into the chloroplast via chloroplast transformation. Such methods of chloroplast transformation are discussed in detail elsewhere herein. Thus, chloroplasts having stably incorporated in their genome a polynucleotide encoding an HPPD polypeptide or an

active variant or fragment thereof lacking a CTP as described herein are provided.

In other embodiments, only the HPPD polypeptides or active variants and fragments thereof are in the chloroplast of a plant or plant cell. In such instances, the HPPD polypeptide can comprise a chloroplast transit peptide and can be expressed from a polynucleotide incorporated into the nuclear genome. In such an instance, the HPPD polypeptide is transported into the chloroplast, the CTP is removed, and the mature form of the HPPD polypeptide is then found within the chloroplast.

In other embodiments, the polynucleotide encoding the HPPD polypeptide or active variant or fragment thereof is incorporated directly into the genome of the chloroplast. In such instances, the HPPD polypeptide need not comprise a CTP.

The sequences 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 polynucleotide of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

As used herein, a "plastid" refers to an organelle present in plant cells that stores and manufactures chemical compounds used by the cell, such as starch, fatty acids, terpenes, and that has been derived from a proplastid. Thus, plastids of plants typically have the same genetic content. Plastids include chloroplasts, which are responsible for photosynthesis, amyloplasts, chromoplasts, statoliths, leucoplasts, elaioplasts, and proteinoplasts.

The plastid genome is circular and varies in size among plant species from about 120 to about 217 kilobase pairs (kb). The genome typically includes a large inverted repeat, which can contain up to about 76 kilobase pairs, but which is more typically in the range of about 20 to about 30 kilobase pairs. The inverted repeat present in the plastid genome of various organisms has been described (Palmer (1990) *Trends Genet* 6:115-120).

Methods are known in the art for introducing genes into the plastid genome. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90: 913-917; Svab and Maliga (1993) *EMBO J.* 12: 601-606; and U.S. Patent Nos. 5,451,513 and 5,545,818; each of which is herein incorporated by reference in its entirety.

One method involves the integration of a polynucleotide of interest into the plastid genome through homologous recombination. Such methods involve the introduction of a polynucleotide of interest flanked by regions of homology with regions of the plastid genome into a plant cell. Delivery of the polynucleotide of interest into the plant cell can be via any method of transformation known in the art, including those described elsewhere herein. These include, but are not limited to, particle gun delivery (Svab, Z. *et al.* (1990) *Proc Natl Acad Sci USA* 87:8526-8530; Svab and Maliga (1993) *Proc Natl Acad Sci USA* 90:913-917; and Staub and Maliga (1993) *EMBO J* 12:601-606; and

U.S. Pat. Nos. 5,451,513 and 5,545,818; each of which is herein incorporated by reference in its entirety). In some species, protoplasts can also be used for chloroplast transformation (O'Neill *et al.* (1993) *Plant J* 3:729-38; and Spoerlein *et al.* (1991) *Theor Appl Gen* 82:717-722; each of which is herein incorporated by reference in its entirety). Once the polynucleotide of interest flanked by the homologous regions enters the cell, the polynucleotide of interest will be integrated within the plastid genome.

The homologous regions flanking the polynucleotide of interest, and in some embodiments, its operably linked promoter, and in particular embodiments, the selectable marker gene as well may vary in length. In some embodiments, the region of homology with the plastid genome is about 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 base pairs or greater in length. In most instances, the frequency of recombination and thus the frequency of obtaining plants having transformed plastids decreases with the decreasing size of the homologous regions. In those embodiments wherein the regions of homology are present in the inverted repeat regions of the plastid genome, two copies of the polynucleotide of interest are expected per transformed plastid.

In some embodiments, the polynucleotide of interest can be co-delivered with a selectable marker gene that is active in the plastid. The selectable marker gene and the polynucleotide of interest can be present on a single DNA construct or on separate constructs. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Genes conferring resistance to kanamycin (NPTII or AphA6) have been used as a selectable marker for plastid transformation (Carrer *et al.* (1993) *Mol Gen Genetics* 241:49-56; and Huang *et al.* (2002) *Mol Gen Genomics* 268:19-27; each of which is herein incorporated by reference in its entirety). Other genes which encode a product involved in chloroplast metabolism may also be used as selectable markers.

Another example of a selectable marker gene for plastid transformation is a selectable marker gene that confers resistance to a substance which inhibits protein synthesis by the plastids, such that cells which have acquired the phenotype are selected for by contacting the cells with a substance which inhibits protein synthesis by the plastids. The plastid DNA encoding the nonlethal selectable phenotype may comprise 16S ribosomal DNA mutated to confer resistance to the effects of streptomycin, or to spectinomycin, or to both antibiotics simultaneously. Expression of heterologous genes that modify non-lethal antibiotics such as streptomycin or spectinomycin by phosphorylation, adenylation or acetylation also are suitable for the selection of plastid transformation events. Another non-limiting example of a gene that confers resistance to streptomycin and spectinomycin is the bacterial *aadA* gene that codes for streptomycin/spectinomycin adenylyltransferase (Svab *et al.* (1993) *Proc Natl Acad Sci USA* 90:913-917). The *aadA* gene product allows for continued growth

and greening of cells in the presence of streptomycin or spectinomycin whose chloroplasts comprise the selectable marker gene product. Cells which do not contain the selectable marker gene product are bleached. Selection for the *aadA* gene marker is thus based on identification of plant cells which are not bleached by the presence of streptomycin or spectinomycin, in the plant growth medium.

5 Other examples of selectable marker genes are those that confer resistance to an herbicide, including a photosystem II herbicide, such as a triazine herbicide, specifically the triazine herbicide atrazine. This phenotype not only provides nonlethal selection, but also provides herbicide resistance. Genes that provide resistance to plant herbicides such as glyphosate, bromoxynil, or imidazolinone may find use as a selectable marker gene. Such genes have been reported (Stalker *et al.* (1985) *J Biol Chem* 260:4724-4728 (glyphosate resistant EPSP); Stalker *et al.* (1985) *J Biol Chem* 10 263:6310-6314 (bromoxynil resistant nitrilase gene); and Sathasivan *et al.* (1990) *Nucl Acids Res* 18:2188 (AHAS imidazolinone resistance gene); each of which is herein incorporated by reference in its entirety).

The selectable marker gene and/or the polynucleotide of interest can be placed under the regulatory control of a chloroplast 5' promoter and 3' transcription termination regions, such as the tobacco 16S rRNA promoter *rrn* region and *rps16* 3' termination region. Numerous additional promoter regions may also be used to drive expression of the selectable marker gene and/or the polynucleotide of interest, including various plastid promoters and bacterial promoters which have been shown to function in plant plastids. Further, if nuclear expression of the selectable marker gene and/or the polynucleotide of interest is not desired, plastid introns can be incorporated into the selectable marker gene and/or the polynucleotide of interest. Certain classes of plastid introns cannot be correctly spliced out in the nucleus, thereby preventing expression of the selectable marker gene and/or the polynucleotide of interest within the nucleus. The polynucleotide of interest and/or the heterologous polynucleotide encoding the cell proliferation factor 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 polynucleotide may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

An additional method of plastid transformation occurs through the transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91: 7301-7305, which is herein incorporated by reference in its entirety. In these methods, the heterologous polynucleotide encoding the cell proliferation factor is introduced into the cell and expressed prior to, during, or immediately after the expression of the plastid-directed RNA polymerase.

In order to select those cells having transformed plastids, following introduction of the chloroplast transformation vectors, the treated tissue is placed on tissue culture medium containing the appropriate selection agent. After a suitable period of incubation on selection medium, transformed cells can be identified and grown to a stage that allows regeneration of the whole plants.

5 The regeneration processes are basically identical to those used for standard nuclear transformation events. Special care must be taken to ensure that selection and regeneration conditions promote the elimination of most wild-type chloroplast genomes. The status of the proportion of wild-type to transformed chloroplast genomes can be monitored by standard molecular techniques including Southern and PCR analysis.

10 For tobacco and a number of other species, leaves are a preferred target for plastid transformation. Chloroplast transformation has been described for tobacco (Svab, Zora; Hajdukiewicz *et al.* (1990) *Proceedings of the National Academy of Sciences of the United States of America* 87(21):8526-30), Arabidopsis (Sikdar, S. R.; Serino, G.; Chaudhuri, S.; Maliga, P (1998) *Plant Cell Reports* 18(1-2):20-24), tomato (Ruf *et al.* (2001) *Nature Biotechnology*:19(9):870-875),
 15 potato (Sidorov *et al.* (1999) *Plant Journal* 19(2): 209-216). For soybean, embryogenic suspension cultures can be targeted for transformation (Dufourmantel *et al.* (2004) *Plant Molecular Biology*:55(4), 479-489; US20070039075 A1).

II. Methods of Use

A. Methods for Increasing Concentration of at Least One HPPD Sequence or an Active Variant or Fragment Therefore in a Plant or Plant Part

20 A method for increasing the concentration of an HPPD polypeptide disclosed herein or an active variant or fragment thereof in plants, plant cells, plant parts, explants, seeds, grain or a chloroplast is provided. HPPD activity and insensitivity to HPPD inhibitors are discussed in detail elsewhere herein. In further embodiments, the concentration/level of the HPPD polypeptide is
 25 increased in a plant or plant part by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 500%, 1000%, 5000%, or 10,000% relative to an appropriate control plant, plant part, or cell which did not have the HPPD sequence. In still other embodiments, the level of the HPPD polypeptide in the plant or plant part is increased by 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 fold or more compared to the level of the native HPPD sequence. Such an increase in the level of the HPPD
 30 polypeptide can be achieved in a variety of ways including, for example, by the expression of multiple copies of one or more HPPD polypeptide and/or by employing a promoter to drive higher levels of expression of the sequence.

In specific embodiments, the polypeptide or the HPPD polynucleotide or active variant or fragment thereof is introduced into plants, plant cells, plant parts, explants, seeds, and grain.

Subsequently, a plant cell having the introduced sequence of the disclosure is selected using methods known to those of skill in the art such as, but not limited to, Southern blot analysis, DNA sequencing, PCR analysis, or phenotypic analysis. A plant cell altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or activity of polypeptides of the present disclosure in the plant. Plant forming conditions are well known in the art and discussed briefly elsewhere herein.

In one embodiment, a method of producing an HPPD herbicide tolerant plant cell is provided and comprises transforming a plant cell with a polynucleotide encoding an HPPD polypeptide or active variant or fragment thereof. In specific embodiments, the method further comprises selecting a plant cell which is resistant or tolerant to an HPPD herbicide by growing the plant cells in a sufficient concentration of an HPPD herbicide, such that the herbicide bleaches the plant cells which do not comprise the HPPD polypeptide of interest.

It is recognized that the incubation of the cells with the HPPD herbicide can occur before or after transformation with the HPPD polynucleotide of interest. For example, in one embodiment, a method comprises culturing a plant cell in the presence of a sufficient concentration of an HPPD herbicide such that said plant cell displays bleaching and then transforming into the bleached plant cells a polynucleotide encoding an HPPD polypeptide as disclosed herein. The plant cells are then grown, wherein the transformed plants cells no longer display bleaching. See, for example, US Patent No. 6,791,014, herein incorporated by reference in its entirety.

It is also recognized that the level and/or activity of the native HPPD sequence in a plant may be altered by employing a polynucleotide that is not capable of directing, in a transformed plant, the expression of a protein or an RNA. For example, the HPPD polynucleotide or active variant or fragment thereof disclosed herein may be used to design polynucleotide constructs that can be employed in methods for altering or mutating a genomic nucleotide sequence in an organism. Such polynucleotide constructs include, but are not limited to, RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use are known in the art. See, U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; herein incorporated by reference.

It is therefore recognized that methods of the present disclosure do not depend on the incorporation of the entire polynucleotide into the genome, only that the plant or cell thereof is altered as a result of the introduction of the polynucleotide into a cell. In one embodiment of the

disclosure, the genome may be altered following the introduction of the polynucleotide into a cell. Alterations to the genome of the present disclosure include, but are not limited to, additions, deletions, and substitutions of nucleotides into the genome. While the methods of the present disclosure do not depend on additions, deletions, and substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprises at least one nucleotide.

B. Method of Producing Crops and Controlling Weeds

Methods for controlling weeds in an area of cultivation, preventing the development or the appearance of herbicide resistant weeds in an area of cultivation, producing a crop, and increasing crop safety are provided. The term “controlling,” and derivations thereof, for example, as in “controlling weeds” refers to one or more of inhibiting the growth, germination, reproduction, and/or proliferation of; and/or killing, removing, destroying, or otherwise diminishing the occurrence and/or activity of a weed.

As used herein, an “area of cultivation” comprises any region in which one desires to grow a plant. Such areas of cultivations include, but are not limited to, a field in which a plant is cultivated (such as a crop field, a sod field, a tree field, a managed forest, a field for culturing fruits and vegetables, etc), a greenhouse, a growth chamber, etc.

As used herein, by “selectively controlled” it is intended that the majority of weeds in an area of cultivation are significantly damaged or killed, while if crop plants are also present in the field, the majority of the crop plants are not significantly damaged. Thus, a method is considered to selectively control weeds when at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more of the weeds are significantly damaged or killed, while if crop plants are also present in the field, less than 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 1% of the crop plants are significantly damaged or killed.

Methods provided comprise planting the an area of cultivation with a transgenic seed derived there from a plant having an HPPD sequence or active variant or fragment thereof disclosed herein or, and in specific embodiments, applying to the crop, seed, weed or area of cultivation thereof an effective amount of a herbicide of interest. It is recognized that the herbicide can be applied before or after the crop is planted in the area of cultivation (e.g., seeds are planted). Herbicide applications can include an application of an HPPD inhibitor including, but not limited to, triketones (such as, mesotrione, sulcotrione, topremezone, and tembotrione) including agriculturally suitable salts (e.g., sodium salts) thereof; isoxazoles (such as, pyrasulfotole and isoxaflutole) including agriculturally suitable salts (e.g., sodium salts) thereof; pyrazoles (such as, benzofenap, pyrazoxyfen, and pyrazolynate) including agriculturally suitable salts (e.g., sodium salts) thereof; and benzobicyclon,

including agriculturally suitable salts (*e.g.*, sodium salts) thereof. See, WO2005/053407. In specific embodiments, a combination of two or more HPPD inhibitors is applied. Generally, the effective amount of herbicide applied to the field is sufficient to selectively control the weeds without significantly affecting the crop.

5 “Weed” as used herein refers to a plant which is not desirable in a particular area. Conversely, a “crop plant” as used herein refers to a plant which is desired in a particular area, such as, for example, a maize or soy plant. Thus, in some embodiments, a weed is a non-crop plant or a non-crop species, while in some embodiments, a weed is a crop species which is sought to be eliminated from a particular area, such as, for example, an inferior and/or non-transgenic soy plant in
10 a field planted with a plant having the HPPD sequence disclosed herein or an active variant or fragment thereof.

Further provided is a method for producing a crop by growing a crop plant that is tolerant to an HPPD herbicide as a result of being transformed with an HPPD polynucleotide or active variant or fragment thereof disclosed herein, under conditions such that the crop plant produces a crop, and
15 harvesting the crop. Preferably, an HPPD inhibitor is applied to the plant, or in the vicinity of the plant, at a concentration effective to control weeds without preventing the transgenic crop plant from growing and producing the crop. The application of the HPPD inhibitor can be before planting, or at any time after planting up to and including the time of harvest. The HPPD inhibitor can be applied once or multiple times. The timing of the HPPD inhibitor application, amount applied, mode of
20 application, and other parameters will vary based upon the specific nature of the crop plant and the growing environment, and can be readily determined by one of skill in the art. The disclosure further provides the crop produced by this method.

Further provided are methods for the propagation of a plant containing a HPPD polypeptide or active variant or fragment thereof. The plant can be, for example, a monocot or a dicot. In one
25 aspect, propagation entails crossing a plant containing a HPPD polynucleotide transgene with a second plant, such that at least some progeny of the cross display HPPD inhibitor tolerance.

The methods of the disclosure further allow for the development of herbicide applications to be used with the plants having the HPPD sequence or active variants or fragments thereof. In such methods, the environmental conditions in an area of cultivation are evaluated. Environmental
30 conditions that can be evaluated include, but are not limited to, ground and surface water pollution concerns, intended use of the crop, crop tolerance, soil residuals, weeds present in area of cultivation, soil texture, pH of soil, amount of organic matter in soil, application equipment, and tillage practices. Upon the evaluation of the environmental conditions, an effective amount of a combination of herbicides can be applied to the crop, crop part, seed of the crop or area of cultivation.

Any herbicide or combination of herbicides can be applied to the plant having the HPPD sequence or active variant or fragment thereof disclosed herein or transgenic seed derived there from, crop part, or the area of cultivation containing the crop plant. By “treated with a combination of” or “applying a combination of” herbicides to a crop, area of cultivation or field” it is intended that a particular field, crop or weed is treated with each of the herbicides and/or chemicals indicated to be part of the combination so that a desired effect is achieved, *i.e.*, so that weeds are selectively controlled while the crop is not significantly damaged. The application of each herbicide and/or chemical may be simultaneous or the applications may be at different times (sequential), so long as the desired effect is achieved. Furthermore, the application can occur prior to the planting of the crop.

Classifications of herbicides (*i.e.*, the grouping of herbicides into classes and subclasses) are well-known in the art and include classifications by HRAC (Herbicide Resistance Action Committee) and WSSA (the Weed Science Society of America) (see also, Retzinger and Mallory-Smith (1997) *Weed Technology* 11: 384-393). An abbreviated version of the HRAC classification (with notes regarding the corresponding WSSA group) is set forth below in Table 1.

Herbicides can be classified by their mode of action and/or site of action and can also be classified by the time at which they are applied (*e.g.*, preemergent or postemergent), by the method of application (*e.g.*, foliar application or soil application), or by how they are taken up by or affect the plant or by their structure. “Mode of action” generally refers to the metabolic or physiological process within the plant that the herbicide inhibits or otherwise impairs, whereas “site of action” generally refers to the physical location or biochemical site within the plant where the herbicide acts or directly interacts. Herbicides can be classified in various ways, including by mode of action and/or site of action (see, *e.g.*, Table 1 which shows an abbreviated version of HRAC Herbicide Classification).

Often, a herbicide-tolerance gene that confers tolerance to a particular herbicide or other chemical on a plant expressing it will also confer tolerance to other herbicides or chemicals in the same class or subclass, for example, a class or subclass set forth in Table 1. Thus, in some embodiments, a transgenic plant is tolerant to more than one herbicide or chemical in the same class or subclass, such as, for example, an HPPD inhibitor, glyphosate, an ALS chemistry, an inhibitor of PPO, a sulfonylurea, and/or a synthetic auxin.

Typically, the plants of the present disclosure can tolerate treatment with different types of herbicides (*i.e.*, herbicides having different modes of action and/or different sites of action) thereby permitting improved weed management strategies that are recommended in order to reduce the incidence and prevalence of herbicide-tolerant weeds.

Table 1.**I. ALS Inhibitors (WSSA Group 2)****A. Sulfonylureas**

1. Azimsulfuron
2. Chlorimuron-ethyl
3. Metsulfuron-methyl
4. Nicosulfuron
5. Rimsulfuron
6. Sulfometuron-methyl
7. Thifensulfuron-methyl
8. Tribenuron-methyl
9. Amidosulfuron
10. Bensulfuron-methyl
11. Chlorsulfuron
12. Cinosulfuron
13. Cyclosulfamuron
14. Ethametsulfuron-methyl
15. Ethoxysulfuron
16. Flazasulfuron
17. Flupyrsulfuron- methyl
18. Foramsulfuron
19. Imazosulfuron
20. Iodosulfuron-methyl
21. Mesosulfuron-methyl
22. Oxasulfuron
23. Primisulfuron-methyl
24. Prosulfuron
25. Pyrazosulfuron-ethyl
26. Sulfosulfuron
27. Triasulfuron
28. Trifloxysulfuron
29. Triflusulfuron-methyl
30. Tritosulfuron
31. Halosulfuron-methyl
32. Flucetosulfuron

B.**Sulfonylaminocarbonyltriazolinones**

1. Flucarbazone
2. Procarbazone

C. Triazolopyrimidines

1. Cloransulam-methyl

2. Flumetsulam
3. Diclosulam
4. Florasulam
5. Metosulam
6. Penoxsulam
7. Pyroxsulam

D. Pyrimidinyloxy(thio)benzoates

1. Bispyribac
2. Pyriftalid
3. Pyribenzoxim
4. Pyriothiobac
5. Pyriminobac-methyl

E. Imidazolinones

1. Imazapyr
2. Imazethapyr
3. Imazaquin
4. Imazapic
5. Imazamethabenz-methyl
6. Imazamox

II. Other Herbicides--Active
Ingredients/ Additional Modes of Action

A. Inhibitors of Acetyl CoA
carboxylase (ACCase) (WSSA Group 1)

1. Aryloxyphenoxypropionates
(‘FOPs’)

- a. Quizalofop-P-ethyl
- b. Diclofop-methyl
- c. Clodinafop-propargyl
- d. Fenoxaprop-P-ethyl
- e. Fluazifop-P-butyl
- f. Propaquizafop
- g. Haloxyfop-P-methyl
- h. Cyhalofop-butyl
- i. Quizalofop-P-ethyl

2. Cyclohexanediones (‘DIMs’)

- a. Alloxydim
- b. Butroxydim
- c. Clethodim

- d. Cycloxydim
- e. Sethoxydim
- f. Tepraloxym
- g. Tralkoxydim

B. Inhibitors of Photosystem II—
HRAC Group C1/ WSSA Group 5

- 1. Triazines
 - a. Ametryne
 - b. Atrazine
 - c. Cyanazine
 - d. Desmetryne
 - e. Dimethametryne
 - f. Prometon
 - g. Prometryne
 - h. Propazine
 - i. Simazine
 - j. Simetryne
 - k. Terbumeton
 - l. Terbutylazine
 - m. Terbutryne
 - n. Trietazine
- 2. Triazinones
 - a. Hexazinone
 - b. Metribuzin
 - c. Metamitron
- 3. Triazolinone
 - a. Amicarbazone
- 4. Uracils
 - a. Bromacil
 - b. Lenacil
 - c. Terbacil
- 5. Pyridazinones
 - a. Pyrazon
- 6. Phenyl carbamates
 - a. Desmedipham
 - b. Phenmedipham

C. Inhibitors of Photosystem II--
HRAC Group C2/WSSA Group 7

1. Ureas

- a. Fluometuron
- b. Linuron
- c. Chlorobromuron
- d. Chlorotoluron
- e. Chloroxuron
- f. Dimefuron
- g. Diuron
- h. Ethidimuron
- i. Fenuron
- j. Isoproturon
- k. Isouron
- l. Methabenzthiazuron
- m. Metobromuron
- n. Metoxuron
- o. Monolinuron
- p. Neburon
- q. Siduron
- r. Tebuthiuron

2. Amides

- a. Propanil
- b. Pentanochlor

D. Inhibitors of Photosystem II--
HRAC Group C3/ WSSA Group 6

1. Nitriles

- a. Bromofenoxim
- b. Bromoxynil
- c. Ioxynil

2. Benzothiadiazinone

(Bentazon)

- a. Bentazon

3. Phenylpyridazines

- a. Pyridate
- b. Pyridafol

E. Photosystem-I-electron
diversion (Bipyridyliums) (WSSA
Group 22)

1. Diquat

2. Paraquat

F. Inhibitors of PPO (protoporphyrinogen oxidase) (WSSA Group 14)

1. Diphenylethers
 - a. Acifluorfen-Na
 - b. Bifenox
 - c. Chlomethoxyfen
 - d. Fluoroglycofen-ethyl
 - e. Fomesafen
 - f. Halosafen
 - g. Lactofen
 - h. Oxyfluorfen
2. Phenylpyrazoles
 - a. Fluazolate
 - b. Pyraflufen-ethyl
3. N-phenylphthalimides
 - a. Cinidon-ethyl
 - b. Flumioxazin
 - c. Flumiclorac-pentyl
4. Thiadiazoles
 - a. Fluthiacet-methyl
 - b. Thidiazimin
5. Oxadiazoles
 - a. Oxadiazon
 - b. Oxadiargyl
6. Triazolinones
 - a. Carfentrazone-ethyl
 - b. Sulfentrazone
7. Oxazolidinediones
 - a. Pentoxazone
8. Pyrimidindiones
 - a. Benzfendizone
 - b. Butafencil

9. Others

- a. Pyrazogyl
- b. Profluazol

G. Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS) (WSSA Group 12)

1. Pyridazinones

- a. Norflurazon

2. Pyridinecarboxamides

- a. Diflufenican
- b. Picolinafen

3. Others

- a. Beflubutamid
- b. Fluridone
- c. Flurochloridone
- d. Flurtamone

H. Bleaching: Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD) (WSSA Group 28)

1. Triketones

- a. Mesotrione
- b. Sulcotrione
- c. topramezone
- d. tembotrione

2. Isoxazoles

- a. Pyrasulfotole
- b. Isoxaflutole

3. Pyrazoles

- a. Benzofenap
- b. Pyrazoxyfen
- c. Pyrazolynate

4. Others

- a. Benzobicyclon

I. Bleaching: Inhibition of
carotenoid biosynthesis (unknown
target) (WSSA Group 11 and 13)

1. Triazoles (WSSA Group
11)

a. Amitrole

2. Isoxazolidinones (WSSA
Group 13)

a. Clomazone

3. Ureas

a. Fluometuron

3. Diphenylether

a. Aclonifen

J. Inhibition of EPSP Synthase

1. Glycines (WSSA Group 9)

a. Glyphosate

b. Sulfosate

K. Inhibition of glutamine
synthetase

1. Phosphinic Acids

a. Glufosinate-ammonium

b. Bialaphos

L. Inhibition of DHP
(dihydropteroate) synthase (WSSA
Group 18)

1 Carbamates

a. Asulam

M. Microtubule Assembly
Inhibition (WSSA Group 3)

1. Dinitroanilines

a. Benfluralin

b. Butralin

c. Dinitramine

d. Ethalfluralin

e. Oryzalin

- f. Pendimethalin
- g. Trifluralin
- 2. Phosphoroamidates
 - a. Amiprofos-methyl
 - b. Butamiphos
- 3. Pyridines
 - a. Dithiopyr
 - b. Thiazopyr
- 4. Benzamides
 - a. Pronamide
 - b. Tebutam
- 5. Benzenedicarboxylic acids
 - a. Chlorthal-dimethyl

N. Inhibition of
mitosis/microtubule organization WSSA
Group 23)

- 1. Carbamates
 - a. Chlorpropham
 - b. Propham
 - c. Carbetamide

O. Inhibition of cell division
(Inhibition of very long chain fatty acids
as proposed mechanism; WSSA Group
15)

- 1. Chloroacetamides
 - a. Acetochlor
 - b. Alachlor
 - c. Butachlor
 - d. Dimethachlor
 - e. Dimethanamid
 - f. Metazachlor
 - g. Metolachlor
 - h. Pethoxamid
 - i. Pretilachlor
 - j. Propachlor
 - k. Propisochlor
 - l. Thenylchlor

2. Acetamides
 - a. Diphenamid
 - b. Napropamide
 - c. Naproanilide
3. Oxyacetamides
 - a. Flufenacet
 - b. Mefenacet
4. Tetrazolinones
 - a. Fentrazamide
5. Others
 - a. Anilofos
 - b. Cafenstrole
 - c. Indanofan
 - d. Piperophos

P. Inhibition of cell wall
(cellulose) synthesis

1. Nitriles (WSSA Group 20)
 - a. Dichlobenil
 - b. Chlorthiamid
2. Benzamides (isoxaben (WSSA Group 21))
 - a. Isoxaben
3. Triazolocarboxamides (flupoxam)
 - a. Flupoxam

Q. Uncoupling (membrane disruption): (WSSA Group 24)

1. Dinitrophenols
 - a. DNOC
 - b. Dinoseb
 - c. Dinoterb

R. Inhibition of Lipid Synthesis
by other than ACC inhibition

1. Thiocarbamates (WSSA
Group 8)

- a. Butylate
- b. Cycloate
- c. Dimepiperate
- d. EPTC
- e. Esprocarb
- f. Molinate
- g. Orbencarb
- h. Pebulate
- i. Prosulfocarb
- j. Benthocarb
- k. Tiocarbazil
- l. Triallate
- m. Vernolate

2. Phosphorodithioates

- a. Bensulide

3. Benzofurans

- a. Benfuresate
- b. Ethofumesate

4. Halogenated alkanolic
acids (WSSA Group 26)

- a. TCA
- b. Dalapon
- c. Flupropanate

S. Synthetic auxins (IAA-like)
(WSSA Group 4)

1. Phenoxy-carboxylic acids

- a. Clomeprop
- b. 2,4-D
- c. Mecoprop

2. Benzoic acids

- a. Dicamba
- b. Chloramben
- c. TBA

3. Pyridine carboxylic acids

- a. Clopyralid

- b. Fluroxypyr
- c. Picloram
- d. Tricyclopyr

- 4. Quinoline carboxylic acids
 - a. Quinclorac
 - b. Quinmerac

- 5. Others (benazolin-ethyl)
 - a. Benazolin-ethyl

T. Inhibition of Auxin Transport

- 1. Phthalamates;
semicarbazones (WSSA Group 19)
 - a. Naptalam
 - b. Diflufenzopyr-Na

U. Other Mechanism of Action

- 1. Arylamino propionic acids
 - a. Flamprop-M-methyl /-
isopropyl
- 2. Pyrazolium
 - a. Difenzoquat
- 3. Organoarsenicals
 - a. DSMA
 - b. MSMA
- 4. Others
 - a. Bromobutide
 - b. Cinmethylin
 - c. Cumyluron
 - d. Dazomet
 - e. Daimuron-methyl
 - f. Dimuron
 - g. Etobenzanid
 - h. Fosamine
 - i. Metam
 - j. Oxaziclomefone
 - k. Oleic acid
 - l. Pelargonic acid

m. Pyributicarb

In still further methods, an HPPD inhibitor can be applied alone or in combination with another herbicide of interest and can be applied to the plants having the HPPD sequence as disclosed herein or their area of cultivation.

5 Additional herbicide treatment that can be applied over the plant or seeds having the HPPD polypeptides or active variants and fragments thereof include, but are not limited to: acetochlor, acifluorfen and its sodium salt, aclonifen, acrolein (2-propenal), alachlor, alloxymid, ametryn, amicarbazone, amidosulfuron, aminopyralid, amitrole, ammonium sulfamate, anilofos, asulam, atrazine, azimsulfuron, beflubutamid, benazolin, benazolin-ethyl, bencarbazone, benfluralin, 10 benfuresate, bensulfuron-methyl, bensulide, bentazone, benzobicyclon, benzofenap, bifenox, bilanafos, bispyribac and its sodium salt, bromacil, bromobutide, bromofenoxim, bromoxynil, bromoxynil octanoate, butachlor, butafenacil, butamifos, butralin, butroxydim, butylate, cafenstrole, carbetamide, carfentrazone-ethyl, catechin, chlomethoxyfen, chloramben, chlorbromuron, chlorflurenol-methyl, chloridazon, chlorimuron-ethyl, chlorotoluron, chlorpropham, chlorsulfuron, 15 chlorthal-dimethyl, chlorthiamid, cinidon-ethyl, cinmethylin, cinosulfuron, clethodim, clodinafop-propargyl, clomazone, clomeprop, clopyralid, clopyralid-olamine, cloransulam-methyl, CUH-35 (2-methoxyethyl 2-[[[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl](3-fluorobenzoyl)-amino]carbonyl]-1-cyclohexene-1-carboxylate), cumyluron, cyanazine, cycloate, cyclosulfamuron, cycloxydim, cyhalofop-butyl, 2,4-D and its butotyl, butyl, isooctyl and isopropyl esters and its 20 dimethylammonium, diolamine and trolamine salts, daimuron, dalapon, dalapon-sodium, dazomet, 2,4-DB and its dimethylammonium, potassium and sodium salts, desmedipham, desmetryn, dicamba and its diglycolammonium, dimethylammonium, potassium and sodium salts, dichlobenil, dichlorprop, diclofop-methyl, diclosulam, difenzoquat metilsulfate, diflufenican, diflufenzopyr, dimefuron, dimepiperate, dimethachlor, dimethametryn, dimethenamid, dimethenamid-P, dimethipin, 25 dimethylarsinic acid and its sodium salt, dinitramine, dinoterb, diphenamid, diquat dibromide, dithiopyr, diuron, DNOC, endothal, EPTC, esprocarb, ethalfluralin, ethametsulfuron-methyl, ethofumesate, ethoxyfen, ethoxysulfuron, etobenzanid, fenoxaprop-ethyl, fenoxaprop-P-ethyl, fentrazamide, fenuron, fenuron-TCA, flamprop-methyl, flamprop-M-isopropyl, flamprop-M-methyl, flazasulfuron, florasulam, fluazifop-butyl, fluazifop-P-butyl, flucarbazone, flucetosulfuron, 30 fluchloralin, flufenacet, flufenpyr, flufenpyr-ethyl, flumetsulam, flumiclorac-pentyl, flumioxazin, fluometuron, fluoroglycofen-ethyl, flupyrsulfuron-methyl and its sodium salt, flurenol, flurenol-butyl, fluridone, flurochloridone, fluroxypyr, flurtamone, fluthiacet-methyl, fomesafen, foramsulfuron, fosamine-ammonium, glufosinate, glufosinate-ammonium, glyphosate and its salts such as

ammonium, isopropylammonium, potassium, sodium (including sesquisodium) and trimesium (alternatively named sulfosate) (See, WO2007/024782, herein incorporated by reference), halosulfuron-methyl, haloxyfop-ethyl, haloxyfop-methyl, hexazinone, HOK-201 (*N*-(2,4-difluorophenyl)-1,5-dihydro-*N*-(1-methylethyl)-5-oxo-1-[(tetrahydro-2*H*-pyran-2-yl)methyl]-4*H*-1,2,4-triazole-4-carboxamide), imazamethabenz-methyl, imazamox, imazapic, imazapyr, imazaquin, imazaquin-ammonium, imazethapyr, imazethapyr-ammonium, imazosulfuron, indanofan, iodosulfuron-methyl, ioxynil, ioxynil octanoate, ioxynil-sodium, isoproturon, isouron, isoxaben, isoxaflutole, pyrasulfotole, lactofen, lenacil, linuron, maleic hydrazide, MCPA and its salts (e.g., MCPA-dimethylammonium, MCPA-potassium and MCPA-sodium, esters (e.g., MCPA-2-ethylhexyl, MCPA-butyl) and thioesters (e.g., MCPA-thioethyl), MCPB and its salts (e.g., MCPB-sodium) and esters (e.g., MCPB-ethyl), mecoprop, mecoprop-P, mefenacet, mefluidide, mesosulfuron-methyl, mesotrione, metam-sodium, metamifop, metamitron, metazachlor, methabenzthiazuron, methylarsonic acid and its calcium, monoammonium, monosodium and disodium salts, methyldymron, metobenzuron, metobromuron, metolachlor, S-metholachlor, metosulam, metoxuron, metribuzin, metsulfuron-methyl, molinate, monolinuron, naproanilide, napropamide, naptalam, neburon, nicosulfuron, norflurazon, orbencarb, oryzalin, oxadiargyl, oxadiazon, oxasulfuron, oxaziclomefone, oxyfluorfen, paraquat dichloride, pebulate, pelargonic acid, pendimethalin, penoxsulam, pentanochlor, pentoxazone, perfluidone, pethoxyamid, phenmedipham, picloram, picloram-potassium, picolinafen, pinoxaden, piperofos, pretilachlor, primisulfuron-methyl, prodiamine, profoxydim, prometon, prometryn, propachlor, propanil, propaquizafop, propazine, propham, propisochlor, propoxycarbazone, propyzamide, prosulfocarb, prosulfuron, pyraclonil, pyraflufen-ethyl, pyrasulfotole, pyrazogyl, pyrazolynate, pyrazoxyfen, pyrazosulfuron-ethyl, pyribenzoxim, pyributicarb, pyridate, pyriftalid, pyriminobac-methyl, pyrimisulfan, pyriothiac, pyriothiac-sodium, pyroxsulam, quinclorac, quinmerac, quinclamine, quizalofop-ethyl, quizalofop-P-ethyl, quizalofop-P-tefuryl, rimsulfuron, sethoxydim, siduron, simazine, simetryn, sulcotrione, sulfentrazone, sulfometuron-methyl, sulfosulfuron, 2,3,6-TBA, TCA, TCA-sodium, tebutam, tebuthiuron, tefuryltrione, tembotrione, tepraloxymid, terbacil, terbutometon, terbuthylazine, terbutryn, thenylchlor, thiazopyr, thienicarbazone, thifensulfuron-methyl, thiobencarb, tiocarbamil, topramezone, tralkoxydim, tri-allate, triasulfuron, triaziflam, tribenuron-methyl, triclopyr, triclopyr-butyl, triclopyr-triethylammonium, tridiphane, trietazine, trifloxysulfuron, trifluralin, triflusulfuron-methyl, tritosulfuron and vernolate.

Additional herbicides include those that are applied over plants having homogentisate solanesyltransferase (HST) polypeptide such as those described in International Publication No. WO 2010/029311(A2), herein incorporated by reference in its entirety.

Other suitable herbicides and agricultural chemicals are known in the art, such as, for example, those described in International Publication No. WO 2005/041654. Other herbicides also include bioherbicides such as those produced by *Streptomyces hygroscopicus*, *Alternaria destruens* Simmons, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Drechslera monoceras* (MTB-951),
 5 *Myrothecium verrucaria* (Albertini & Schweinitz) Ditmar: Fries, *Phytophthora palmivora* (Butl.) Butl. and *Puccinia thlaspeos* Schub. Combinations of various herbicides can result in a greater-than-additive (*i.e.*, synergistic) effect on weeds and/or a less-than-additive effect (*i.e.* safening) on crops or other desirable plants. In certain instances, combinations of HPPD herbicides with other herbicides having a similar spectrum of control but a different mode of action will be particularly
 10 advantageous for preventing the development of resistant weeds.

The time at which a herbicide is applied to an area of interest (and any plants therein) may be important in optimizing weed control. The time at which a herbicide is applied may be determined with reference to the size of plants and/or the stage of growth and/or development of plants in the area of interest, *e.g.*, crop plants or weeds growing in the area.

15 Ranges of the effective amounts of herbicides can be found, for example, in various publications from University Extension services. See, for example, Bernards *et al.* (2006) *Guide for Weed Management in Nebraska* (www.ianrpubs.url.edu/sendlt/ec130); Regher *et al.* (2005) *Chemical Weed Control for Fields Crops, Pastures, Rangeland, and Noncropland*, Kansas State University Agricultural Extension Station and Corporate Extension Service; Zollinger *et al.* (2006)
 20 *North Dakota Weed Control Guide*, North Dakota Extension Service, and the Iowa State University Extension at www.weeds.iastate.edu, each of which is herein incorporated by reference.

Many plant species can be controlled (*i.e.*, killed or damaged) by the herbicides described herein. Accordingly, the methods of the disclosure are useful in controlling these plant species where they are undesirable (*i.e.*, where they are weeds). These plant species include crop plants as
 25 well as species commonly considered weeds, including but not limited to species such as: blackgrass (*Alopecurus myosuroides*), giant foxtail (*Setaria faberi*), large crabgrass (*Digitaria sanguinalis*), Surinam grass (*Brachiaria decumbens*), wild oat (*Avena fatua*), common cocklebur (*Xanthium pensylvanicum*), common lambsquarters (*Chenopodium album*), morning glory (*Ipomoea coccinea*), pigweed (*Amaranthus spp.*), velvetleaf (*Abutilion theophrasti*), common barnyardgrass (*Echinochloa crus-galli*), bermudagrass (*Cynodon dactylon*), downy brome (*Bromus tectorum*), goosegrass (*Eleusine indica*), green foxtail (*Setaria viridis*), Italian ryegrass (*Lolium multiflorum*), Johnsongrass (*Sorghum halepense*), lesser canarygrass (*Phalaris minor*), windgrass (*Apera spica-venti*), woolly cupgrass (*Erichloa villosa*), yellow nutsedge (*Cyperus esculentus*), common chickweed (*Stellaria media*), common ragweed (*Ambrosia artemisiifolia*), *Kochia scoparia*, horseweed (*Conyza*
 30

canadensis), rigid ryegrass (*Lolium rigidum*), goosegrass (*Eleusine indica*), hairy fleabane (*Conyza bonariensis*), buckhorn plantain (*Plantago lanceolata*), tropical spiderwort (*Commelina benghalensis*), field bindweed (*Convolvulus arvensis*), purple nutsedge (*Cyperus rotundus*), redvine (*Brunnichia ovata*), hemp sesbania (*Sesbania exaltata*), sicklepod (*Senna obtusifolia*), Texas
5 blueweed (*Helianthus ciliaris*), and Devil's claws (*Proboscidea louisianica*). In other embodiments, the weed comprises a herbicide-resistant ryegrass, for example, a glyphosate resistant ryegrass, a paraquat resistant ryegrass, a ACCase-inhibitor resistant ryegrass, and a non-selective herbicide resistant ryegrass.

In some embodiments, a plant having the HPPD sequence disclosed herein or active variants
10 and fragments thereof is not significantly damaged by treatment with an HPPD inhibitor applied to that plant, whereas an appropriate control plant is significantly damaged by the same treatment.

Generally, an HPPD inhibitor is applied to a particular field (and any plants growing in it) no more than 1, 2, 3, 4, 5, 6, 7, or 8 times a year, or no more than 1, 2, 3, 4, or 5 times per growing season.

Thus, methods of the disclosure encompass applications of herbicide which are
15 "preemergent," "postemergent," "preplant incorporation" and/or which involve seed treatment prior to planting.

In one embodiment, methods are provided for coating seeds. The methods comprise coating a seed with an effective amount of a herbicide or a combination of herbicides (as disclosed elsewhere
20 herein). The seeds can then be planted in an area of cultivation. Further provided are seeds having a coating comprising an effective amount of a herbicide or a combination of herbicides (as disclosed elsewhere herein). In other embodiments, the seeds can be coated with at least one fungicide and/or at least one insecticide and/or at least one herbicide or any combination thereof.

"Preemergent" refers to a herbicide which is applied to an area of interest (*e.g.*, a field or area
25 of cultivation) before a plant emerges visibly from the soil. "Postemergent" refers to a herbicide which is applied to an area after a plant emerges visibly from the soil. In some instances, the terms "preemergent" and "postemergent" 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 only a particular type of weed or species of weed
30 that is present or believed to be present in the area of interest. While any herbicide may be applied in a preemergent and/or postemergent treatment, some herbicides are known to be more effective in controlling a weed or weeds when applied either preemergence or postemergence. For example, rimsulfuron has both preemergence and postemergence activity, while other herbicides have predominately preemergence (metolachlor) or postemergence (glyphosate) activity. These properties

of particular herbicides are known in the art and are readily determined by one of skill in the art. Further, one of skill in the art would readily be able to select appropriate herbicides and application times for use with the transgenic plants of the disclosure and/or on areas in which transgenic plants of the disclosure are to be planted. “Preplant incorporation” involves the incorporation of
5 compounds into the soil prior to planting.

Thus, improved methods of growing a crop and/or controlling weeds such as, for example, “pre-planting burn down,” are provided wherein an area is treated with herbicides prior to planting the crop of interest in order to better control weeds. The disclosure also provides methods of growing a crop and/or controlling weeds which are “no-till” or “low-till” (also referred to as
10 “reduced tillage”). In such methods, the soil is not cultivated or is cultivated less frequently during the growing cycle in comparison to traditional methods; these methods can save costs that would otherwise be incurred due to additional cultivation, including labor and fuel costs.

The term “safener” refers to a substance that when added to a herbicide formulation eliminates or reduces the phytotoxic effects of the herbicide to certain crops. One of ordinary skill in
15 the art would appreciate that the choice of safener depends, in part, on the crop plant of interest and the particular herbicide or combination of herbicides. Exemplary safeners suitable for use with the presently disclosed herbicide compositions include, but are not limited to, those disclosed in U.S. Patent Nos. 4,808,208; 5,502,025; 6,124,240 and U.S. Patent Application Publication Nos. 2006/0148647; 2006/0030485; 2005/0233904; 2005/0049145; 2004/0224849; 2004/0224848;
20 2004/0224844; 2004/0157737; 2004/0018940; 2003/0171220; 2003/0130120; 2003/0078167, the disclosures of which are incorporated herein by reference in their entirety. The methods of the disclosure can involve the use of herbicides in combination with herbicide safeners such as benoxacor, BCS (1-bromo-4-[(chloromethyl) sulfonyl]benzene), cloquintocet-mexyl, cyometrinil, dichlormid, 2-(dichloromethyl)-2-methyl-1,3-dioxolane (MG 191), fenchlorazole-ethyl, fenclorim,
25 flurazole, fluxofenim, furilazole, isoxadifen-ethyl, mefenpyr-diethyl, methoxyphenone ((4-methoxy-3-methylphenyl)(3-methylphenyl)-methanone), naphthalic anhydride (1,8-naphthalic anhydride) and oxabetrinil to increase crop safety. Antidotally effective amounts of the herbicide safeners can be applied at the same time as the compounds of this disclosure, or applied as seed treatments. Therefore an aspect of the present disclosure relates to the use of a mixture comprising an HPPD
30 inhibitor, at least one other herbicide, and an antidotally effective amount of a herbicide safener.

Seed treatment is useful for selective weed control, because it physically restricts antidoting to the crop plants. Therefore in one embodiment, a method for selectively controlling the growth of weeds in a field comprising treating the seed from which the crop is grown with an antidotally effective amount of safener and treating the field with an effective amount of herbicide to control

weeds.

An antidotally effective amount of a safener is present where a desired plant is treated with the safener so that the effect of a herbicide on the plant is decreased in comparison to the effect of the herbicide on a plant that was not treated with the safener; generally, an antidotally effective amount of safener prevents damage or severe damage to the plant treated with the safener. One of skill in the art is capable of determining whether the use of a safener is appropriate and determining the dose at which a safener should be administered to a crop.

As used herein, an “adjuvant” is any material added to a spray solution or formulation to modify the action of an agricultural chemical or the physical properties of the spray solution. See, for example, Green and Foy (2003) “Adjuvants: Tools for Enhancing Herbicide Performance,” in *Weed Biology and Management*, ed. Inderjit (Kluwer Academic Publishers, The Netherlands). Adjuvants can be categorized or subclassified as activators, acidifiers, buffers, additives, adherents, antiflocculants, antifoamers, defoamers, antifreezes, attractants, basic blends, chelating agents, cleaners, colorants or dyes, compatibility agents, cosolvents, couplers, crop oil concentrates, deposition agents, detergents, dispersants, drift control agents, emulsifiers, evaporation reducers, extenders, fertilizers, foam markers, formulants, inerts, humectants, methylated seed oils, high load COCs, polymers, modified vegetable oils, penetrators, repellants, petroleum oil concentrates, preservatives, rainfast agents, retention aids, solubilizers, surfactants, spreaders, stickers, spreader stickers, synergists, thickeners, translocation aids, uv protectants, vegetable oils, water conditioners, and wetting agents.

In addition, methods of the disclosure can comprise the use of a herbicide or a mixture of herbicides, as well as, one or more other insecticides, fungicides, nematocides, bactericides, acaricides, growth regulators, chemosterilants, semiochemicals, repellents, attractants, pheromones, feeding stimulants or other biologically active compounds or entomopathogenic bacteria, virus, or fungi to form a multi-component mixture giving an even broader spectrum of agricultural protection. Examples of such agricultural protectants which can be used in methods of the disclosure include: insecticides such as abamectin, acephate, acetamiprid, amidoflomet (S-1955), avermectin, azadirachtin, azinphos-methyl, bifenthrin, bifenazate, buprofezin, carbofuran, cartap, chlorfenapyr, chlorfluazuron, chlorpyrifos, chlorpyrifos-methyl, chromafenozide, clothianidin, cyflumetofen, cyfluthrin, beta-cyfluthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyromazine, deltamethrin, diafenthiuron, diazinon, dieldrin, diflubenzuron, dimefluthrin, dimethoate, dinotefuran, diofenolan, emamectin, endosulfan, esfenvalerate, ethiprole, fenothiocarb, fenoxycarb, fenpropathrin, fenvalerate, fipronil, flonicamid, flubendiamide, flucythrinate, tau-fluvalinate, flufenoxuron, fonophos, halofenozide, hexaflumuron, hydramethylnon, imidacloprid, indoxacarb,

isofenphos, lufenuron, malathion, metaflumizone, metaldehyde, methamidophos, methidathion, methomyl, methoprene, methoxychlor, metofluthrin, monocrotophos, methoxyfenozide, nitenpyram, nithiazine, novaluron, noviflumuron (XDE-007), oxamyl, parathion, parathion-methyl, permethrin, phorate, phosalone, phosmet, phosphamidon, pirimicarb, profenofos, profluthrin, pymetrozine, 5 pyrafluprole, pyrethrin, pyridalyl, pyriprole, pyriproxyfen, rotenone, ryanodine, spinosad, spirotetramat, sulprofos, tebufenozide, teflubenzuron, tefluthrin, terbufos, tetrachlorvinphos, thiacloprid, thiamethoxam, thiodicarb, thiosultap-sodium, tralomethrin, triazamate, trichlorfon and triflumuron; fungicides such as acibenzolar, aldimorph, amisulbrom, azaconazole, azoxystrobin, benalaxyl, benomyl, benthiavalicarb, benthiavalicarb-10 isopropyl, binomial, biphenyl, bitertanol, blasticidin-S, Bordeaux mixture (Tribasic copper sulfate), boscalid/nicobifen, bromuconazole, bupirimate, buthiobate, carboxin, carpropamid, captafol, captan, carbendazim, chloroneb, chlorothalonil, chlozolate, clotrimazole, copper oxychloride, copper salts such as copper sulfate and copper hydroxide, cyazofamid, cyflunamid, cymoxanil, cyproconazole, cyprodinil, dichlofluanid, diclocymet, diclomezone, dicloran, diethofencarb, difenoconazole, 15 dimethomorph, dimoxystrobin, diniconazole, diniconazole-M, dinocap, discostrobin, dithianon, dodemorph, dodine, econazole, etaconazole, edifenphos, epoxiconazole, ethaboxam, ethirimol, ethridiazole, famoxadone, fenamidone, fenarimol, fenbuconazole, fencaramid, fenfuram, fenhexamide, fenoxanil, fenciclonil, fenpropidin, fenpropimorph, fentin acetate, fentin hydroxide, ferbam, ferfurazate, ferimzone, fluazinam, fludioxonil, flumetover, fluopicolide, fluoxastrobin, 20 fluquinconazole, fluquinconazole, flusilazole, flusulfamide, flutolanil, flutriafol, folpet, fosetyl-aluminum, fuberidazole, furalaxyl, furametapir, hexaconazole, hymexazole, guazatine, imazalil, imibenconazole, iminoctadine, iodicarb, ipconazole, iprobenfos, iprodione, iprovalicarb, isoconazole, isoprothiolane, kasugamycin, kresoxim-methyl, mancozeb, mandipropamid, maneb, mapanipyrim, mefenoxam, mepronil, metalaxyl, metconazole, methasulfocarb, metiram, 25 metominostrobin/fenominostrobin, mepanipyrim, metrafenone, miconazole, myclobutanil, neo-asozin (ferric methanearsonate), nuarimol, oethilinone, ofurace, orysastrobin, oxadixyl, oxolinic acid, oxpoconazole, oxycarboxin, paclobutrazol, penconazole, pencycuron, penthiopyrad, perfurazate, phosphonic acid, phthalide, picobenzamid, picoxystrobin, polyoxin, probenazole, prochloraz, procymidone, propamocarb, propamocarb-hydrochloride, propiconazole, propineb, proquinazid, 30 prothioconazole, pyraclostrobin, pryazophos, pyrifenoxy, pyrimethanil, pyrifenoxy, pyrolytrine, pyroquilon, quinconazole, quinoxifen, quintozone, silthiofam, simeconazole, spiroxamine, streptomycin, sulfur, tebuconazole, techrazene, tecloftalam, tecnazene, tetraconazole, thiabendazole, thifluzamide, thiophanate, thiophanate-methyl, thiram, tiadinil, tolclofos-methyl, tolyfluanid, triadimefon, triadimenol, triarimol, triazoxide, tridemorph, trimopramide, tricyclazole,

trifloxystrobin, triforine, triticonazole, uniconazole, validamycin, vinclozolin, zineb, ziram, and zoxamide; nematocides such as aldicarb, oxamyl and fenamiphos; bactericides such as streptomycin; acaricides such as amitraz, chinomethionat, chlorobenzilate, cyhexatin, dicofol, dienochlor, etoxazole, fenazaquin, fenbutatin oxide, fenpropathrin, fenpyroximate, hexythiazox, propargite, pyridaben and tebufenpyrad; and biological agents including entomopathogenic bacteria, such as *Bacillus thuringiensis* subsp. *Aizawai*, *Bacillus thuringiensis* subsp. *Kurstaki*, and the encapsulated delta-endotoxins of *Bacillus thuringiensis* (e.g., Cellcap, MPV, MPVII); entomopathogenic fungi, such as green muscardine fungus; and entomopathogenic virus including baculovirus, nucleopolyhedro virus (NPV) such as HzNPV, AfNPV; and granulosis virus (GV) such as CpGV.

The methods of controlling weeds can further include the application of a biologically effective amount of a herbicide of interest or a mixture of herbicides, and an effective amount of at least one additional biologically active compound or agent and can further comprise at least one of a surfactant, a solid diluent or a liquid diluent. Examples of such biologically active compounds or agents are: insecticides such as abamectin, acephate, acetamiprid, amidoflomet (S-1955), avermectin, azadirachtin, azinphos-methyl, bifenthrin, binfenazate, buprofezin, carbofuran, chlorfenapyr, chlorfluazuron, chlorpyrifos, chlorpyrifos-methyl, chromafenozide, clothianidin, cyfluthrin, beta-cyfluthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyromazine, deltamethrin, diafenthiuron, diazinon, diflubenzuron, dimethoate, diofenolan, emamectin, endosulfan, esfenvalerate, ethiprole, fenothicarb, fenoxycarb, fenpropathrin, fenvalerate, fipronil, flonicamid, flucythrinate, tau-fluvalinate, flufenimer (UR-50701), flufenoxuron, fonophos, halofenozide, hexaflumuron, imidacloprid, indoxacarb, isofenphos, lufenuron, malathion, metaldehyde, methamidophos, methidathion, methomyl, methoprene, methoxychlor, monocrotophos, methoxyfenozide, nithiazin, novaluron, noviflumuron (XDE-007), oxamyl, parathion, parathion-methyl, permethrin, phorate, phosalone, phosmet, phosphamidon, pirimicarb, profenofos, pymetrozine, pyridalyl, pyriproxyfen, rotenone, spinosad, spiromesifin (BSN 2060), sulprofos, tebufenozide, teflubenzuron, tefluthrin, terbufos, tetrachlorvinphos, thiacloprid, thiamethoxam, thiodicarb, thiosultap-sodium, tralomethrin, trichlorfon and triflumuron; fungicides such as acibenzolar, azoxystrobin, benomyl, blasticidin-S, Bordeaux mixture (tribasic copper sulfate), bromuconazole, carpropamid, captafol, captan, carbendazim, chloroneb, chlorothalonil, copper oxychloride, copper salts, cyflufenamid, cymoxanil, cyproconazole, cyprodinil, (*S*)-3,5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide (RH 7281), diclocymet (S-2900), diclomezine, dicloran, difenoconazole, (*S*)-3,5-dihydro-5-methyl-2-(methylthio)-5-phenyl-3-(phenyl-amino)-4*H*-imidazol-4-one (RP 407213), dimethomorph, dimoxystrobin, diniconazole, diniconazole-M, dodine, edifenphos, epoxiconazole, famoxadone, fenamidone, fenarimol, fenbuconazole, fencaramid (SZX0722), fencpiclonil,

fenpropidin, fenpropimorph, fentin acetate, fentin hydroxide, fluazinam, fludioxonil, flumetover (RPA 403397), flumorf/flumorlin (SYP-L190), fluoxastrobin (HEC 5725), fluquinconazole, flusilazole, flutolanil, flutriafol, folpet, fosetyl-aluminum, furalaxyl, furametapyr (S-82658), hexaconazole, ipconazole, iprobenfos, iprodione, isoprothiolane, kasugamycin, kresoxim-methyl, mancozeb, maneb, mefenoxam, mepronil, metalaxyl, metconazole, metominostrobin/fenominostrobin (SSF-126), metrafenone (AC375839), myclobutanil, neo-asozin (ferric methane-arsonate), nicobifen (BAS 510), oryastrobin, oxadixyl, penconazole, pencycuron, probenazole, prochloraz, propamocarb, propiconazole, proquinazid (DPX-KQ926), prothioconazole (JAU 6476), pyrifenoxy, pyraclostrobin, pyrimethanil, pyroquilon, quinoxifen, spiroxamine, sulfur, tebuconazole, tetraconazole, thiabendazole, thifluzamide, thiophanate-methyl, thiram, tiadinil, triadimefon, triadimenol, tricyclazole, trifloxystrobin, triticonazole, validamycin and vinclozolin; nematocides such as aldicarb, oxamyl and fenamiphos; bactericides such as streptomycin; acaricides such as amitraz, chinomethionat, chlorobenzilate, cyhexatin, dicofol, dinoclor, etoxazole, fenazaquin, fenbutatin oxide, fenpropathrin, fenpyroximate, hexythiazox, propargite, pyridaben and tebufenpyrad; and biological agents including entomopathogenic bacteria, such as *Bacillus thuringiensis* subsp. *Aizawai*, *Bacillus thuringiensis* subsp. *Kurstaki*, and the encapsulated delta-endotoxins of *Bacillus thuringiensis* (e.g., Celcapp, MPV, MPVII); entomopathogenic fungi, such as green muscardine fungus; and entomopathogenic virus including baculovirus, nucleopolyhedro virus (NPV) such as HzNPV, AfNPV; and granulosis virus (GV) such as CpGV. Methods of the disclosure may also comprise the use of plants genetically transformed to express proteins (such as *Bacillus thuringiensis* delta-endotoxins) toxic to invertebrate pests. In such embodiments, the effect of exogenously applied invertebrate pest control compounds may be synergistic with the expressed toxin proteins. General references for these agricultural protectants include *The Pesticide Manual, 13th Edition*, C. D. S. Tomlin, Ed., British Crop Protection Council, Farnham, Surrey, U.K., 2003 and *The BioPesticide Manual, 2nd Edition*, L. G. Copping, Ed., British Crop Protection Council, Farnham, Surrey, U.K., 2001.

In certain instances, combinations with other invertebrate pest control compounds or agents having a similar spectrum of control but a different mode of action will be particularly advantageous for resistance management. Thus, compositions of the present disclosure can further comprise a biologically effective amount of at least one additional invertebrate pest control compound or agent having a similar spectrum of control but a different mode of action. Contacting a plant genetically modified to express a plant protection compound (e.g., protein) or the locus of the plant with a biologically effective amount of a compound of this disclosure can also provide a broader spectrum of plant protection and be advantageous for resistance management.

Thus, methods of controlling weeds can employ a herbicide or herbicide combination and may further comprise the use of insecticides and/or fungicides, and/or other agricultural chemicals such as fertilizers. The use of such combined treatments of the disclosure can broaden the spectrum of activity against additional weed species and suppress the proliferation of any resistant biotypes.

5 Methods can further comprise the use of plant growth regulators such as aviglycine, *N*-(phenylmethyl)-1*H*-purin-6-amine, ethephon, epocholeone, gibberellic acid, gibberellin A₄ and A₇, harpin protein, mepiquat chloride, prohexadione calcium, prohydrojasmon, sodium nitrophenolate and trinexapac-methyl, and plant growth modifying organisms such as *Bacillus cereus* strain BP01.

C. Method of Detections

10 Methods for detecting an HPPD polypeptide or an active variant or fragment thereof are provided. Such methods comprise analyzing plant tissues to detect such polypeptides or the polynucleotides encoding the same. The detection methods can directly assay for the presence of the HPPD polypeptide or polynucleotide or the detection methods can indirectly assay for the sequences by assaying the phenotype of the cell plant, plant cell or plant explant expressing the sequence.

15 In one embodiment, the HPPD polypeptide is detected in the plant tissue using an immunoassay comprising an antibody or antibodies that specifically recognizes an HPPD polypeptide or active variant or fragment thereof. In specific embodiments, the antibody or antibodies which are used are raised to an HPPD polypeptide or active variant or fragment thereof as disclosed herein.

20 By “specifically or selectively binds” is intended that the binding agent has a binding affinity for a given HPPD polypeptide or fragment or variant disclosed herein, which is greater than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the binding affinity for a known HPPD sequence. One of skill will be aware of the proper controls that are needed to carry out such a determination

By “antibodies that specifically bind” is intended that the antibodies will not substantially
25 cross react with another polypeptide. By “not substantially cross react” is intended that the antibody or fragment thereof has a binding affinity for the other polypeptide which is less than 10%, less than 5%, or less than 1%, of the binding affinity for the HPPD polypeptide or active fragment or variant thereof.

30 In an aspect, the HPPD polypeptide can be detected in plant tissue extracts by subjecting the extract to trypsin digestion, followed by LC-MS. For example, tryptic fragments can be partially separated from each other by reverse-phase liquid chromatography and the column effluent directed into a mass spectrometer. A specific peptide unique to the transgenically expressed HPPD is detected by adjusting the instrument setting so as to record and quantify the peptide according to the exact

mass of the signature peptide. Such an exemplary method is described in Example 1 herein.

In still other embodiments, the HPPD polypeptide or active variant or fragment thereof can be inferred in a plant tissue by detecting the presence of a polynucleotide encoding any of the various HPPD polypeptides or active variants and fragments thereof. In one embodiment, the detection
5 method comprises assaying plant tissue using PCR amplification.

As used herein, “primers” are isolated polynucleotides that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the disclosure refer to their use for amplification of a target polynucleotide, e.g., by
10 the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods. “PCR” or “polymerase chain reaction” is a technique used for the amplification of specific DNA segments (see, U.S. Pat. Nos. 4,683,195 and 4,800,159; herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence and specifically detect and/or identify a polynucleotide encoding an HPPD polypeptide or active variant
15 or fragment thereof as describe elsewhere herein. It is recognized that the hybridization conditions or reaction conditions can be determined by the operator to achieve this result. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Such probes and primers can hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers according to embodiments of the present disclosure may have
20 complete DNA sequence identity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to specifically detect and/or identify a target DNA sequence may be designed by conventional methods. Accordingly, probes and primers can share about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity or complementarity to the target polynucleotide.

25 Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual*, 2nd ed, vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, “Sambrook *et al.*, 1989”); *Current Protocols in Molecular Biology*, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, “Ausubel *et al.*, 1992”); and Innis *et al.*, PCR
30 Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 10 (Invitrogen); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer (Version 0.5.COPYRGT., 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be

visually scanned and primers manually identified using guidelines known to one of skill in the art.

D. Method of Identifying HPPD Variants.

Various methods can be employed to identify further HPPD variants. The polynucleotides of the disclosure are optionally used as substrates for a variety of diversity generating procedures, e.g., mutation, recombination and recursive recombination reactions, in addition to their use in standard cloning methods as set forth in, e.g., Ausubel, Berger and Sambrook, i.e., to produce additional HPPD polynucleotides and polypeptides with desired properties. A variety of diversity generating protocols can be used. The procedures can be used separately, and/or in combination to produce one or more variants of a polynucleotide or set of polynucleotides, as well variants of encoded proteins. Individually and collectively, these procedures provide robust, widely applicable ways of generating diversified polynucleotides and sets of polynucleotides (including, e.g., polynucleotide libraries) useful, e.g., for the engineering or rapid evolution of polynucleotides, proteins, pathways, cells and/or organisms with new and/or improved characteristics. The process of altering the sequence can result in, for example, single nucleotide substitutions, multiple nucleotide substitutions, and insertion or deletion of regions of the nucleic acid sequence.

While distinctions and classifications are made in the course of the ensuing discussion for clarity, it will be appreciated that the techniques are often not mutually exclusive. Indeed, the various methods can be used singly or in combination, in parallel or in series, to access diverse sequence variants.

The result of any of the diversity generating procedures described herein can be the generation of one or more polynucleotides, which can be selected or screened for polynucleotides that encode proteins with or which confer desirable properties. Following diversification by one or more of the methods herein, or otherwise available to one of skill, any polynucleotides that are produced can be selected for a desired activity or property, e.g. altered Km, use of alternative cofactors, increased kcat, etc. This can include identifying any activity that can be detected, for example, in an automated or automatable format, by any of the assays in the art. For example, modified HPPD polypeptides can be detected by assaying for an increased insensitivity to HPPD inhibitor. Assays to measure such activity are described elsewhere herein. A variety of related (or even unrelated) properties can be evaluated, in serial or in parallel, at the discretion of the practitioner.

Descriptions of a variety of diversity generating procedures, including family shuffling and methods for generating modified nucleic acid sequences encoding multiple enzymatic domains, are found in the following publications and the references cited therein: Soong N. et al. (2000) *Nat Genet* 25(4):436-39; Stemmer *et al.* (1999) *Tumor Targeting* 4:1-4; Ness *et al.* (1999) *Nature*

Biotechnology 17:893-896; Chang *et al.* (1999) *Nature Biotechnology* 17:793-797; Minshull and Stemmer (1999) *Current Opinion in Chemical Biology* 3:284-290; Christians *et al.* (1999) *Nature Biotechnology* 17:259-264; Cramer *et al.* (1998) *Nature* 391:288-291; Cramer *et al.* (1997) *Nature Biotechnology* 15:436-438; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Patten *et al.* (1997) *Current Opinion in Biotechnology* 8:724-733; Cramer *et al.* (1996) *Nature Medicine* 2:100-103; Cramer *et al.* (1996) *Nature Biotechnology* 14:315-319; Gates *et al.* (1996) *Journal of Molecular Biology* 255:373-386; Stemmer (1996) "Sexual PCR and Assembly PCR" In: The Encyclopedia of Molecular Biology. VCH Publishers, New York. pp.447-457; Cramer and Stemmer (1995) *BioTechniques* 18:194-195; Stemmer *et al.* (1995) *Gene*: 164:49-53; Stemmer (1995) *Science* 270: 1510; Stemmer (1995) *Bio/Technology* 13:549-553; Stemmer (1994) *Nature* 370:389-391; and Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751. See also WO2008/073877 and US 20070204369, both of which are herein incorporated by reference in their entirety.

Mutational methods of generating diversity include, for example, site-directed mutagenesis (Ling *et al.* (1997) *Anal Biochem.* 254(2): 157-178; Dale *et al.* (1996) *Methods Mol. Biol.* 57:369-374; Smith (1985) *Ann. Rev. Genet.* 19:423-462; Botstein & Shortle (1985) *Science* 229:1193-1201; Carter (1986) *Biochem. J.* 237:1-7; and Kunkel (1987) *Nucleic Acids & Molecular Biology* (Eckstein, F. and Lilley, D.M.J. eds., Springer Verlag, Berlin)); mutagenesis using uracil containing templates (Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154, 367-382; and Bass *et al.* (1988) *Science* 242:240-245); oligonucleotide-directed mutagenesis (*Methods in Enzymol.* 100: 468-500 (1983); *Methods in Enzymol.* 154: 329-350 (1987); Zoller & Smith (1982) *Nucleic Acids Res.* 10:6487-6500; Zoller & Smith (1983) *Methods in Enzymol.* 100:468-500; and Zoller & Smith (1987) *Methods in Enzymol.* 154:329-350); phosphorothioate-modified DNA mutagenesis (Taylor *et al.* (1985) *Nucl. Acids Res.* 13: 8749-8764; Taylor *et al.* (1985) *Nucl. Acids Res.* 13: 8765-8787 (1985); Nakamaye & Eckstein (1986) *Nucl. Acids Res.* 14: 9679-9698; Sayers *et al.* (1988) *Nucl. Acids Res.* 16:791-802; and Sayers *et al.* (1988) *Nucl. Acids Res.* 16: 803-814); mutagenesis using gapped duplex DNA (Kramer *et al.* (1984) *Nucl. Acids Res.* 12: 9441-9456; Kramer & Fritz (1987) *Methods in Enzymol.* 154:350-367; Kramer *et al.* (1988) *Nucl. Acids Res.* 16: 7207; and Fritz *et al.* (1988) *Nucl. Acids Res.* 16: 6987-6999).

Additional suitable methods include, but are not limited to, point mismatch repair (Kramer *et al.* (1984) *Cell* 38:879-887), mutagenesis using repair-deficient host strains (Carter *et al.* (1985) *Nucl. Acids Res.* 13: 4431-4443; and Carter (1987) *Methods in Enzymol.* 154: 382-403), deletion mutagenesis (Eghtedarzadeh & Henikoff (1986) *Nucl. Acids Res.* 14: 5115), restriction-selection and restriction-purification (Wells *et al.* (1986) *Phil. Trans. R. Soc. Lond. A* 317: 415-423), mutagenesis by total gene synthesis (Nambiar *et al.* (1984) *Science* 223: 1299-1301; Sakamar and Khorana (1988)

Nucl. Acids Res. 14: 6361-6372; Wells *et al.* (1985) *Gene* 34:315-323; and Grundström *et al.* (1985) *Nucl. Acids Res.* 13: 3305-3316), and double-strand break repair (Mandecki (1986); Arnold (1993) *Current Opinion in Biotechnology* 4:450-455 and *Proc. Natl. Acad. Sci. USA*, 83:7177-7181). Additional details on many of the above methods can be found in *Methods in Enzymology* Volume 5 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

Additional details regarding various diversity generating methods can be found in the following U.S. patents, PCT publications, and EPO publications: U.S. Pat. No. 5,605,793, U.S. Pat. No. 5,811,238, U.S. Pat. No. 5,830,721, U.S. Pat. No. 5,834,252, U.S. Pat. No. 5,837,458, WO 10 95/22625, WO 96/33207, WO 97/20078, WO 97/35966, WO 99/41402, WO 99/41383, WO 99/41369, WO 99/41368, EP 752008, EP 0932670, WO 99/23107, WO 99/21979, WO 98/31837, WO 98/27230, WO 98/13487, WO 00/00632, WO 00/09679, WO 98/42832, WO 99/29902, WO 98/41653, WO 98/41622, WO 98/42727, WO 00/18906, WO 00/04190, WO 00/42561, WO 00/42559, WO 00/42560, WO 01/23401, and, PCT/US01/06775. See, also WO20074303, herein 15 incorporated by reference.

In brief, several different general classes of sequence modification methods, such as mutation, recombination, etc. are applicable to the present disclosure and set forth, e.g., in the references above. That is, alterations to the component nucleic acid sequences to produced modified gene fusion constructs can be performed by any number of the protocols described, either before 20 cojoining of the sequences, or after the cojoining step. The following exemplify some of the different types of preferred formats for diversity generation in the context of the present disclosure, including, e.g., certain recombination based diversity generation formats.

Nucleic acids can be recombined *in vitro* by any of a variety of techniques discussed in the references above, including e.g., DNase digestion of nucleic acids to be recombined followed by 25 ligation and/or PCR reassembly of the nucleic acids. For example, sexual PCR mutagenesis can be used in which random (or pseudo random, or even non-random) fragmentation of the DNA molecule is followed by recombination, based on sequence similarity, between DNA molecules with different but related DNA sequences, *in vitro*, followed by fixation of the crossover by extension in a polymerase chain reaction. This process and many process variants are described in several of the 30 references above, e.g., in Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751.

Similarly, nucleic acids can be recursively recombined *in vivo*, e.g., by allowing recombination to occur between nucleic acids in cells. Many such *in vivo* recombination formats are set forth in the references noted above. Such formats optionally provide direct recombination between nucleic acids of interest, or provide recombination between vectors, viruses, plasmids, etc.,

comprising the nucleic acids of interest, as well as other formats. Details regarding such procedures are found in the references noted above.

Whole genome recombination methods can also be used in which whole genomes of cells or other organisms are recombined, optionally including spiking of the genomic recombination mixtures with desired library components (e.g., genes corresponding to the pathways of the present disclosure). These methods have many applications, including those in which the identity of a target gene is not known. Details on such methods are found, e.g., in WO 98/31837 and in PCT/US99/15972. Thus, any of these processes and techniques for recombination, recursive recombination, and whole genome recombination, alone or in combination, can be used to generate the modified nucleic acid sequences and/or modified gene fusion constructs of the present disclosure.

Synthetic recombination methods can also be used, in which oligonucleotides corresponding to targets of interest are synthesized and reassembled in PCR or ligation reactions which include oligonucleotides which correspond to more than one parental nucleic acid, thereby generating new recombined nucleic acids. Oligonucleotides can be made by standard nucleotide addition methods, or can be made, e.g., by tri-nucleotide synthetic approaches. Details regarding such approaches are found in the references noted above, including, e.g., WO 00/42561, WO 01/23401, WO 00/42560, and, WO 00/42559.

In silico methods of recombination can be affected in which genetic algorithms are used in a computer to recombine sequence strings which correspond to homologous (or even non-homologous) nucleic acids. The resulting recombined sequence strings are optionally converted into nucleic acids by synthesis of nucleic acids which correspond to the recombined sequences, e.g., in concert with oligonucleotide synthesis/ gene reassembly techniques. This approach can generate random, partially random or designed variants. Many details regarding in silico recombination, including the use of genetic algorithms, genetic operators and the like in computer systems, combined with generation of corresponding nucleic acids (and/or proteins), as well as combinations of designed nucleic acids and/or proteins (e.g., based on cross-over site selection) as well as designed, pseudo-random or random recombination methods are described in WO 00/42560 and WO 00/42559.

Many methods of accessing natural diversity, e.g., by hybridization of diverse nucleic acids or nucleic acid fragments to single-stranded templates, followed by polymerization and/or ligation to regenerate full-length sequences, optionally followed by degradation of the templates and recovery of the resulting modified nucleic acids can be similarly used. In one method employing a single-stranded template, the fragment population derived from the genomic library(ies) is annealed with partial, or, often approximately full length ssDNA or RNA corresponding to the opposite strand.

Assembly of complex chimeric genes from this population is then mediated by nuclease-base removal of non-hybridizing fragment ends, polymerization to fill gaps between such fragments and subsequent single stranded ligation. The parental polynucleotide strand can be removed by digestion (e.g., if RNA or uracil-containing), magnetic separation under denaturing conditions (if labeled in a manner conducive to such separation) and other available separation/purification methods. Alternatively, the parental strand is optionally co-purified with the chimeric strands and removed during subsequent screening and processing steps. Additional details regarding this approach are found, e.g., in PCT/US01/06775.

In another approach, single-stranded molecules are converted to double-stranded DNA (dsDNA) and the dsDNA molecules are bound to a solid support by ligand-mediated binding. After separation of unbound DNA, the selected DNA molecules are released from the support and introduced into a suitable host cell to generate a library enriched sequences which hybridize to the probe. A library produced in this manner provides a desirable substrate for further diversification using any of the procedures described herein.

Any of the preceding general recombination formats can be practiced in a reiterative fashion (e.g., one or more cycles of mutation/recombination or other diversity generation methods, optionally followed by one or more selection methods) to generate a more diverse set of recombinant nucleic acids.

Mutagenesis employing polynucleotide chain termination methods have also been proposed (*see e.g.*, U.S. Patent No. 5,965,408 and the references above), and can be applied to the present disclosure. In this approach, double stranded DNAs corresponding to one or more genes sharing regions of sequence similarity are combined and denatured, in the presence or absence of primers specific for the gene. The single stranded polynucleotides are then annealed and incubated in the presence of a polymerase and a chain terminating reagent (e.g., ultraviolet, gamma or X-ray irradiation; ethidium bromide or other intercalators; DNA binding proteins, such as single strand binding proteins, transcription activating factors, or histones; polycyclic aromatic hydrocarbons; trivalent chromium or a trivalent chromium salt; or abbreviated polymerization mediated by rapid thermocycling; and the like), resulting in the production of partial duplex molecules. The partial duplex molecules, e.g., containing partially extended chains, are then denatured and reannealed in subsequent rounds of replication or partial replication resulting in polynucleotides which share varying degrees of sequence similarity and which are diversified with respect to the starting population of DNA molecules. Optionally, the products, or partial pools of the products, can be amplified at one or more stages in the process. Polynucleotides produced by a chain termination method, such as described above, are suitable substrates for any other described recombination

format.

Diversity also can be generated in nucleic acids or populations of nucleic acids using a recombinational procedure termed “incremental truncation for the creation of hybrid enzymes” (“ITCHY”) described in Ostermeier *et al.* (1999) *Nature Biotech* 17:1205. This approach can be used to generate an initial a library of variants which can optionally serve as a substrate for one or more in vitro or in vivo recombination methods. See, also, Ostermeier *et al.* (1999) *Proc. Natl. Acad. Sci. USA*, 96: 3562-67; Ostermeier *et al.* (1999), *Biological and Medicinal Chemistry* 7: 2139-44.

Mutational methods which result in the alteration of individual nucleotides or groups of contiguous or non-contiguous nucleotides can be favorably employed to introduce nucleotide diversity into the nucleic acid sequences and/or gene fusion constructs of the present disclosure. Many mutagenesis methods are found in the above-cited references; additional details regarding mutagenesis methods can be found in following, which can also be applied to the present disclosure.

For example, error-prone PCR can be used to generate nucleic acid variants. Using this technique, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Examples of such techniques are found in the references above and, e.g., in Leung *et al.* (1989) *Technique* 1:11-15 and Caldwell *et al.* (1992) *PCR Methods Applic.* 2:28-33. Similarly, assembly PCR can be used, in a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions can occur in parallel in the same reaction mixture, with the products of one reaction priming the products of another reaction.

Oligonucleotide directed mutagenesis can be used to introduce site-specific mutations in a nucleic acid sequence of interest. Examples of such techniques are found in the references above and, e.g., in Reidhaar-Olson *et al.* (1988) *Science* 241:53-57. Similarly, cassette mutagenesis can be used in a process that replaces a small region of a double stranded DNA molecule with a synthetic oligonucleotide cassette that differs from the native sequence. The oligonucleotide can contain, e.g., completely and/or partially randomized native sequence(s).

Recursive ensemble mutagenesis is a process in which an algorithm for protein mutagenesis is used to produce diverse populations of phenotypically related mutants, members of which differ in amino acid sequence. This method uses a feedback mechanism to monitor successive rounds of combinatorial cassette mutagenesis. Examples of this approach are found in Arkin & Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815.

Exponential ensemble mutagenesis can be used for generating combinatorial libraries with a high percentage of unique and functional mutants. Small groups of residues in a sequence of interest

are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Examples of such procedures are found in Delegrave & Youvan (1993) *Biotechnology Research* 11:1548-1552.

In vivo mutagenesis can be used to generate random mutations in any cloned DNA of interest by propagating the DNA, e.g., in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Such procedures are described in the references noted above.

Other procedures for introducing diversity into a genome, e.g. a bacterial, fungal, animal or plant genome can be used in conjunction with the above described and/or referenced methods. For example, in addition to the methods above, techniques have been proposed which produce nucleic acid multimers suitable for transformation into a variety of species (see, e.g., U.S. Patent No. 5,756,316 and the references above). Transformation of a suitable host with such multimers, consisting of genes that are divergent with respect to one another, (e.g., derived from natural diversity or through application of site directed mutagenesis, error prone PCR, passage through mutagenic bacterial strains, and the like), provides a source of nucleic acid diversity for DNA diversification, e.g., by an *in vivo* recombination process as indicated above.

Alternatively, a multiplicity of monomeric polynucleotides sharing regions of partial sequence similarity can be transformed into a host species and recombined *in vivo* by the host cell. Subsequent rounds of cell division can be used to generate libraries, members of which, include a single, homogenous population, or pool of monomeric polynucleotides. Alternatively, the monomeric nucleic acid can be recovered by standard techniques, e.g., PCR and/or cloning, and recombined in any of the recombination formats, including recursive recombination formats, described above.

Methods for generating multispecies expression libraries have been described (in addition to the reference noted above, see, e.g., U.S. Pat. No. 5,783,431 and U.S. Pat. No. 5,824,485) and their use to identify protein activities of interest has been proposed (In addition to the references noted above, see, U.S. Pat. No. 5,958,672. Multispecies expression libraries include, in general, libraries comprising cDNA or genomic sequences from a plurality of species or strains, operably linked to appropriate regulatory sequences, in an expression cassette. The cDNA and/or genomic sequences are optionally randomly ligated to further enhance diversity. The vector can be a shuttle vector suitable for transformation and expression in more than one species of host organism, e.g., bacterial species, eukaryotic cells. In some cases, the library is biased by preselecting sequences which encode a protein of interest, or which hybridize to a nucleic acid of interest. Any such libraries can

be provided as substrates for any of the methods herein described.

The above described procedures have been largely directed to increasing nucleic acid and/ or encoded protein diversity. However, in many cases, not all of the diversity is useful, e.g., functional, and contributes merely to increasing the background of variants that must be screened or selected to identify the few favorable variants. In some applications, it is desirable to preselect or prescreen libraries (e.g., an amplified library, a genomic library, a cDNA library, a normalized library, etc.) or other substrate nucleic acids prior to diversification, e.g., by recombination-based mutagenesis procedures, or to otherwise bias the substrates towards nucleic acids that encode functional products. For example, in the case of antibody engineering, it is possible to bias the diversity generating process toward antibodies with functional antigen binding sites by taking advantage of in vivo recombination events prior to manipulation by any of the described methods. For example, recombined CDRs derived from B cell cDNA libraries can be amplified and assembled into framework regions (e.g., Jirholt *et al.* (1998) *Gene* 215: 471) prior to diversifying according to any of the methods described herein.

Libraries can be biased towards nucleic acids which encode proteins with desirable enzyme activities. For example, after identifying a variant from a library which exhibits a specified activity, the variant can be mutagenized using any known method for introducing DNA alterations. A library comprising the mutagenized homologues is then screened for a desired activity, which can be the same as or different from the initially specified activity. An example of such a procedure is proposed in U.S. Patent No. 5,939,250. Desired activities can be identified by any method known in the art. For example, WO 99/10539 proposes that gene libraries can be screened by combining extracts from the gene library with components obtained from metabolically rich cells and identifying combinations which exhibit the desired activity. It has also been proposed (e.g., WO 98/58085) that clones with desired activities can be identified by inserting bioactive substrates into samples of the library, and detecting bioactive fluorescence corresponding to the product of a desired activity using a fluorescent analyzer, e.g., a flow cytometry device, a CCD, a fluorometer, or a spectrophotometer.

Libraries can also be biased towards nucleic acids which have specified characteristics, e.g., hybridization to a selected nucleic acid probe. For example, application WO 99/10539 proposes that polynucleotides encoding a desired activity (e.g., an enzymatic activity, for example: a lipase, an esterase, a protease, a glycosidase, a glycosyl transferase, a phosphatase, a kinase, an oxygenase, a peroxidase, a hydrolase, a hydratase, a nitrilase, a transaminase, an amidase or an acylase) can be identified from among genomic DNA sequences in the following manner. Single stranded DNA molecules from a population of genomic DNA are hybridized to a ligand-conjugated probe. The genomic DNA can be derived from either a cultivated or uncultivated microorganism, or from an

environmental sample. Alternatively, the genomic DNA can be derived from a multicellular organism, or a tissue derived there from. Second strand synthesis can be conducted directly from the hybridization probe used in the capture, with or without prior release from the capture medium or by a wide variety of other strategies known in the art. Alternatively, the isolated single-stranded genomic DNA population can be fragmented without further cloning and used directly in, e.g., a recombination-based approach, that employs a single-stranded template, as described above.

“Non-Stochastic” methods of generating nucleic acids and polypeptides are found in WO 00/46344. These methods, including proposed non-stochastic polynucleotide reassembly and site-saturation mutagenesis methods be applied to the present disclosure as well. Random or semi-random mutagenesis using doped or degenerate oligonucleotides is also described in, e.g., Arkin and Youvan (1992) *Biotechnology* 10:297-300; Reidhaar-Olson *et al.* (1991) *Methods Enzymol.* 208:564-86; Lim and Sauer (1991) *J. Mol. Biol.* 219:359-76; Breyer and Sauer (1989) *J. Biol. Chem.* 264:13355-60; and US Patents 5,830,650 and 5,798,208, and EP Patent 0527809 B1.

It will readily be appreciated that any of the above described techniques suitable for enriching a library prior to diversification can also be used to screen the products, or libraries of products, produced by the diversity generating methods. Any of the above described methods can be practiced recursively or in combination to alter nucleic acids, e.g., HPPD encoding polynucleotides.

The above references provide many mutational formats, including recombination, recursive recombination, recursive mutation and combinations or recombination with other forms of mutagenesis, as well as many modifications of these formats. Regardless of the diversity generation format that is used, the nucleic acids of the present disclosure can be recombined (with each other, or with related (or even unrelated) sequences) to produce a diverse set of recombinant nucleic acids for use in the gene fusion constructs and modified gene fusion constructs of the present disclosure, including, e.g., sets of homologous nucleic acids, as well as corresponding polypeptides.

Many of the above-described methodologies for generating modified polynucleotides generate a large number of diverse variants of a parental sequence or sequences. In some embodiments, the modification technique (e.g., some form of shuffling) is used to generate a library of variants that is then screened for a modified polynucleotide or pool of modified polynucleotides encoding some desired functional attribute, e.g., improved HPPD inhibitor insensitivity and/or maintained or improved HPPD activity.

One example of selection for a desired enzymatic activity entails growing host cells under conditions that inhibit the growth and/or survival of cells that do not sufficiently express an enzymatic activity of interest, e.g. the HPPD activity. Using such a selection process can eliminate from consideration all modified polynucleotides except those encoding a desired enzymatic activity.

For example, in some embodiments of the disclosed host cells are maintained under conditions that inhibit cell growth or survival in the absence of sufficient levels of HPPD, e.g., a concentration of an HPPD inhibitor that is lethal or inhibits the growth of a wild-type plant of the same variety that lacks or does not express the HPPD polynucleotide or active variant or fragment thereof. Under these conditions, only a host cell harboring a modified nucleic acid that encodes enzymatic activity or activities able to catalyze production of sufficient levels of the product will survive and grow. Some embodiments of the disclosure employ multiples rounds of screening at increasing concentrations of an HPPD inhibitor.

For convenience and high throughput it will often be desirable to screen/select for desired modified nucleic acids in a microorganism, e.g., a bacteria such as *E. coli*. On the other hand, screening in plant cells or plants can in some cases be preferable where the ultimate aim is to generate a modified nucleic acid for expression in a plant system.

In some preferred embodiments of the disclosure throughput is increased by screening pools of host cells expressing different modified nucleic acids, either alone or as part of a gene fusion construct. Any pools showing significant activity can be deconvoluted to identify single variants expressing the desirable activity.

In high throughput assays, it is possible to screen up to several thousand different variants in a single day. For example, each well of a microtiter plate can be used to run a separate assay, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single variant.

In addition to fluidic approaches, it is possible, as mentioned above, simply to grow cells on media plates that select for the desired enzymatic or metabolic function. This approach offers a simple and high-throughput screening method.

A number of well known robotic systems have also been developed for solution phase chemistries useful in assay systems. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, MA.; Orca, Hewlett-Packard, Palo Alto, CA) which mimic the manual synthetic operations performed by a scientist. Any of the above devices are suitable for application to the present disclosure. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein with reference to the integrated system will be apparent to persons skilled in the relevant art.

High throughput screening systems are commercially available (*see*, e.g., Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of

the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization.

The manufacturers of such systems provide detailed protocols for the various high throughput devices. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like. Microfluidic approaches to reagent manipulation have also been developed, e.g., by Caliper Technologies (Mountain View, CA).

Non-limiting embodiments include:

1. A recombinant polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity comprises:

	5	10	15
	Met Gly Pro Thr	Pro Thr Ala Thr Ala Ala Gly Ala Ala Val Ala	
	20	25	30
	Ala Ala Ser Ala	Ala Glu Gln Ala Ala Phe Arg Leu Val Gly His	
15	35	40	45
	Arg Asn Phe Val	Arg Phe Asn Pro Arg Ser Asp Arg Phe Gln Thr	
	50	55	60
	Leu Ala Phe His	His Val Xaa Xaa Xaa Xaa Xaa Asp Xaa Ala Ser	
	65	70	75
20	Ala Ala Gly Arg	Phe Ser Phe Ala Leu Gly Val Pro Leu Ala Ala	
	80	85	90
	Arg Ser Asp Leu	Ser Thr Gly Asn Ser Ala His Ala Ser Leu Leu	
	95	100	105
	Leu Arg Ser Gly	Ser Leu Ser Leu Leu Phe Thr Ala Pro Tyr Ala	
25	110	115	120
	His Gly Ala Asp	Ala Ala Thr Ala Ala Leu Pro Ser Phe Ser Ala	
	125	130	135
	Ala Ala Ala Arg	Xaa Phe Ala Ala Asp His Gly Leu Ala Val Arg	
	140	145	150
30	Ala Val Ala Leu	Arg Val Ala Asp Ala Glu Asp Xaa Xaa Xaa Ala	
	155	160	165
	Ser Xaa Xaa Xaa	Gly Ala Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	
	170	175	180
	Xaa Xaa Gly Xaa	Xaa Xaa Xaa Xaa Xaa Xaa Xaa Tyr Gly Asp Val	

				185				190					195
	Xaa	Xaa	Arg	Tyr	Val	Ser	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
				200				205					210
	Xaa	Leu	Pro	Gly	Xaa	Glu	Xaa	Val	Xaa	Xaa	Xaa	Xaa	Xaa
5				215				220					225
	Xaa	Xaa	Xaa	Xaa	Xaa	Phe	Xaa	His	Ile	Xaa	Xaa	Xaa	Val
				230				235					240
	Leu	Xaa	Pro	Xaa	Xaa	Xaa	Tyr	Xaa	Xaa	Gly	Phe	Thr	Xaa
				245				250					255
10	Xaa	Xaa	Ala	Glu	Phe	Thr	Thr	Glu	Asp	Val	Gly	Thr	Thr
				260				265					270
	Gly	Leu	Asn	Ser	Met	Ala	Leu	Ala	Asn	Asn	Ser	Glu	Asn
				275				280					285
	Leu	Pro	Xaa	Asn	Glu	Pro	Val	His	Gly	Thr	Lys	Arg	Arg
15				290				295					300
	Ile	Gln	Thr	Phe	Leu	Asp	Xaa	His	Gly	Gly	Pro	Gly	Val
				305				310					315
	Ile	Ala	Leu	Ala	Ser	Asp	Asp	Val	Leu	Arg	Thr	Leu	Arg
				320				325					330
20	Arg	Ala	Arg	Ser	Ala	Met	Gly	Gly	Phe	Glu	Phe	Leu	Pro
				335				340					345
	Xaa	Xaa	Asp	Tyr	Tyr	Asp	Gly	Val	Xaa	Xaa	Cys	Xaa	Xaa
				350				355					360
	Leu	Xaa	Xaa	Xaa	Gln	Xaa	Xaa	Xaa	Cys	Gln	Xaa	Xaa	Xaa
25				365				370					375
	Val	Asp	Arg	Xaa	Xaa	Xaa	Gly	Xaa	Xaa	Leu	Gln	Xaa	Xaa
				380				385					390
	Xaa	Xaa	Gly	Xaa	Arg	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
				395				400					405
30	Xaa	Xaa	Cys	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Glu	Tyr
				410				415					420
	Xaa	Xaa	Xaa	Gly	Gly	Xaa	Gly	Xaa	Gly	Xaa	Xaa	Xaa	Leu
				425				430					435
	Lys	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa

440

Ala Xaa Xaa Xaa Thr Ala Gln Gly Ser (SEQ ID NO:80);

wherein

- 5 Xaa at position 52 is Glu, Asp, Gly, His, Arg, Ser or Thr;
Xaa at position 53 is Leu, Phe, Ile or Met;
Xaa at position 54 is Trp, Cys, Leu or Gln;
Xaa at position 55 is Cys, Ala, Gly, Thr or Val;
Xaa at position 56 is Ala, His, Leu, Asn, Gln, Ser or Thr;
Xaa at position 58 is Ala, Gly or Thr;
- 10 Xaa at position 125 is Arg or Leu;
Xaa at position 147 is Ala or Ser;
Xaa at position 148 is Phe, Trp or Tyr;
Xaa at position 149 is Arg, Ala, Ile, Lys, Met, Pro, Ser, Thr, Val or Trp;
Xaa at position 152 is Val or Glu;
- 15 Xaa at position 153 is Ala, Phe, Ile, Lys, Leu, Gln, Arg, Thr or Val;
Xaa at position 154 is Ala, Cys, Gly, Asn, Arg or Thr;
Xaa at position 157 is Arg, His, Asn, Thr or Val;
Xaa at position 158 is Pro, Ala, Glu, Gly or Lys;
Xaa at position 159 is Ala, Cys, Met, Ser, Thr or Val;
- 20 Xaa at position 160 is Phe, Leu, Met or Tyr;
Xaa at position 161 is Gly, Ala, Glu, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Trp
or Tyr;
Xaa at position 162 is Pro or Thr;
Xaa at position 163 is Val, Ala, Cys, Met or Thr;
- 25 Xaa at position 164 is Asp, Ala, Glu, His, Ser or Thr;
Xaa at position 165 is Leu, Cys, Met or Val;
Xaa at position 166 is Gly, Ala or Pro;
Xaa at position 167 is Arg or Val;
Xaa at position 169 is Phe, Ala, His, Trp or Tyr;
- 30 Xaa at position 170 is Arg, Cys, Gly, Ile, Lys, Leu, Met, Pro, Gln, Ser, Thr, Val or
Trp;
Xaa at position 171 is Leu, Phe, Ile, Met or Val;
Xaa at position 172 is Ala, Pro, Arg or Ser;
Xaa at position 173 is Glu or Pro;

Xaa at position 174 is Val, Cys or Ile;
Xaa at position 175 is Glu, Asp, Val or Trp;
Xaa at position 176 is Leu, Ala or Met;
Xaa at position 181 is Val, Phe, Ile, Met or Asn;
5 Xaa at position 182 is Leu or Met;
Xaa at position 187 is Tyr, Cys, Glu, Gly, His, Met or Val;
Xaa at position 188 is Pro, Asp, Gly or Gln;
Xaa at position 189 is Asp, Glu, Pro, Ser or Thr;
Xaa at position 190 is Gly, Ala, Cys, Asp, Glu, Leu, Met, Asn, Pro, Gln, Arg, Ser,
10 Thr, Val or Trp;
Xaa at position 191 is Ala, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Arg, Ser,
Thr or Val;
Xaa at position 192 is Ala or Val;
Xaa at position 193 is Gly, Ala, His, Ile, Leu, Pro, Gln, Arg, Ser, Thr or Val;
15 Xaa at position 194 is Glu, Asp or Leu;
Xaa at position 195 is Pro, Ala, Cys, Asp, Glu, Gln, Ser, Thr or Val;
Xaa at position 196 is Phe or Trp;
Xaa at position 200 is Phe or Cys;
Xaa at position 202 is Gly, Ala, Glu, Lys, Leu, Thr, Val or Tyr;
20 Xaa at position 204 is Ala, Cys, Asp, Glu, Gly, His, Lys, Leu, Met, Asn, Gln, Thr
or Val;
Xaa at position 205 is Ser, Asp, Gly, Leu, Gln, Thr or Val;
Xaa at position 206 is Pro, Cys, Asp, Lys, Leu, Gln, Ser or Thr;
Xaa at position 207 is Gly, Gln, Ser or Val;
25 Xaa at position 208 is Ala, Gly, His, Gln, Arg or Trp;
Xaa at position 209 is Ala, Cys, Asp, Glu, Gly, Ile, Lys, Leu, Met, Pro, Gln, Ser,
Thr or Trp;
Xaa at position 210 is Asp, Glu, Gly, Ser or Thr;
Xaa at position 211 is Tyr, Cys, Phe, Leu, Ser or Trp;
30 Xaa at position 212 is Gly or Lys;
Xaa at position 213 is Leu or Trp;
Xaa at position 214 is Ser, Lys, Met, Asn, Gln, Arg or Thr;
Xaa at position 215 is Arg, Ala or Gly;
Xaa at position 217 is Asp, Gln or Tyr;

Xaa at position 220 is Val or Thr;
Xaa at position 221 is Gly, Ala, Phe, His, Ile, Leu, Met, Thr or Val;
Xaa at position 222 is Asn, Gly or Val;
Xaa at position 227 is Ala, Asp, Glu, Gly, Ile, Lys, Leu, Met, Asn, Gln, Arg, Ser,
5 Thr or Val;
Xaa at position 229 is Ala or Thr;
Xaa at position 230 is Ala, Arg or Val;
Xaa at position 231 is Ala, Cys, His, Leu, Ser or Thr;
Xaa at position 233 is Met or Val;
10 Xaa at position 234 is Ala, Cys, Glu, Gly, Lys, Leu, Met, Gln, Ser or Val;
Xaa at position 238 is Gly or Ser;
Xaa at position 240 is His or Arg;
Xaa at position 241 is Glu, Ala, Asp, Gly, Asn, Pro, Arg, Ser or Thr;
Xaa at position 242 is Phe, Ala, Asp or Ser;
15 Xaa at position 273 is Leu or Val;
Xaa at position 292 is His or Asn;
Xaa at position 331 is Leu, Asp, Gly, Asn or Arg;
Xaa at position 332 is Ser or Ala;
Xaa at position 339 is Arg or Lys;
20 Xaa at position 340 is Arg, Glu, Lys or Asn;
Xaa at position 342 is Ala, Cys, Leu, Met, Asn, Arg or Val;
Xaa at position 343 is Gly or Arg;
Xaa at position 345 is Val or Ile;
Xaa at position 347 is Thr or Ser;
25 Xaa at position 348 is Glu or Tyr;
Xaa at position 349 is Ala, Glu, Gln, Arg or Ser;
Xaa at position 351 is Ile, Cys or Val;
Xaa at position 352 is Asn, Glu, Lys, Leu, Gln or Arg;
Xaa at position 353 is Glu, Leu, Met, Ser or Thr;
30 Xaa at position 356 is Glu, Lys or Arg;
Xaa at position 357 is Leu or Tyr;
Xaa at position 358 is Gly, Glu or Arg;
Xaa at position 360 is Met, Leu or Thr;
Xaa at position 364 is Asp, Gly, Ser or Val;

Xaa at position 365 is Asp or Ala;
Xaa at position 366 is Gln or Glu;
Xaa at position 368 is Val or Leu;
Xaa at position 369 is Leu, Met or Val;
5 Xaa at position 372 is Ile, Ala, Lys, Gln, Ser or Thr;
Xaa at position 373 is Phe, Gly, Leu, Arg or Val;
Xaa at position 375 is Lys, Leu or Arg;
Xaa at position 376 is Pro, Cys, Gly, Ser, Val or Trp;
Xaa at position 377 is Val, Glu, Gly or Leu;
10 Xaa at position 379 is Asp or Lys;
Xaa at position 381 is Pro or Asn;
Xaa at position 382 is Thr, Ala, Phe or Ser;
Xaa at position 383 is Phe, Leu or Met;
Xaa at position 384 is Phe, Trp or Tyr;
15 Xaa at position 385 is Leu, Ile or Val;
Xaa at position 386 is Glu, Cys, Ile or Val;
Xaa at position 387 is Ile, Gly or Leu;
Xaa at position 388 is Ile, Leu, Ser or Val;
Xaa at position 389 is Gln, Gly, Lys or Thr;
20 Xaa at position 390 is Arg or Asn;
Xaa at position 391 is Ile, Leu or Val;
Xaa at position 392 is Gly, Arg or Val;
Xaa at position 394 is Met, Ile, Lys, Leu, Gln, Val or Tyr;
Xaa at position 395 is Glu, Lys, Gln, Ser or Val;
25 Xaa at position 396 is Lys, Ala, Leu, Met, Gln, Arg, Thr or Val;
Xaa at position 397 is Asp, Gly or Ser;
Xaa at position 398 is Glu, Ala, Asp, Gly, Pro or Ser;
Xaa at position 399 is Lys, Ala, Asp, Gly, His, Ile, Met, Asn, Gln, Arg, Ser, Thr,
Val or Tyr;
30 Xaa at position 400 is Gly, Glu, Lys or Ser;
Xaa at position 401 is Gln, Ala, Glu, Ser or Val;
Xaa at position 406 is Gly or Cys;
Xaa at position 407 is Gly, Ala, Lys, Leu, Arg, Ser or Thr;
Xaa at position 408 is Cys, Gly, Arg, Thr, Val or Trp;

Xaa at position 411 is Phe, Ala or Leu;
 Xaa at position 413 is Lys, Ala, Pro, Arg or Ser;
 Xaa at position 415 is Asn or Ala;
 Xaa at position 416 is Phe, Arg or Val;
 5 Xaa at position 417 is Gly, Gln or Ser;
 Xaa at position 418 is Gln, Ala, Cys, Glu, Gly, Leu or Thr;
 Xaa at position 422 is Ser or Met;
 Xaa at position 423 is Ile, Val or Trp;
 Xaa at position 424 is Glu or Gln;
 10 Xaa at position 425 is Asp, Ala, Glu, Gly, Met, Ser or Thr;
 Xaa at position 426 is Tyr, Leu or Trp;
 Xaa at position 427 is Glu, Ala, Leu or Met;
 Xaa at position 428 is Lys, Arg or Val;
 Xaa at position 429 is Ser, Cys, Asp, Gly or Thr;
 15 Xaa at position 430 is Leu, Met or Val;
 Xaa at position 431 is Glu, Ala, Phe, Gly, Leu or Asn;
 Xaa at position 432 is Ala, Asp, Gly, Lys, Leu, Arg, Ser or Val;
 Xaa at position 433 is Lys, Glu, His, Leu, Pro, Arg, Ser or Val;
 Xaa at position 434 is Gln, Ala, Cys, Asp, Phe, Gly, His, Lys, Leu or Arg;
 20 Xaa at position 437 is Ala, Phe, Lys or Val;
 Xaa at position 438 is Ala, Gly or Arg;
 Xaa at position 439 is Ala or Arg;

wherein one or more amino acid(s) designated by Xaa in SEQ ID NO:80 is an amino acid
 different from the corresponding amino acid of SEQ ID NO:1; and wherein the polypeptide
 25 having 4-hydroxyphenylpyruvate dioxygenase activity has improved insensitivity to an
 HPPD inhibitor compared to the polypeptide of SEQ ID NO:1.

2. A recombinant polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD)
 activity; wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity comprises:

	5	10	15
30	Met Gly Pro Thr Pro Thr Ala Thr Ala Ala Gly Ala Ala Val Ala		
	20	25	30
	Ala Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg Leu Val Gly His		
	35	40	45
	Arg Asn Phe Val Arg Phe Asn Pro Arg Ser Asp Arg Phe Gln Thr		

					50					55					60
	Leu	Ala	Phe	His	His	Val	Glu	Xaa	Trp	Cys	Xaa	Asp	Ala	Ala	Ser
					65					70					75
	Ala	Ala	Gly	Arg	Phe	Ser	Phe	Ala	Leu	Gly	Val	Pro	Leu	Ala	Ala
5					80					85					90
	Arg	Ser	Asp	Leu	Ser	Thr	Gly	Asn	Ser	Ala	His	Ala	Ser	Leu	Leu
					95					100					105
	Leu	Arg	Ser	Gly	Ser	Leu	Ser	Leu	Leu	Phe	Thr	Ala	Pro	Tyr	Ala
					110					115					120
10	His	Gly	Ala	Asp	Ala	Ala	Thr	Ala	Ala	Leu	Pro	Ser	Phe	Ser	Ala
					125					130					135
	Ala	Ala	Ala	Arg	Xaa	Phe	Ala	Ala	Asp	His	Gly	Leu	Ala	Val	Arg
					140					145					150
	Ala	Val	Ala	Leu	Arg	Val	Ala	Asp	Ala	Glu	Asp	Ala	Phe	Arg	Ala
15					155					160					165
	Ser	Val	Ala	Ala	Gly	Ala	Arg	Xaa	Xaa	Phe	Xaa	Pro	Val	Asp	Leu
					170					175					180
	Xaa	Arg	Gly	Xaa	Arg	Xaa	Ala	Glu	Val	Glu	Leu	Tyr	Gly	Asp	Val
					185					190					195
20	Val	Xaa	Arg	Tyr	Val	Ser	Tyr	Pro	Xaa	Xaa	Xaa	Ala	Xaa	Glu	Pro
					200					205					210
	Phe	Leu	Pro	Gly	Phe	Glu	Gly	Val	Ala	Ser	Xaa	Gly	Ala	Ala	Asp
					215					220					225
	Xaa	Gly	Leu	Xaa	Xaa	Phe	Xaa	His	Ile	Val	Xaa	Asn	Val	Pro	Glu
25					230					235					240
	Leu	Xaa	Pro	Ala	Ala	Ala	Tyr	Met	Ala	Gly	Phe	Thr	Gly	Phe	His
					245					250					255
	Glu	Phe	Ala	Glu	Phe	Thr	Thr	Glu	Asp	Val	Gly	Thr	Thr	Glu	Ser
					260					265					270
30	Gly	Leu	Asn	Ser	Met	Ala	Leu	Ala	Asn	Asn	Ser	Glu	Asn	Val	Leu
					275					280					285
	Leu	Pro	Leu	Asn	Glu	Pro	Val	His	Gly	Thr	Lys	Arg	Arg	Ser	Gln
					290					295					300
	Ile	Gln	Thr	Phe	Leu	Asp	His	His	Gly	Gly	Pro	Gly	Val	Gln	His

				305					310					315
	Ile	Ala	Leu	Ala	Ser	Asp	Asp	Val	Leu	Arg	Thr	Leu	Arg	Met
				320					325					330
	Arg	Ala	Arg	Ser	Ala	Met	Gly	Gly	Phe	Glu	Phe	Leu	Pro	Pro
5				335					340					345
	Xaa	Xaa	Asp	Tyr	Tyr	Asp	Gly	Val	Arg	Xaa	Cys	Xaa	Gly	Val
				350					355					360
	Leu	Thr	Glu	Xaa	Gln	Ile	Xaa	Glu	Cys	Gln	Xaa	Leu	Xaa	Val
				365					370					375
10	Val	Asp	Arg	Asp	Asp	Xaa	Gly	Val	Leu	Leu	Gln	Ile	Xaa	Thr
				380					385					390
	Xaa	Val	Gly	Asp	Arg	Xaa	Xaa	Xaa	Phe	Xaa	Glu	Xaa	Ile	Gln
				395					400					405
	Ile	Gly	Cys	Met	Glu	Xaa	Asp	Xaa	Xaa	Gly	Gln	Glu	Tyr	Gln
15				410					415					420
	Gly	Gly	Cys	Gly	Gly	Phe	Gly	Xaa	Gly	Asn	Phe	Xaa	Xaa	Leu
				425					430					435
	Lys	Ser	Xaa	Glu	Asp	Tyr	Glu	Lys	Xaa	Leu	Xaa	Xaa	Lys	Xaa
				440										
20	Ala	Xaa	Xaa	Xaa	Thr	Ala	Gln	Gly	Ser	(SEQ ID NO:81);				

wherein

- | | |
|----|---|
| | Xaa at position 53 is Leu or Phe; |
| | Xaa at position 56 is Ala or Ser; |
| | Xaa at position 125 is Arg or Leu; |
| 25 | Xaa at position 158 is Pro or Lys; |
| | Xaa at position 159 is Ala or Cys; |
| | Xaa at position 161 is Gly, Glu, Gln, Ser, Tyr, Asn or Leu; |
| | Xaa at position 166 is Gly, Pro or Ala; |
| | Xaa at position 169 is Phe or His; |
| 30 | Xaa at position 171 is Leu or Phe; |
| | Xaa at position 182 is Leu or Met; |
| | Xaa at position 189 is Asp or Pro; |
| | Xaa at position 190 is Gly or Thr; |
| | Xaa at position 191 is Ala, Ser, Leu or Gly; |

Xaa at position 193 is Gly or Gln;
Xaa at position 206 is Pro, Gln, Ser, Lys or Leu;
Xaa at position 211 is Tyr, Leu or Ser;
Xaa at position 214 is Ser, Asn, Thr, Arg or Met;
5 Xaa at position 215 is Arg or Gly;
Xaa at position 217 is Asp or Tyr;
Xaa at position 221 is Gly, Val or Ala;
Xaa at position 227 is Ala or Gly;
Xaa at position 331 is Leu, Asn, Arg, Gly or Asp;
10 Xaa at position 332 is Ser or Ala;
Xaa at position 340 is Arg or Lys;
Xaa at position 342 is Ala, Val, Met or Cys;
Xaa at position 349 is Ala, Gln or Glu;
Xaa at position 352 is Asn or Arg;
15 Xaa at position 356 is Glu, Lys or Arg;
Xaa at position 358 is Gly or Glu;
Xaa at position 360 is Met or Leu;
Xaa at position 366 is Gln or Glu;
Xaa at position 373 is Phe or Val;
20 Xaa at position 375 is Lys, Leu or Arg;
Xaa at position 376 is Pro or Trp;
Xaa at position 381 is Pro or Asn;
Xaa at position 382 is Thr or Ser;
Xaa at position 383 is Phe, Met or Leu;
25 Xaa at position 385 is Leu, Val or Ile;
Xaa at position 387 is Ile or Leu;
Xaa at position 390 is Arg or Asn;
Xaa at position 396 is Lys, Thr, Met or Leu;
Xaa at position 398 is Glu, Asp or Ala;
30 Xaa at position 399 is Lys, Asn, Arg, Gln or Ser;
Xaa at position 413 is Lys or Ala;
Xaa at position 417 is Gly, Ser or Gln;
Xaa at position 418 is Gln or Glu;
Xaa at position 423 is Ile or Trp;

Xaa at position 429 is Ser, Gly or Thr;

Xaa at position 431 is Glu or Phe;

Xaa at position 432 is Ala, Arg, Lys or Ser;

Xaa at position 434 is Gln, Arg, His, Leu or Lys;

5 Xaa at position 437 is Ala, Lys or Phe;

Xaa at position 438 is Ala, Gly or Arg;

Xaa at position 439 is Ala or Arg;

10 wherein one or more amino acid(s) designated by Xaa in SEQ ID NO:81 is an amino acid different from the corresponding amino acid of SEQ ID NO:1; and wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity has improved insensitivity to an HPPD inhibitor compared to the polypeptide of SEQ ID NO:1.

3. A recombinant polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity comprises:

	5	10	15
15	Met Gly Pro Thr Pro Thr Ala Thr Ala Ala Gly Ala Ala Val Ala		
	20	25	30
	Ala Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg Leu Val Gly His		
	35	40	45
	Arg Asn Phe Val Arg Phe Asn Pro Arg Ser Asp Arg Phe Gln Thr		
20	50	55	60
	Leu Ala Phe His His Val Glu Leu Trp Cys Ala Asp Ala Ala Ser		
	65	70	75
	Ala Ala Gly Arg Phe Ser Phe Ala Leu Gly Val Pro Leu Ala Ala		
	80	85	90
25	Arg Ser Asp Leu Ser Thr Gly Asn Ser Ala His Ala Ser Leu Leu		
	95	100	105
	Leu Arg Ser Gly Ser Leu Ser Leu Leu Phe Thr Ala Pro Tyr Ala		
	110	115	120
	His Gly Ala Asp Ala Ala Thr Ala Ala Leu Pro Ser Phe Ser Ala		
30	125	130	135
	Ala Ala Ala Arg Arg Phe Ala Ala Asp His Gly Leu Ala Val Arg		
	140	145	150
	Ala Val Ala Leu Arg Val Ala Asp Ala Glu Asp Ala Phe Arg Ala		
	155	160	165

	Ser	Val	Ala	Ala	Gly	Ala	Arg	Xaa	Ala	Phe	Gly	Pro	Val	Asp	Leu
					170					175					180
	Gly	Arg	Gly	Phe	Arg	Leu	Ala	Glu	Val	Glu	Leu	Tyr	Gly	Asp	Val
					185					190					195
5	Val	Xaa	Arg	Tyr	Val	Ser	Tyr	Pro	Xaa	Gly	Xaa	Ala	Gly	Glu	Pro
					200					205					210
	Phe	Leu	Pro	Gly	Phe	Glu	Gly	Val	Ala	Xaa	Xaa	Gly	Ala	Ala	Asp
					215					220					225
	Xaa	Gly	Leu	Xaa	Arg	Phe	Asp	His	Ile	Val	Gly	Asn	Val	Pro	Glu
10					230					235					240
	Leu	Xaa	Pro	Ala	Ala	Ala	Tyr	Met	Ala	Gly	Phe	Thr	Gly	Phe	His
					245					250					255
	Glu	Phe	Ala	Glu	Phe	Thr	Thr	Glu	Asp	Val	Gly	Thr	Thr	Glu	Ser
					260					265					270
15	Gly	Leu	Asn	Ser	Met	Ala	Leu	Ala	Asn	Asn	Ser	Glu	Asn	Val	Leu
					275					280					285
	Leu	Pro	Leu	Asn	Glu	Pro	Val	His	Gly	Thr	Lys	Arg	Arg	Ser	Gln
					290					295					300
	Ile	Gln	Thr	Phe	Leu	Asp	His	His	Gly	Gly	Pro	Gly	Val	Gln	His
20					305					310					315
	Ile	Ala	Leu	Ala	Ser	Asp	Asp	Val	Leu	Arg	Thr	Leu	Arg	Glu	Met
					320					325					330
	Arg	Ala	Arg	Ser	Ala	Met	Gly	Gly	Phe	Glu	Phe	Leu	Pro	Pro	Pro
					335					340					345
25	Leu	Ser	Asp	Tyr	Tyr	Asp	Gly	Val	Arg	Xaa	Cys	Xaa	Gly	Asp	Val
					350					355					360
	Leu	Thr	Glu	Ala	Gln	Ile	Asn	Glu	Cys	Gln	Xaa	Leu	Gly	Val	Xaa
					365					370					375
	Val	Asp	Arg	Asp	Asp	Xaa	Gly	Val	Leu	Leu	Gln	Ile	Phe	Thr	Lys
30					380					385					390
	Xaa	Val	Gly	Asp	Arg	Pro	Thr	Phe	Phe	Xaa	Glu	Xaa	Ile	Gln	Xaa
					395					400					405
	Ile	Gly	Cys	Met	Glu	Lys	Asp	Glu	Lys	Gly	Gln	Glu	Tyr	Gln	Lys
					410					415					420

Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Xaa Gln Leu Phe
425 430 435
Lys Ser Ile Glu Asp Tyr Glu Lys Ser Leu Xaa Xaa Lys Xaa Ala
440

5 Ala Ala Ala Ala Thr Ala Gln Gly Ser (SEQ ID NO:82);

wherein

Xaa at position 158 is Pro or Lys;

Xaa at position 182 is Leu or Met;

Xaa at position 189 is Asp or Pro;

10 Xaa at position 191 is Ala, Gly or Leu;

Xaa at position 205 is Ser or Thr;

Xaa at position 206 is Pro, Gln or Ser;

Xaa at position 211 is Tyr or Leu;

Xaa at position 214 is Ser, Asn or Thr;

15 Xaa at position 227 is Ala or Gly;

Xaa at position 340 is Arg or Lys;

Xaa at position 342 is Ala or Val;

Xaa at position 356 is Glu, Lys or Arg;

Xaa at position 360 is Met or Leu;

20 Xaa at position 366 is Gln or Glu;

Xaa at position 376 is Pro or Trp;

Xaa at position 385 is Leu, Ile or Val;

Xaa at position 387 is Ile or Leu;

Xaa at position 390 is Arg or Asn;

25 Xaa at position 417 is Gly, Gln or Ser;

Xaa at position 431 is Glu or Phe;

Xaa at position 432 is Ala, Arg or Lys;

Xaa at position 434 is Gln, His or Arg;

wherein one or more amino acid(s) designated by Xaa in SEQ ID NO:82 is an amino acid
30 different from the corresponding amino acid of SEQ ID NO:1; and wherein the polypeptide
having 4-hydroxyphenylpyruvate dioxygenase activity has improved insensitivity to an
HPPD inhibitor compared to the polypeptide of SEQ ID NO:1.

4. A recombinant polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity comprises:

				5					10					15	
	Met	Gly	Pro	Thr	Pro	Thr	Ala	Thr	Ala	Ala	Gly	Ala	Ala	Val	Ala
				20					25					30	
	Ala	Ala	Ser	Ala	Ala	Glu	Gln	Ala	Ala	Phe	Arg	Leu	Val	Gly	His
5				35					40					45	
	Arg	Asn	Phe	Val	Arg	Phe	Asn	Pro	Arg	Ser	Asp	Arg	Phe	Gln	Thr
				50					55					60	
	Leu	Ala	Phe	His	His	Val	Glu	Leu	Trp	Cys	Ala	Asp	Ala	Ala	Ser
				65					70					75	
10	Ala	Ala	Gly	Arg	Phe	Ser	Phe	Ala	Leu	Gly	Val	Pro	Leu	Ala	Ala
				80					85					90	
	Arg	Ser	Asp	Leu	Ser	Thr	Gly	Asn	Ser	Ala	His	Ala	Ser	Leu	Leu
				95					100					105	
	Leu	Arg	Ser	Gly	Ser	Leu	Ser	Leu	Leu	Phe	Thr	Ala	Pro	Tyr	Ala
15				110					115					120	
	His	Gly	Ala	Asp	Ala	Ala	Thr	Ala	Ala	Leu	Pro	Ser	Phe	Ser	Ala
				125					130					135	
	Ala	Ala	Ala	Arg	Arg	Phe	Ala	Ala	Asp	His	Gly	Leu	Ala	Val	Arg
				140					145					150	
20	Ala	Val	Ala	Leu	Arg	Val	Ala	Asp	Ala	Glu	Asp	Ala	Phe	Arg	Ala
				155					160					165	
	Ser	Val	Ala	Ala	Gly	Ala	Arg	Xaa	Ala	Phe	Gly	Pro	Val	Asp	Leu
				170					175					180	
	Gly	Arg	Gly	Phe	Arg	Leu	Ala	Glu	Val	Glu	Leu	Tyr	Gly	Asp	Val
25				185					190					195	
	Val	Xaa	Arg	Tyr	Val	Ser	Tyr	Pro	Asp	Gly	Xaa	Ala	Gly	Glu	Pro
				200					205					210	
	Phe	Leu	Pro	Gly	Phe	Glu	Gly	Val	Ala	Ser	Xaa	Gly	Ala	Ala	Asp
				215					220					225	
30	Xaa	Gly	Leu	Xaa	Arg	Phe	Asp	His	Ile	Val	Gly	Asn	Val	Pro	Glu
				230					235					240	
	Leu	Ala	Pro	Ala	Ala	Ala	Tyr	Xaa	Ala	Gly	Phe	Thr	Gly	Phe	His
				245					250					255	
	Glu	Phe	Ala	Glu	Phe	Thr	Thr	Glu	Asp	Val	Gly	Thr	Thr	Glu	Ser

		260		265		270									
	Gly	Leu	Asn	Ser	Met	Ala	Leu	Ala	Asn	Asn	Ser	Glu	Asn	Val	Leu
		275		280		285									
	Leu	Pro	Leu	Asn	Glu	Pro	Val	His	Gly	Thr	Lys	Arg	Arg	Ser	Gln
5		290		295		300									
	Ile	Gln	Thr	Phe	Leu	Asp	His	His	Gly	Gly	Pro	Gly	Val	Gln	His
		305		310		315									
	Ile	Ala	Leu	Ala	Ser	Asp	Asp	Val	Leu	Arg	Thr	Leu	Arg	Glu	Met
		320		325		330									
10	Arg	Ala	Arg	Ser	Ala	Met	Gly	Gly	Phe	Glu	Phe	Leu	Pro	Pro	Pro
		335		340		345									
	Leu	Ser	Asp	Tyr	Tyr	Asp	Gly	Val	Arg	Xaa	Cys	Ala	Gly	Asp	Val
		350		355		360									
	Leu	Thr	Glu	Ala	Gln	Ile	Asn	Glu	Cys	Gln	Xaa	Leu	Gly	Val	Met
15		365		370		375									
	Val	Asp	Arg	Asp	Asp	Xaa	Gly	Val	Leu	Leu	Gln	Ile	Phe	Thr	Lys
		380		385		390									
	Pro	Val	Gly	Asp	Arg	Pro	Thr	Phe	Phe	Xaa	Glu	Xaa	Ile	Gln	Arg
		395		400		405									
20	Ile	Gly	Cys	Xaa	Glu	Lys	Asp	Glu	Lys	Gly	Gln	Glu	Tyr	Gln	Lys
		410		415		420									
	Gly	Gly	Cys	Gly	Gly	Phe	Gly	Lys	Gly	Asn	Phe	Xaa	Gln	Leu	Phe
		425		430		435									
	Lys	Ser	Ile	Glu	Asp	Tyr	Glu	Xaa	Ser	Leu	Glu	Xaa	Lys	Xaa	Ala
25		440													

Ala Ala Ala Ala Thr Ala Gln Gly Ser (SEQ ID NO:83);

wherein

Xaa at position 158 is Pro or Lys;

Xaa at position 182 is Leu or Met;

30 Xaa at position 191 is Ala or Gly;

Xaa at position 206 is Pro, Ser or Gln;

Xaa at position 211 is Tyr or Leu;

Xaa at position 214 is Ser or Asn;

Xaa at position 233 is Met or Val;

Xaa at position 340 is Arg or Lys;
Xaa at position 356 is Glu, Lys or Arg;
Xaa at position 366 is Gln or Glu;
Xaa at position 385 is Leu, Ile or Val;
5 Xaa at position 387 is Ile or Leu;
Xaa at position 394 is Met, Val or Lys;
Xaa at position 417 is Gly, Ser or Gln;
Xaa at position 428 is Lys or Arg;
Xaa at position 432 is Ala, Lys or Arg;
10 Xaa at position 434 is Gln, Arg or His;

wherein one or more amino acid(s) designated by Xaa in SEQ ID NO:83 is an amino acid different from the corresponding amino acid of SEQ ID NO:1; and wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity has improved insensitivity to an HPPD inhibitor compared to the polypeptide of SEQ ID NO:1.

15 5. The polypeptide of embodiments 1-4, wherein the polypeptide further comprises substitution of one or more conservative amino acids, insertion of one or more amino acids, deletion of one or more amino acids, and combinations thereof.

6. The polypeptide of any of embodiments 1-5, wherein the polypeptide has an ON rate ratio of at least about 0.5; wherein the ON rate ratio is the ratio of the reaction rate with herbicidal
20 inhibitor to the reaction rate without herbicidal inhibitor; and wherein the reaction rates are determined in an *in vitro* assay.

7. The polypeptide of embodiment 6, wherein the herbicidal inhibitor is mesotrione or tembrione.

8. The polypeptide of embodiment 6 or 7, wherein *in vitro* assay is carried out in the
25 presence of 60 or 120 nM of the 4-hydroxyphenylpyruvate dioxygenase protein.

9. The polypeptide of any of embodiments 6-8, wherein *in vitro* assay is carried out in the presence of 100 μ M 4-hydroxyphenylpyruvate.

10. The polypeptide of any of embodiments 6-9, wherein the polypeptide has an OFF rate ratio of at least about 0.3; wherein the OFF rate ratio is the ratio of the steady state rate in the
30 presence of inhibitor to the initial reaction rate in the absence of inhibitor; and wherein the reaction rates are determined in an *in vitro* assay.

11. The polypeptide of embodiment 10, wherein the herbicidal inhibitor is mesotrione or tembrione.

12. The polypeptide of embodiment 10 or 11, wherein the *in vitro* assay is carried out in

the presence of 60 or 120 nM of the 4-hydroxyphenylpyruvate dioxygenase protein.

13. The polypeptide of any of embodiments 10-12, wherein the *in vitro* assay is carried out in the presence of 100 μ M 4-hydroxyphenylpyruvate.

14. The polypeptide of any of embodiments 1-13, wherein the polypeptide has a fitness parameter of about 2 to about 200 $\text{min}^{-1}\mu\text{M}^{-1}$; and wherein

$$\text{Fitness Parameter} = \frac{k_{cat}}{K_m} \times (\text{ON rate ratio} \times \text{OFF rate ratio}).$$

15. A nucleic acid construct comprising a polynucleotide sequence encoding a polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide encoded is the polypeptide of any of embodiments 1-14.

16. The nucleic acid construct of embodiment 15, further comprising a promoter operably linked to the polynucleotide sequence.

17. A plant cell comprising a nucleic acid construct comprising a polynucleotide sequence encoding a polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide encoded is the polypeptide of any of embodiments 1-14.

18. The plant cell of embodiment 17, wherein the nucleic acid construct further comprises a promoter operably linked to the polynucleotide sequence.

19. The plant cell of embodiments 17 or 18, wherein the plant cell exhibits has an improved insensitivity to an HPPD inhibitor compared to a wild type plant cell of the same species, strain or cultivar.

20. The plant cell of any of embodiments 17-19, wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity is present in an amount from about 1000 ppm to about 5000 ppm.

21. The plant cell of any of embodiments 17-19, wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity is present in an amount from about 200 ppm to about 4000 ppm.

22. The plant cell of any of embodiments 17-21, wherein the nucleic acid construct is stably incorporated into the genome of the plant cell.

23. The plant cell of any of embodiments 17-22, wherein the plant cell is from a monocot.

24. The plant cell of embodiment 23, wherein the monocot is maize, wheat, rice, barley, sugarcane, sorghum, or rye.

25. The plant cell of any of embodiments 17-22, wherein the plant cell is from a dicot.

26. The plant cell of embodiment 24, wherein the dicot is soybean, *Brassica*, sunflower, cotton, or alfalfa.

27. The plant cell of any of embodiments 17-26, wherein the plant cell further comprises at least one additional polypeptide imparting tolerance to an additional herbicide.

28. The plant cell of embodiment 27, wherein the at least one polypeptide imparting tolerance to an additional herbicide comprises: (a) a sulfonylurea-tolerant acetolactate synthase; (b) an imidazolinone-tolerant acetolactate synthase; (c) a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase; (d) a glyphosate-tolerant glyphosate oxido-reductase; (e) a glyphosate-N-acetyltransferase; (f) a phosphinothricin acetyl transferase; (g) a protoporphyrinogen oxidase, (h) an auxin enzyme; (i) a P450 polypeptide; or, (j) an acetyl coenzyme A carboxylase (ACCase).

29. The plant cell of embodiment 27, wherein the at least one polypeptide imparting tolerance to an additional herbicide comprises a high resistance allele of acetolactate synthase (HRA) and/or a glyphosate-N-acetyltransferase polypeptide.

30. The plant cell of embodiment 27, wherein the plant cell further comprises at least one additional polypeptide imparting tolerance to an HPPD herbicide.

31. The plant cell of embodiment 30, wherein the at least one additional polypeptide imparting tolerance to an HPPD herbicide comprises a P450 polypeptide or NSF1.

32. A plant comprising the plant cell of any of embodiments 17-31.

33. An explant comprising the plant cell of any of embodiments 17-31.

34. A transgenic seed produced by the plant of embodiment 32.

35. A method of producing a 4-hydroxyphenylpyruvate dioxygenase (HPPD) herbicide tolerant plant cell comprising transforming a plant cell with a polynucleotide encoding the polypeptide of any of embodiments 1-14 or the nucleic acid construct of embodiments 15-16.

36. The method of embodiment 35, further comprising selecting a plant cell which is resistant to an HPPD herbicide by growing plant cells in the presence of a concentration of an HPPD herbicide that bleaches the plant cell which does not comprise a polynucleotide encoding the polypeptide of any of embodiments 1-14 or the nucleic acid construct of embodiments 15-16.

37. The method of embodiment 36, wherein the method comprises

(a) culturing the plant cell in the presence of a sufficient concentration of an HPPD herbicide such that the plant cells display bleaching;

(b) transforming into the plant cells of step (a) a polynucleotide encoding the polypeptide of any of embodiments 1-14 or the nucleic acid construct of embodiments 15-16; and

(c) growing the plant cells of (b), wherein transformed plants cells no longer display bleaching.

38. The method of embodiment 35, wherein the method further comprises regenerating a

transgenic plant from the plant cell.

39. The method of embodiment 35, wherein the transforming the plant cell results in the sTable 2ntegration of the polynucleotide into the genome of the plant cell.

40. The method of embodiment 35, wherein the transforming the plant cell results in the sTable 2ntegration of the polynucleotide into the genome of a chloroplast in the plant cell.

41. A method for controlling weeds in a field containing a crop comprising:

- (a) planting the field with the transgenic seeds of embodiment 34; and,
- (b) applying to any crop and weeds in the field a sufficient amount of an HPPD herbicide to control weeds without significantly affecting the crop.

42. The method of embodiment 41, wherein the HPPD herbicide comprises at least one of triketones, isoxazoles, pyrazoles, or benzobicyclon or active derivatives thereof or an agriculturally acceptable salt thereof.

43. The method of embodiment 42, wherein the HPPD herbicide comprises at least one of mesotrione, sulcotrione, topremezone, and tembotrione, pyrasulfotole, isoxaflutole, benzofenap, pyrazoxyfen, or pyrazolynate or active derivative thereof or an agriculturally acceptable salt thereof.

44. The method of embodiment 41, further comprising applying to the crop and weeds in the field a sufficient amount of at least one additional herbicide comprising glyphosate, bialaphos, phosphinothricin, azafenidin, butafenacil, sulfosate, glufosinate, an ALS inhibitor, or a protox inhibitor.

45. A method for detecting an HPPD polypeptide comprising analyzing plant tissues using an immunoassay comprising at least one antibody that recognizes the polypeptide of any of embodiments 1-14.

46. A method for detecting the presence of a polynucleotide encoding the polypeptide of any of embodiments 1-14 comprising assaying plant tissue using PCR amplification and detecting the polynucleotide.

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EXAMPLES

Example 1. Experimental Methods

Immuno-Localization Electron Microscopy

Briefly, native or heterologously expressed HPPD was tagged with affinity purified rabbit anti-HPPD antibodies, which were labeled with goat anti-rabbit F(ab') conjugated with μ ltrasmall gold particles (Aurion, The Netherlands). Gold labeling was followed by silver enhancement (Aurion). Sections were counterstained with 4% uranyl acetate (aqueous) followed by Reynold's lead citrate and examined by electron microscopy.

Genomic and Transcript Analysis

A synthetic maize wild-type HPPD cDNA (NCBI Reference Sequence: NM_00112312.1) was assembled from commercially synthesized oligonucleotides. During the synthesis of the gene, an *NcoI* restriction site was engineered into the start of the sequence to facilitate cloning. The change of codons (C)ATG CCC to (C)ATG GGT resulted in the substitution of glycine for proline at position 2. An EST (sgc5c.pk001.j9) coding for soybean HPPD was identified from a DuPont-Pioneer proprietary *G. max* EST database using conventional bioinformatic tools including BLAST of the HPPD sequence described in Cahoon and Coughlan (2007). The soybean HPPD coding region sequence was cloned from the EST cDNA into a T7-based bacterial expression vector. Expression, purification and characterization of the *G. max* HPPD protein proved that the encoded protein was a functional HPPD, able to catalyze the reaction from 4-hydroxyphenylpyruvate to homogentisate. Using this soybean HPPD coding sequence as query, Pioneer Unigene PSO409914 was identified. Search of the genome assembly database with the unigene as query resulted in identification of approximately 2kb genomic sequence upstream of the EST. To validate the genomic sequence, polymerase chain reaction (PCR) primers (forward primer: GCAAGTATTTCAATACAATAGC (SEQ ID NO:84) and reverse primer: GTTATCTGATATGATGTTGC (SEQ ID NO:85)) were designed and used to amplify the HPPD locus from genomic DNA isolated from an elite soybean variety and the common Jack variety, following protocols for isolation of plant genomic DNA (Qiagen, Germantown, MD). PCR reaction parameters were: Cycle1: 94°C, 2 min; Cycle 2 to 30: 94°C, 30 s; 65°C, 1 min; 72°C, 5 min; Cycle 31: 72°C, 10 min. A proof-reading DNA polymerase, *pfu* Turbo (Stratagene, La Jolla, CA) was used for PCR amplification. A 4306 bp fragment and a 4310 bp fragment were obtained from elite and Jack, respectively. These fragments were cloned into Zero blunt TOPO PCR cloning vectors (Invitrogen, Carlsbad, CA) and fully sequenced. Each locus comprises 3' sequences, HPPD coding region, and upstream genomic sequence. The loci are highly conserved, with an overall 99% sequence identity at the nucleotide level. A 462 bp intron and a 459 bp intron in the HPPD coding region were identified in elite and Jack, respectively.

RNA ligation mediated 5' rapid cloning of cDNA ends (5' RACE) was used to validate transcription start sites for the native HPPD promoter using total RNA extracted from young soybean leaves and First Choice RLM-RACE kit (Ambion) per manufacturer's protocol. Total RNA was isolated with the Qiagen RNeasy mini kit. Linked transcription-translation was performed in wheat germ extracts using 3' truncated *G. max* HPPD transcription vectors. Tissue specific patterns of expression for *G. max* HPPD, EPSPS, ALS, and ACT2/7 genes were compiled from expression data in SoyBase (soybase.org) using the Soybean Breeder's Toolbox search.

Expression Vectors for Localization Microscopy

Vectors for transient expression in monocot species were constructed in which N-terminal fragments of monocot HPPD proteins or the soybean HPPD protein and the synthetic monocot consensus peptide were fused to the gene coding for *Discosoma sp.* red fluorescence protein 2 (DsRed2; Clontech, Mountain View CA) and inserted into a binary expression vector under control of the maize rubisco activase promoter (Liu *et al.*, 1996) or Arabidopsis Ubiquitin 10 promoter (Norris, *et al.*, 1993) and terminated with the *Solanum tuberosum* proteinase inhibitor II (*pinII*) terminator region (An *et al.*, 1989) with a hygromycin selection cassette. The vector also contained an untargeted Zs Green cassette to provide cytoplasmic contrast and a kanamycin selection cassette. All three genes were between left and right border sequences of *Agrobacterium* T-DNA. A positive control vector was identical except that the insert was DsRed2 fused to the chloroplast targeting peptide of Arabidopsis rubisco activase, while a negative control was DsRed2 with no targeting sequence.

A second set of florescent vectors was made with N-terminal fragments encoding either the maize or soybean N-terminal HPPD protein fragments fused to a gene encoding *Aequorea coerulescens* green fluorescent protein 1 (AcGFP1) and inserted into a binary expression vector under control of the Arabidopsis Ubiquitin 10 promoter (Norris *et al.*, 1993) and terminated with the *G. max* Kunitz trypsin inhibitor 3 terminator region (NCBI accession S45092). Such vectors were used for either stable or transient gene expression in plant cells. Fusions for testing the soybean CTP function contained the sequence encoding amino acid residues 1-42 or 1-86 of the long *G. max* HPPD protein. Another contained the sequence encoding residues 1-44 of the short HPPD protein (corresponding to residues 42-86 of the long protein). A positive control vector was identical except that the AcGFP1 coding region was fused to the 6H1 synthetic chloroplast targeting peptide (Lassner and Wilkinson, 2008), while a negative control was AcGFP1 with no targeting sequence.

Promoter Constructs

Cassettes for testing transient expression of promoters were similar to the DsRed N-terminal test vectors except that *G. max* HPPD promoter-derived fragments replaced the rubisco and UBQ10

promoters. A 2061 bp fragment comprising the native HPPD promoter of elite *G. max* was created from the upstream genomic sequence. The PCR reaction used (forward primer hp0234: GTTTTCCGCGGGTGTGATCC (SEQ ID NO:86) and reverse primer hp2296: TCATTGGTACCTGGTGTGGTGTGATGCTGC (SEQ ID NO:87)) introduced *SacII* and *KpnI* restriction sites. This fragment was isolated by gel-purification, digested with restriction enzymes *SacII* and *KpnI*, and ligated to a DsRed2 marker gene to form the native *G. max* HPPD promoter expression cassette. Introduction of the *KpnI* site between the promoter fragments and DsRed2 resulted in the introduction of a two amino acid linker (GGTACC gly-thr) in the fusion proteins containing the long protein CTP region.

The 1225 bp genomic sequence at the 3' end of the promoter fragment was subjected to *in silico* promoter analysis using Promoter REAPER and Promoter Delineator (Simmons and Navarro Acevedo, 2007). Genomic sequence from other species including *Arabidopsis thaliana*, *Medicago truncatula*, *Populus trichocarpa*, *Brassica rapa*, *Vitis vinifera*, and the monocot *Sorghum bicolor* were included for comparison in this analysis. With the program Promoter REAPER, regions were identified in the soybean HPPD promoter that are predicted to be important for its activity based on the sequence conservation of a set of DNA motifs across the seven plant species. To evaluate the predicted TATA boxes in promoter activity, a deletion series was created with the 2061 bp template using PCR with forward primer hp0234: GTTTTCCGCGGGTGTGATCC and the reverse primers as follows: SHP103C, hp2154: AGCATGGTACCTTGCCTCTGGGTTGAG (SEQ ID NO:88); SHP110C, hp2048: ATCTGGTACCTGATGTTGATGCGGC (SEQ ID NO:89); SHP000C, hp1962: AGGAGGTACCGTCAAATCCACCTAG (SEQ ID NO:90); SHP102C, hp1791: AGCCTGGTACCTTGTGTGTAAAAAAGATAAGAC (SEQ ID NO:91); and SHP101C, hp1663: TCCTTGGTACCTGATGCACTATATAACG (SEQ ID NO:92). Single or triple mutations in the putative TATA boxes (see Table 4) were created using site-directed mutagenesis (Quick Change, Stratagene). Resultant deletion and mutated promoter fragments were fused with DsRed2 to create cassettes for analysis of expression activity in agro-infiltrated leaf tissues.

SHP101C and SHP102C lack promoter activity due to deletion of active TATA binding sites. To create synthetic promoters using these non-promoter DNA fragments, synthetic element I or II, each flanked by restriction sites *XhoI* and *KpnI*, were synthesized and ligated with the 3' ends of the HPPD promoter-derived DNA fragments to create SHP101 and 201 and SHP102 and 202, respectively. Synthetic element I comprises the SynII core (Bowen et al., 2000; sequence 1) sequence followed by the 45 bp putative 5'UTR sequence including the predicted transcription start site from the soybean native HPPD gene (ACAACCACCAAGCTCAATCTCAAGCAGCAGCATCACACCACCA (SEQ ID NO:93),

nucleotides between TATA3 and ATG). Synthetic element II contains the Rsyn7 region (Bowen et al., 2000, sequence 2) immediately upstream of synthetic element I. In a similar manner, promoters SHP103, 104, 105, 106, 107, 108, and 109 were created with element I and SHP203, 204, 205, 206, 207, 208, and 209 were created with synthetic element II. In SHP110, a partial SynII core sequence is inserted in place of TATA5. In SHP210, TATA5 through transcription start site at -237 is deleted and replaced with Rsyn7 plus the partial SynII core sequence. SHP110e is the same as SHP110 with the addition of a CaMV enhancer (Ow et al., 1987) at the 5' end of the promoter fragment. In SHP111, the sequence encompassing TATA3 through transcription start site at +1 is deleted and replaced with the partial SynII core sequence. In SHP120C an A to T mutation eliminates the start codon of the upORF element.

Agrobacterium-Infiltration and Florescence Measurements

All seedling plants were grown in growth chambers with 16 hr light at 375-450 $\mu\text{m}^{-2} \text{s}^{-1}$, 26°C day and 22°C night. Expression plasmids for localization studies were transformed into *Agrobacterium tumefaciens* AGL-1 via electroporation according to Shen and Forde (1989) and agro-infiltration (Kapila et al., 1997) used to introduce the constructs into plant cells. *Agrobacterium* cultures were grown overnight in LB with 40 mg/L kanamycin and a working suspension normalized to 1.0 OD₆₀₀ in 10 mM MgSO₄, 400 μM acetosyringone and 1 mM DTT. Leaves of 3-week old maize or sorghum seedlings were infiltrated with the *Agrobacterium*, and examined by fluorescence microscopy two days later (Nikon Eclipse 80i, DsRed2 filter set). Infiltrated leaf samples were derived from plants of uniform developmental stage grown under the same conditions. Leaves of 4-week old *Nicotiana benthamiana*, 8-day old *Phaseolus vulgaris*, (variety Shade) and 10-day old *Glycine max* seedlings were infiltrated with the *Agrobacterium*, and examined by fluorescence microscopy 4 and 5 days later.

Agrobacterium strains expressing plasmids for promoter characterization were infiltrated into bush bean leaf tissues. Visual analysis confirmed that the native *G. max* HPPD promoter DNA fragment was able to drive the expression of DsRed2 in infiltrated leaf tissues compared with fluorescent background from leaf tissues infiltrated with a negative control construct consisting of the dMMV promoter (Dey and Maiti, 1999) driving a beta-glucuronidase reporter. Relative promoter strength was determined by quantitative measurement of the red fluorescence generated from expressed DsRed2 protein in infiltrated leaf tissues. Red fluorescence from 50 μg of protein extracted from infiltrated leaf discs was quantified using a Typhoon Trio+ Variable Mode Imager. Infiltration experiments for each construct were repeated at least three times. The background red fluorescence detected in leaves infiltrated with the negative control vector was subtracted for data normalization. The DsRed2 readouts were used to calculate the level of DsRed2 from each construct

relative to the DsRed2 expressed from the full native promoter construct (2061 nucleotides 5' to the ATG of the short protein), which was set to 100%. Up to 30 leaf discs infected with the same culture were pooled for analysis. Each pool of infiltrated leaf samples represented (about 260 mg fresh weight) tissue equally derived from 15 plants of uniform developmental stage.

Qualitative assessment of promoter strength was determined 4-5 days post-infection by visually inspecting treated samples under a stereo fluorescent microscope (Leica Microsystems - Wetzlar, Germany; M165 FC with DsRed2 Filter set; no. 10447412), and acquiring images (Leica Microsystems - Wetzlar, Germany; DFC300 FX R2) of representative examples at fixed exposure time of 7 seconds.

HPPD expression and purification

A synthetic maize wild-type HPPD gene (referred to herein as the maize wild-type sequence) was assembled from commercially synthesized oligonucleotides to deliver the amino acid sequence of SEQ ID NO:1. During the synthesis of the gene, an *NcoI* restriction site was engineered into the start of the sequence to facilitate cloning. The change of codons (C)ATG CCC to (C)ATG GGT resulted in the substitution of Glycine for Proline at position 2 in SEQ ID NO:1 compared to the maize wild-type protein of WO1997049816 SEQ ID NO:11.

Wild type and shuffled variant genes were cloned into pVER7062, a modified version of pET24a(+)(Novagen), which places six histidine residues at the N-terminus of the expressed protein. Vectors were electroporated into *E. coli* host strain BL21(DE3). Cells were grown at 30 C in rich medium such as 2xYT containing the selection antibiotic, kanamycin. At a density of about 0.6 OD600, IPTG was added to 0.2 mM, the temperature was reduced to 16 C and growth continued for another 24 hrs. Cells were harvested by centrifugation and stored at -80 C. Cell pellets were lysed in BPER (Pierce) protein extraction reagent containing 0.2 mg/ml lysozyme, 1 mM dithiothreitol, protease inhibitor cocktail (Sigma, bacterial cocktail) and endonuclease. Insoluble cellular debris was removed by centrifugation. HPPD protein was purified from the soluble protein solution by affinity chromatography on the nickel form of nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Protein concentrations were determined by the Bradford method, as supplied by Bio-Rad.

Medium browning assay

The homogenisate produced by the HPPD reaction is not further metabolised by *E. coli*, but is converted to a brown ochronotic pigment (Zannoni VG et al., Biochimica Et Biophysica Acta 177: 94-105, 1969) Therefore, *E. coli* cells expressing active shuffled maize HPPD enzyme turn the medium brown, which constituted a convenient first tier screen. The screen was made more informative by including an HPPD inhibitor in the medium, requiring that the HPPD variant be desensitized to inhibition as well as active. The intensity of browning was quantified by measuring

absorbance at 360 nm. Those wells with absorbance greater than the appropriate standard were advanced to more refined screens involving *in vitro* enzyme assay.

HPPD activity assay

HPPD catalyzes the conversion of 4-hydroxyphenylpyruvate (HPP) to homogentisate (FIG. 17). Substrate and product do not differ in absorbance of light at any useful wavelength. However, the product of the ensuing reaction in tyrosine metabolism, maleylacetoacetate, absorbs strongly at 320 nm. Furthermore, the enzyme catalyzing that reaction is also a dioxygenase having similar mechanism and buffer requirements as HPPD, making homogentisate dioxygenase (HGD) an ideal partner in a coupled assay, as shown.

The HDG gene from *Pseudomonas aeruginosa* (Amaya (2004) *Arch Biochem Biophys* 421, 135-142) was cloned into pVER7062 and electroporated into *E. coli* strain BL21(DE3). The enzyme was produced by methods similar to those described for HPPD. The purified enzyme has a K_M for homogentisate of 23 μ M and a k_{cat} of 100/sec, properties highly suited for instantly converting the homogentisate produced by HPPD to maleylacetoacetate.

HPPD activity was measured by placing an aliquot (e.g., 6 μ l) of the substrate HPP at 50-fold the desired final concentration into the wells of a low UV-absorbing assay plate. Reactions were started by adding a mixture (e.g., 294 μ l) containing 25 mM Hepes, pH 7, 2 mM ascorbate, 10 μ M FeSO₄, 1 to 100 μ M HPP, 50 nM HGD and 5 to 240 nM HPPD. Absorbance at 320 nm was monitored continuously in a plate-reading spectrophotometer (Spectramax, Molecular Devices).

HPPD kinetic parameters

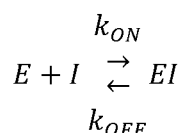
The catalytic performance of wild-type HPPD and variants generated by shuffling was assessed by determining the substrate saturation kinetic parameters. The Michaelis-Menten kinetics protocol of the Spectramax software was customized for HPP concentrations ranging from 3.33 μ M to 100 μ M. Reaction rates were measured as described above for each concentration of HPP. The software returns values of K_M and V_{max} using the Lineweaver-Burke transformation of the Michaelis-Menten equation.

HPPD inhibitor sensitivity parameters

The major HPPD inhibitors used in agriculture today are mesotrione, isoxaflutole, tembotrione, and topramezone. As seen in FIG. 1, the structures share an aromatic ring and a pair of keto oxygens in common with the substrate 4-hydroxyphenylpyruvate, for which they are competitive inhibitors (Secor, 1994). The elements in which the inhibitors differ from the substrates (e.g., cyclohexane ring, substituents on the aromatic ring) present the opportunity to use gene shuffling to enable the enzyme to discriminate between the desired and undesired ligands. All herbicidal inhibitors of HPPD form a tight complex with the enzyme by the dual mechanisms of

coordination to the active site iron atom through a pair of keto oxygens and a pi stack of the aromatic ring of the inhibitor between a pair of active site phenylalanines (Neidig et al., 2005). As a result, conventional I_{50} determinations are not able to distinguish differences in binding affinity among various forms of HPPD and the inhibitor. All values will similarly approximate 50% of the enzyme concentration.

To devise a parameter for detecting changes in inhibitor binding affinity, K_D , one can utilize the relationship between K_D and the rates of binding and release of inhibitor to and from the enzyme.



At equilibrium, rates of binding and release are equal. Thus,

$$k_{ON} [E] [I] = k_{OFF} [EI]$$

Written as a dissociation (products over reactants), the equation can be re-arranged to:

$$\frac{[E][I]}{[EI]} = \frac{k_{OFF}}{k_{ON}} = K_D$$

Higher K_D (reduced affinity or increased insensitivity) can be attained with a numerically smaller ON rate, a larger OFF rate or both. To detect and quantify changes in k_{ON} and k_{OFF} , observations of the time course of an HPPD reaction as inhibitor binds to and inactivates the enzyme (k_{ON}), or is released from a pre-formed enzyme-inhibitor complex (k_{OFF}) were used, as shown in FIGS. 13A and 13B. In practice, a quantitative indicator of k_{ON} was obtained by monitoring the time courses of HPPD reactions containing 60 or 120 nM HPPD and 100 μ M HPP in the presence and absence of 4 μ M inhibitor (e.g., mesotrione or tembotrione). The ratio of the reaction rate with inhibitor to that without inhibitor during the 70 to 90 second interval of the reaction was termed the “ON rate ratio”. The smaller the actual k_{ON} , the more slowly the HPPD reaction decelerates and the higher the value is for the ON rate ratio. FIGS. 13A and 13B illustrate the contrasting reaction velocity in the 70-90 sec interval for maize wild-type HPPD (FIG. 13A) *versus* an improved variant (SEQ ID NO:78) (FIG. 13B).

A quantitative indicator of k_{OFF} can be obtained by observing the time course of an HPPD reaction as inhibitor is released from a pre-formed enzyme-inhibitor complex. HPPD and inhibitor were incubated together at concentrations of 7.2 and 8 μ M, respectively. Incubations with the same concentrations of enzyme but no inhibitor were done in parallel. After 1 hr at room temperature, 5 to

10 μl of the enzyme-inhibitor complex was dispensed into the wells of the assay plate. Reactions were started with the addition of 290 to 295 μl of 25 mM Hepes, pH 7, 2 mM ascorbate, 10 μM FeSO_4 , 100 μM HPP and 50 nM HGD. The reactions were monitored at 320 nm for 12 min. Reaction velocity accelerates as inhibitor is released from the enzyme until a steady state is reached,
 5 during which the reaction velocity is constant. The ratio of the steady state rate in mixtures containing herbicidal inhibitor to the initial velocity of mixtures lacking inhibitor is termed the “OFF rate ratio”.

Another parameter to record is the time span required for the reactions with inhibitor to reach the steady state. FIGS. 13C and 13D illustrate the contrasting time span in which maize wild-type
 10 HPPD (FIG. 13C) and an improved variant (SEQ ID NO:78) (FIG. 13D) dissociated from mesotrione, as indicated by the accelerating reaction rates. The plateau of absorbance is due to exhaustion of the substrate. With the improved variant, not only was the steady state attained in a shorter time span (80 sec for the variant versus 280 sec for wild type), the velocity attained was twice as high as with wild type, though the enzymes were present at the same concentration (240 nM). This
 15 is because a larger fraction of the variant enzyme is free of inhibitor compared to the wild-type enzyme, and the k_{cat} for both is nearly the same, about 220 min^{-1} .

It is not certain that the steady state attained in the OFF rate reactions actually represents an equilibrium where OFF rates are now equal to ON rates. Because substrate and inhibitors bind in the same site, the high concentration of substrate present may competitively trap the inhibitor and
 20 prevent it from binding again. To be sure that improvement in ON rates is being taken into account, the ON and OFF rate ratios were multiplied, creating an “insensitivity parameter” that is a surrogate for K_D . K_D is expressed as $k_{\text{OFF}}/k_{\text{ON}}$, but because the ON rate ratio is an inverse parameter (the higher the value of ON rate ratio, the lower the value of the represented phenomenon, k_{ON}), it is appropriate to multiply ON and OFF rate ratios rather than divide OFF rate ratio by ON rate ratio. Another
 25 parameter, which quantifies the combined catalytic and insensitivity properties of the enzyme is $k_{\text{cat}}/K_M \times \text{ON rate ratio} \times \text{OFF rate ratio}$, and is termed “trait fitness”. This parameter is intended to have a meaning similar to the commonly used expression $k_{\text{cat}}/K_M \times K_i$.

The diketonitrile form of isoxaflutole was synthesized by DuPont chemists. All other inhibitors were obtained from Sigma-Aldrich. The compounds shown in FIG. 16 can be synthesized
 30 by previously described methods known to one skilled in the art.

HPPD Protein Detection in planta

HPPD protein concentration in young soybean leaves was measured using LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) according Hu and Owens (2011) with some modifications. 600 μL of PBST buffer were added to extract 10 leaf punches after being

lyophilized and ground with Spex Certiprep 2000 GenoGrinder. The total extracted proteins (TEP) were measured with Bradford assay and all the samples were normalized to 0.8 µg/µL TEP. 50 µL normalized transgenic extracts were used for analysis and different amount of protein standards were spiked into 50 µL of negative tissue extracts to prepare standard curves. All samples were then heated for 15 min at 95 °C oven and 80 µL digestion buffer ABCT (100mM ammonium bicarbonate with 0.05% Tween-20) was added. The samples were reduced with 6 µL of 0.25 M dithiothreitol at 50 °C for 30 min and then were alkylated with 6 µL of 0.3 M iodoacetamide at room temperature in the dark for 30 min. 1 µg of trypsin (in 10 µL) was added to each sample and digestion lasted about 18 hours before 10 µL 10% (v/v) formic acid and 10 µL internal standard (peptide SIEDYEK where K was labeled with stable isotopes ¹³C and ¹⁵N) were added. HPPD protein was quantified by its signature tryptic peptide SIEDYEK using Waters UPLC coupled with AB SCIEX QTRAP 4000 or 5500. Autosampler temperature was kept at 5°C during analysis. 10 µL were injected onto an Aquasil 100 x 2.1mm 3µ C18 column (ThermoFisher) kept at 60 °C. Mobile phases consisted of 0.1% formic acid (MPA) and 0.1% formic acid in acetonitrile (MPB), and LC was performed at a flow rate of 0.6 mL/min with linear gradient of 5-16% MPB in 2.5 min. The MRM transitions of 442.3/683.3 and 446.3/691.3 were monitored for peptide SIEDYEK and its internal standard peptide, respectively. Chromatogram peak area ratios to the corresponding IS were plotted against protein concentrations. The limit of quantifiable detection of HPPD by mass spec analysis is about 0.003% of total extractable protein (30 ppm, Hu and Owens, 2011)

Stable Soybean Transformation

Soybean plants expressing HPPD variant transgenes were produced using the method of particle gun bombardment (Klein *et al.*, 1987) using a DuPont Biolistic PDS1000/He instrument. A selectable marker used to facilitate soybean transformation was a chimeric gene composed of the S-adenosylmethionine synthase (SAMS) promoter (Falco and Li, 2010) from soybean, a highly resistant allele of soybean acetolactate synthase (Bedbrook *et al.*, 1997), and the native soybean acetolactate synthase terminator region. The selection agent used during the transformation process was chlorsulfuron. HPPD genes were expressed with a synthetic constitutive promoter (Bowen *et al.*, 2000) or the *G. max* HPPD derived promoters, an insensitive HPPD variant, and an Arabidopsis ubiquitin3 gene terminator (Callis *et al.*, 1995). Bombardments were carried out with linear DNA fragments purified away from any bacterial vector DNA. The selectable marker gene cassette was in the same DNA fragment as the HPPD cassette. In some cases the characterized CTP sequences 6H1 (Wilkinson and Lassner, 2008) and the Arabidopsis rubisco large subunit CTP (Lee *et al.*, 2006) were fused to a truncated maize HPPD variant. Bombarded soybean embryogenic suspension tissue was cultured for one week in the absence of selection agent, then placed in liquid selection medium

for 6 weeks. Putative transgenic suspension tissue was sampled for PCR analysis to determine the presence of the HPPD gene. Putative transgenic suspension culture tissue was maintained in selection medium for 3 weeks to obtain enough tissue for plant regeneration. Suspension tissue was matured for 4 weeks using standard procedures; matured somatic embryos were desiccated for 4-7 days and then placed on germination induction medium for 2-4 weeks. Germinated plantlets were transferred to soil in cell pack trays for 3 weeks for acclimatization. Plantlets were potted to 10-inch pots in the greenhouse for evaluation of herbicide resistance.

Herbicide Tolerance Testing

T0 plants with HPPD transgenes were grown to the V2 to V8 growth stage and then sprayed with commercial mesotrione formulation at rate 210 g ai/ha (two times the labeled rate for corn in the field). All mesotrione treatments were applied with 0.25% nonionic surfactant and 1% ammonium sulfate in a spray volume of 374 L/ha. Individual plants were compared to untreated plants of similar genetic background, evaluated for herbicide response at eight days after treatment and assigned a visual response rating from 0 (no effect) to 100 (dead plant). Sibling plants were similarly evaluated with tembotrione at 93 g ai/ha. Protein expression level was determined by mass spectrometry (Hu and Owens, 2011). In the T0 generation, plants that had improved tolerance compared to controls based upon low injury scores ($\leq 30\%$) were advanced to the T1 generation for more extensive herbicide testing. When sibling clones of the events were available, the same process was carried out on T0 plants using tembotrione.

T1 plants were evaluated for zygosity. Homozygous single-locus transgenic plants and their corresponding null segregants were identified and T2 true breeding seed was obtained from each. Plants were grown to the V1 to V2 growth stage and then sprayed with the commercial formulation of mesotrione at 420 g ai/ha. All treatments were applied with nonionic surfactant and ammonium sulfate in a spray volume of 374 L/ha. Ratings of response to the herbicide were made eight days after treatment, as described for T0 plants.

Homozygous T3 generation seeds were planted in short rows in Johnston, IA at a DuPont Pioneer regulated field location. Herbicides were applied at the V4 stage with a backpack sprayer in a spray volume of 140 L/ha at two or four times the labeled rates for corn in the field, which are: mesotrione, 118 g ai/ha; tembotrione, 90 g ai/ha; isoxaflutole, 67 g ai/ha. All treatments included 0.25% v/v nonionic surfactant and 8 lb/100 gal ammonium sulfate. As negative controls, rows of non-transformed plants of the same variety were sprayed at the same rates. Positive control rows received no spray. Visual injury was rated at 3, 7, and 14 days after treatment (DAT).

Example 2. Bioinformatic and Functional Localization Analysis of *Z. mays* (Maize) HPPD

A single HPPD gene was identified in *Z. mays* via genomic and EST database searches (NCBI Reference Sequence: NM_001112312.1) giving rise to a 444 amino acid encoded protein (FIG. 3A). ProtComp 6.1 (linux1.softberry.com/berry.phtml) indicated a cytosolic location of maize HPPD (cytoplasmic score = 14470; chloroplastic score = 1.4). Similarly, WoLF PSORT (Horton et al., 2007) and TargetP (Emanuelsson et al., 2000) predict a cytosolic location of the HPPD protein. PCLR (Schein et al., 2001) predicts non-chloroplast localization with either the N-terminal 55 residues or the whole protein (0.25 with 0.42 threshold), Protein Prowler (Hawkins and Boden, 2006) predicts either a mitochondrial (0.34) or chloroplast (0.39) location and Multiloc (Hoglund et al., 2006) predicts an extracellular (0.74) localization with the first 50 amino acids of maize HPPD, but a strong chloroplast localization (0.97) for the full maize HPPD sequence.

Immuno-localization electron microscopy of maize tissue sections was carried out to detect native HPPD localization. Gold labeling was observed mainly in bundle sheath chloroplasts (range; 5-28 particles per chloroplast, 50 chloroplasts observed (FIG. 10). Particles were also found in mesophyll chloroplasts, but in no other structures. Observations with direct detection of HPPD in maize tissues by labeling, protein purification using antibody pull-down, and mass-spectrometric protein detection all indicated that HPPD accumulation in maize leaves is very low.

To functionally corroborate the localization of maize HPPD, a binary plant transformation vector was constructed in which the portion of the maize HPPD gene coding for the N-terminal 50 amino acids was fused to the gene coding for DsRed2 all under control of the maize rubisco activase promoter. A positive control vector was identical except that the DsRed2 insert was fused to the chloroplast targeting peptide (CTP) of maize rubisco activase, while a negative control was DsRed2 with no targeting sequence. The plasmids were transformed into *Agrobacterium tumefaciens* AGL-1 and agrobacterium-infiltration was used to introduce the constructs into plant cells for transient expression. Agrobacterium-infiltration is a well described method (Kapila et al., 1997) of introducing an *Agrobacterium* cell suspension to plant cells of intact tissues so that reproducible infection and subsequent plant derived transgene expression may be measured or studied. Leaves of 3-week old maize seedlings were infiltrated with the *Agrobacterium*, and examined by fluorescence microscopy of hand sections two days later. With the vector where DsRed2 was fused to rubisco activase CTP, red fluorescence was seen in discrete packets in a pattern resembling peri-nuclear chloroplasts, as expected (FIG. 3B). A similar pattern was seen when DsRed2 was fused to the N-terminal 50 amino acids of maize HPPD (FIG. 3C). Without targeting, fluorescence was diffuse with some concentration in the nucleus (FIG. 3D). In another experiment confirming these results, maize leaf tissue was co-bombarded with DNA from both the DsRed2-containing test plasmids and a

plasmid encoding untargeted cycle 3 green fluorescence protein (C3GFP). Transformation of guard cells with vectors encoding either rubisco activase CTP-DsRed2 or the N-terminal 50 amino acids of maize HPPD fused to DsRed2 clearly resulted in plastid targeting of the DsRed2 reporter, whereas untargeted C3CFP showed no overlap with the DsRed2 signal (FIG. 11).

To determine the length of the functional CTP for maize HPPD, vectors were constructed in which the portion of the maize HPPD gene coding for the N-terminal 0, 10, 20, 30, 40 or 50 amino acids was fused to the gene coding for DsRed2 and evaluated with transient expression following agro-infiltration of maize leaves. Microscopy revealed that 50 amino acids of the maize HPPD N-terminus effectively targeted DsRed2 to plastids (FIG. 3G), but 40 amino acids or fewer failed to do so, with DsRed2 fluorescence visible only in the cytoplasm (FIGS. 3E and 3F). This result indicates that more than 40 amino acids of the N-terminus are required for chloroplast localization and that 50 amino acids are sufficient for targeting.

To investigate if the maize HPPD protein is unique, several monocot sequences were compared. FIG. 3J provides an N-terminal alignment of monocot HPPD proteins having identities of 59-85%. A vector constructed in which the *Oryza sativa* HPPD N-terminal sequence was fused to the gene coding for DsRed2 was assayed in maize leaves as described. Microscopy revealed that the 53 amino acid rice N-terminal sequence effectively targeted DsRed2 to maize plastids. In view of the conservation among the sequence of the monocot HPPD proteins, a consensus monocot HPPD chloroplast targeting peptide sequences was determined. The consensus sequence, MPPTPTTAAATGAGAAA VTP EHA AFR LVGHRRFVR FNPRSDRFHTLAFHHVE (SEQ ID NO:94), targeted the DsRed2 protein to the maize chloroplasts. To further confirm the monocot functional similarity, the vectors targeting DsRed2 with 0, 10, 20, 30, 40, and 50 amino acids of the maize HPPD N-terminus were tested by agro-infiltration in sorghum (*Sorghum bicolor*) leaves. The results matched those obtained in maize. The data indicate that the monocot HPPD proteins share a homologous chloroplast targeting motif and function.

The function of the monocot HPPD sequence in dicots was explored by transient expression in bush bean (*Phaseolus vulgaris*), tobacco (*Nicotiana benthamiana*), and soybean cells. The sequence encoding amino acids 1-50 of the maize HPPD protein was fused to a gene encoding AcGFP1 and inserted into a binary expression vector under control of the *Arabidopsis* Ubiquitin 10 promoter. A positive control vector was identical except that the AcGFP1 coding region was fused to the 6H1 synthetic chloroplast targeting peptide (Lassner and Wilkinson, 2008), while a negative control was AcGFP1 with no targeting sequence. The first 50 amino acids of maize HPPD were sufficient to drive chloroplast import of AcGFP1 in epidermal cells of *P. vulgaris*, although some green fluorescence remained in the cytoplasm. In *N. benthamiana* the AcGFP1 remained in the

cytoplasm with none apparent in the chloroplasts. Results by bombardment in soybean showed AcGFP1 in both plastids and cytoplasm (FIG. 3H). This shows that the maize HPPD CTP is recognized in dicot plant cells, but may be inefficiently processed. Stably transformed soybean plants expressing the 50 amino acid N-terminal CTP fused to AcGFP1 showed strong fluorescence in the chloroplasts (FIG. 3I), indicating that the inefficient translocation can be due to the high template concentration that occurs in transient expression conditions.

Example 3. Bioinformatic and Functional Localization Analysis of *G. max* (Soybean) HPPD

G. max is a polyploid derived from 2 evolutionary genome duplications (Schmutz et al, 2010) and as such appears to have multiple paralogous HPPD genes. As some of the annotated genes appear incomplete or have rare ESTs, glyma14g03410.1 on chromosome 14 was studied. This soybean HPPD protein has been previously annotated as a 449 amino acid protein with N-terminal sequence MPIPMCNEIQ (Cahoon and Coughlin, 2007; sequence 36) and as a 443 amino acid protein with N-terminal sequence MCNEIQAQAAQ (Genbank ABQ96868). Analysis of genomic and EST data revealed that an in-frame N-terminal extension of the previously annotated coding region exists, adding 41 amino acids to produce a 490 amino acid full-length HPPD protein as shown in FIG. 4A. Furthermore, EST data reveal two major mRNAs, one of which is able to support translation of the 490 amino acid protein and a smaller EST that is able to support translation of the 449 amino acid protein.

5' cap-dependent rapid cloning of cDNA ends (5' RACE) revealed two major transcripts for the native soybean HPPD gene. Cloning and sequencing of these PCR products revealed a shorter transcript that was designated as starting at position +1. The longer transcript began at position -237 relative to the shorter transcription start site (FIG. 4A). Sequence of multiple clones for each 5' RACE product indicated an approximately 4 nucleotide variation between individual transcripts for both sites. Linked *in vitro* transcription and translation indicated that both mRNAs are translated and make the predicted size protein (truncated for convenience, FIG. 4B). The +1 transcript is translated to produce a single protein, designated the "short" protein. The -237 transcript yields two proteins, with the lower molecular weight band at the same MW as the single protein produced from the +1 transcript, indicating that translation can initiate at both positions on the long transcript. The higher MW protein produced from the -237 transcript is designated the "long" protein.

Bioinformatic evaluation of the shorter soybean HPPD sequence did not predict a chloroplast or other targeting sequence. However, a chloroplast targeting function was predicted for the N-terminally extended form by ProtComp 9.0 (linux1.softberry.com), WoLF PSORT (Horton et al., 2007) and PCLR (Schein et al., 2001). TargetP (Emanuelsson et al., 2000) indicates chloroplast localization, but gave a higher score to 'other' while MultiLoc2 (Blum et al., 2009) predicted a

cytoplasmic localization for both the first 42 and the first 86 amino acids of the long protein. Functional analysis was required to clarify the predictions.

Transient expression experiments indicated that the long HPPD protein is imported to chloroplasts, while the short protein remains in the cytosol. Plant expression cassettes were constructed fusing portions of the N-terminus of the soybean HPPD to either DsRed2 or AcGFP1. The first fusion contained amino acid residues 1-42 of the long protein while the second fusion contained residues 1-44 of the short HPPD protein (corresponding to residues 42-86 of the long protein). The third fusion contained the full 1-86 amino acids of the long HPPD protein. These cassettes, under control of the Arabidopsis Ubiquitin 10 promoter, were used to infect soybean seedling leaves. As shown in FIG. 4C-E, fluorescence is clearly visible in the chloroplasts of infected cells only when the marker gene is fused to amino acid residues 1-86. When the fusion is made with the shorter sequences of both proteins, fluorescence is visible only in the cytoplasm. The pattern of dual localization seen with the reporter constructs was confirmed by immuno-localization electron microscopy (FIGs. 12A and 12B). Thus, the N-terminal 42 amino acids of the long protein is predicted to be a CTP, but to be functional, a longer sequence is required.

Although the N-terminal sequences of maize and soybean HPPD differ considerably (FIG. 4F), they are both able to target proteins in the heterologous species. Leaves of 4-week old maize seedlings were infiltrated with the *Agrobacterium* carrying the soybean constructs and examined by fluorescence microscopy. Transient expression indicated that the long HPPD protein N-terminus did target the marker protein to maize cell chloroplasts, while the protein variant with the shorter N-terminus delivered the protein to the cytosol. Thus, the dicot chloroplast targeting region of soybean HPPD is able to function in monocot cells.

Because the reported HPPD sequences were mis-annotated for soybean, available sequence data for Arabidopsis and another legume, *Medicago trunculata* (alfalfa), were examined to determine if other related dicot species showed the same N-terminal extension. The Arabidopsis chromosome 1 HPPD gene NC_003070.9 (Genbank) encodes a 418 amino acid open reading frame with no upstream open reading frames extending the HPPD protein. The lack of N-terminal extension is punctuated by two in-frame stop codons 15 nucleotides upstream of the ATG start. On the other hand, a search of the *M. trunculata* genome returned a predicted HPPD gene located on chromosome 5 (NC_016411.1; Genbank). The annotated predicted protein of 437 amino acids is preceded by a long open reading frame that could initiate at methionines adding 74 or 115 amino acids. The 74 N-terminal extension showed a very strong chloroplast localization score with PCLR (Schein et al., 2001) while the 115 amino acid extension did not.

Example 4. Directed Evolution of the Maize HPPD Protein for Increased Insensitivity to Herbicide

Inhibitors

Directed evolution is an iterative process whereby beneficial diversity is discovered and iteratively recombined to evolve variants that achieve a desired protein engineering goal. A goal was to create an HPPD enzyme that retained kinetic activity yet was insensitive to most or all HPPD-inhibiting herbicides. Characterization of the maize and soybean HPPD proteins showed that the former was 40-fold less sensitive to mesotrione than the latter (see Table 2 below, which shows kinetic parameters of *E. coli* purified proteins of wild-type HPPD enzymes from maize and soybean). Inhibition measurements are for the herbicidal compound mesotrione. The maize enzyme was, therefore, chosen for use in soybean plants. Final evaluation of shuffled HPPD enzymes was carried out with multiple HPPD herbicides to select the most robust variant.

Table 2.

HPPD Variant	SEQ ID NO.	Kinetic parameters			Insensitivity parameters				Fitness	
		k_{cat} , min ⁻¹	K_M , μ M	k_{cat}/K_M	ON rate ratio	OFF rate ratio	ON x OFF	Fold vs wt	ONxOFF x k_{cat}/K_M	Fold vs Zm wt
<i>Z. mays</i> wt	1	219	6.4	34.2	0.20	0.31	0.06		2.10	1
<i>G. max</i> wt	---	100	3.0	33.3	0.20	0.007	0.0014	0.023	0.047	0.02
R1 Exemplar	---	86	4.0	21.6	0.44	0.12	0.05	0.9	1.2	0.6
R2 Exemplar	---	50	2.7	18.9	0.70	0.18	0.13	2.1	2.4	1.1
R3 Exemplar	---	95.6	2.6	36.8	0.58	0.13	0.08	1.3	2.9	1.4
R4 Exemplar	---	117	4.3	27.5	0.61	0.16	0.10	1.6	2.7	1.3
R5-9043	---	146	5.9	24.7	0.60	0.63	0.38	6.2	9.3	4.4
R6-9075	79	114	4.0	28.9	0.68	0.68	0.46	7.6	13.4	6.4
R7-9070	2	88.9	2.9	30.7	0.67	0.83	0.56	9.11	16.6	7.9
R8-1973	8	70.8	2.0	35.2	0.73	1.00	0.73	11.9	25.5	12.2

In the first 6 rounds, shuffled gene variant libraries were made based on the maize HPPD protein template using techniques including family shuffling, single-gene shuffling, back-crossing, semi-synthetic and synthetic shuffling (Zhang J-H et al., 1997; Cramer et al., 1998; Ness et al., 2002). The amino acid diversity for those libraries originated from phylogenetic sequence diversity, random mutagenesis, site-directed mutagenesis, and structural features based on crystal structures of proteins in Protein Data Bank (PDB; pdb.org/pdb/home/home.do). Typically, four to five thousand variants per library were screened for kinetic and insensitivity parameters. Genes coding for shuffled variants of HPPD were expressed in *E. coli*. The library was plated out on rich agar medium, and individual colonies were picked and grown in rich medium. Colonies with active HPPD enzyme cause the medium to turn brown due to the conversion of homogentisate to a brown ochronotic pigment (Zannoni VG et al., 1969). Proteins with the ability to turn the medium brown in the

presence of 100 μ M mesotrione in rounds 1 to 6 or tembotrione in rounds 7 and 8 were subjected to detailed analysis. Kinetic characteristics of k_{ON} , k_{OFF} , k_{cat} and K_m were determined (Table 2).

The diversity of substitutions identified through phylogenetic sequence diversity, random mutagenesis, site-directed mutagenesis, and structural features based on crystal structures of proteins in Protein Data Bank (PDB; pdb.org/pdb/home/home.do) in the improved HPPD enzymes of this study is listed in Table 3 and 3b. Diversity not accessible through these methods but identified by saturation mutagenesis are shown in Example 7. Table 3 shows exemplary diversity contributing to improvement of insensitive maize HPPD. Indicated amino acids are neutral or beneficial to HPPD fitness. Table 3b shows diversity identified by saturation mutagenesis of Round 7, variant 9070 (SEQ ID NO:2) and fitness (ON rate ratio x OFF rate ratio x k_{cat}/K_m) relative to Round 7, variant 9070 (SEQ ID NO:2) for the inhibitors indicated therein. Native maize wild-type sequence is shown for comparison. In many cases a single substitution was identified, while at other positions, many neutral or beneficial substitutions were identified. The additional increment of enzyme fitness seen in round 8 (Table 2; FIGS. 5 and 6) was attained through mutations not present in the natural diversity, but accessed only through saturation mutagenesis. The variant with the highest fitness parameter, Round 8, variant 1973 (SEQ ID NO:8), had 26 amino acid substitutions relative to native maize HPPD.

Table 3.

Position	32	40	42	44	46	47	68	71	98	114
Maize WT	N	S	R	H	L	A	G	A	F	A
Diversity	R	A	H	VG QSC LI	V	S	A	V	L	S

Position	120	122	125	137	144	146	150	161	167	175
Maize WT	A	A	R	V	A	D	A	G	R	E
Diversity	P	T	S	I	V	V	S	S	G	G

Position	184	187	193	202	207	209	211	219	221	225
Maize WT	Y	Y	G	G	G	A	Y	I	G	E
Diversity	F	H	D	R	D	V	C	VL M	VAS C	D

Position	226	233	241	253	260	261	262	268	278	282
Maize WT	L	F	E	A	M	V	L	N	H	R
Diversity	M	VCL MI	G	T	VLI	A	WI	G	R	K

Position	289	291	301	303	316	320	327	328	330	331
Maize WT	F	D	M	L	Q	A	M	A	P	T
Diversity	Y	E	I	V	RK	S	L	P	R	GQPL H

Position	341	347	352	360	377	382	383	387	395	405
Maize WT	R	T	K	L	V	T	L	I	E	K
Diversity	DEA CI	S	DEN	M	LI	A	F	M	G	E

Position	414	417	418	425	437	438	440	442	443	444
Maize WT	G	S	Q	D	A	A	A	Q	G	S
Diversity	DTFV QASR MHY K	G	E	E	P	E	TVK	A	Q	G

Table 3b.

Mutation*	ON x OFF x kcat/km fold vs Round 7, variant 9070				
	tembo	meso	DKN	Topra	Sulco
Q366E	1.8	1.8	1.7	1.9	1.4
G417S	1.6	1.3	0.9	1.2	1.1
A432R	1.6	1.5	2.1	1.4	1.3
G417Q	1.5	1.5	1.0	1.4	1.2
L385V	1.4	1.0	1.1	1.3	1.0
P206Q	1.4	1.1	1.2	1.1	1.0
A432K	1.4	1.6	1.6	1.5	1.5
S214N	1.3	1.1	1.4	1.2	1.1
L385I	1.3	1.1	1.2	1.2	1.1
P158K	1.3	1.0	1.0	1.1	0.9
E356R	1.2	1.1	1.1	1.1	1.1
E356K	1.2	1.2	1.1	0.9	1.1
R340K	1.2	1.3	2.2	1.1	1.3
I387L	1.1	1.3	1.6	1.0	1.3
Y211L	1.1	0.9	1.1	1.1	0.9
Q434R	1.1	1.1	1.1	1.1	1.1
P206S	1.1	0.9	1.1	1.0	0.8
Q434H	1.1	1.3	1.4	1.1	1.3
A191G	1.0	1.1	1.1	1.1	1.1
L182M	1.0	0.8	0.9	0.9	0.9

* Mutation shown relative to Round 7, variant 9070 (SEQ ID NO:2)

FIG. 5 illustrates the progression of desensitization to mesotrione from wild type to the best variant that emerged from the round 8 library. Improved (slower) rates of association of enzyme and mesotrione, manifested in the assay as a higher value for ON rate ratio, were readily attained. Values

ranged from that of the wild type, 0.2, to the theoretical maximum value of 1.0, meaning that the reaction with inhibitor did not measurably slow compared to the reaction with no inhibitor during the first 90 seconds. Improved (faster) rates of dissociation were less common, being attained in the early rounds either not at all or at the expense of reduced catalytic efficiency. In the examples shown in FIG. 5, catalytic efficiency was retained, but in the first 4 rounds of shuffling, OFF rate ratio actually decreased. However, variants with OFF rate ratios up to 0.7 were observed in rounds 5 to 8. Variants sufficiently improved in ON rate, OFF rate or both so as to result in improved insensitivity parameter (ON rate ratio X OFF rate ratio) included many with values in the range of 0.27 to 0.7, representing more than a 14-fold improved insensitivity compared to maize wild-type HPPD (FIG. 5 and Table 2). No attempt was made to improve k_{cat} , but rather to find a sequence context in which improved insensitivity could be attained with little or no loss of enzyme turnover rate (k_{cat}) or catalytic efficiency (k_{cat}/K_M). The number of mutations required was such that enzyme fitness (ON x OFF x k_{cat}/K_M) improved only 2-fold until round 5, but improved another 7- to 15-fold, depending on the inhibitor, in rounds 6 through 8, for overall improvements of 20- and 35-fold for mesotrione and tembotrione, respectively (Table 2, FIG. 6).

Because there are diverse chemotypes among the registered herbicidal inhibitors of HPPD, a commercially valuable trait should confer tolerance to most or all of these registered chemistries. To ensure that directed evolution met that criterion, parents for each successive library were tested for insensitivity to the other inhibitors shown in FIG. 1, using the same procedures as for mesotrione. The result was that improved insensitivity, and hence enzyme fitness, obtained by selection with mesotrione or tembotrione was accompanied by improved insensitivity to all other inhibitors (FIG. 6).

Example 5. HPPD Expression Analysis in Soybean Plants

To mimic the pattern of expression with regard to growth stage, tissue and subcellular location, and uncharacterized signaling cues, the transgene promoters were based on the native soybean HPPD promoter. Attempts to directly isolate or measure native HPPD protein accumulation indicated low abundance in leaves. This was confirmed by EST analysis. Profiles of transcript abundance in multiple tissues from soybean (SoyBase.org) were compared to other well-known herbicide target genes, EPSPS (glyphosate target) and ALS (ALS-inhibitor target) (FIG. 7). HPPD and EPSPS appear to be expressed at a low level in all tissues other than flowers compared to the well-characterized ACT2/7 gene (Hu et al., 2009). Preserving the native expression pattern for HPPD while substituting the improved insensitive maize HPPD variant genes can confer robust herbicide tolerance with only moderate overexpression of the transgene.

Example 6. Isolation and Characterization of the Soybean HPPD Promoter

A 1225 bp genomic sequence at the 5' end of the *G. max* HPPD gene was isolated, sequenced and subjected to promoter analysis using Promoter REAPER and Promoter Delineator (Simmons and Navarro Acevaedo, 2010). Five putative TATA boxes, GTATAAATAA (SEQ ID NO:95; TATA1), CCAATATATG (SEQ ID NO:96; TATA2), CCTTATATATC (SEQ ID NO:97; TATA3), TATATAATAA (SEQ ID NO:98; TATA4), and GAATATAAG (SEQ ID NO:99; TATA5) were identified as shown in FIG. 8A. The open reading frame starting from the first ATG after TATA3 encodes the 449 amino acid short protein. TATA2 is within the long protein transcript and TATA3 is embedded in the coding region for the long protein. Based on the known transcripts, TATA5 and TATA3 appear to be best situated for the long and short transcripts, respectively. Each is situated approximately 35 nucleotides from the transcription initiation as expected for eukaryotic promoters (Smale and Kadonaga, 2003). Open reading frame (ORF) analysis of the long transcript indicates a short ORF encoding 7 AAs starting at position -195. Downstream of this, the long ORF begins at position -99 and continues in frame comprising the short protein, which starts at +25. A schematic of the *G. max* HPPD promoter, transcripts, and translation products is shown in FIG. 8B.

To evaluate the predicted TATA boxes for activity, 3' deletions and various mutations of the promoter region were constructed and the fragments fused with DsRed2 for expression analysis using transient expression in bushbean leaf tissue (Table 4). Table 4 shows representative HPPD promoter activity data. DsRed2 measurements are relative to full *G. max* HPPD promoter in extracts of bush bean leaves 3 days after agro-infiltration with described promoter:DsRed2 fusion constructs. The full promoter:DsRed2 construct with both transcripts intact should produce both a 41 residue N-terminal HPPD:DsRed2 fusion protein and the DsRed2 protein alone. The fluorescence value of the fusion protein was not measured in comparison to the DsRed2 protein. It was previously shown that the 41 amino acids fused to DsRed2 did produce red fluorescence without conferring chloroplast localization (FIG. 4C). Deletion of a 613 bp DNA fragment upstream of the short protein transcription start site, including all 5 putative TATA boxes, resulted in a DNA fragment that was not able to drive DsRed2 expression to a level above the background. When TATA3 and the short transcript region were deleted with the DsRed2 initiating at the same position as the long protein, 90% of the full promoter region accumulation of DsRed2 was measured, showing the contribution of the upstream transcript. To eliminate translation of the fusion protein and leave only the contribution of the short protein, a stop codon was introduced upstream of the DsRed2 start. Only the short transcript DsRed2 would be translated in this construct. The same amount of DsRed2 accumulated as when both the fusion and the downstream DsRed2 could be translated. Thus, it appears that both transcripts are capable of supporting translation activity but this activity is not additive. Total HPPD

product from the native promoter could be regulated via competition for transcription and/or translation. Deletions of TATA2 and 3, TATA2, 3, and 5, or TATA2, 3, 4, and 5 abolished promoter activity. It was unexpected that deletion of TATA2 and 3 did not default to TATA5 and the upstream (-237) transcription initiation for DsRed2 expression (see FIG. 8 and Table 4).

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Table 4.

Promoter Description	Promoter Variant	Average Relative Promoter Strength (%)
Native <i>G. max</i> HPPD promoter region	Gm HPPD	100
Deletions series		
Deletion of TATA2, TATA3, and TATA5	SHP000C	2
Deletion of all 5 TATA	SHP101C	0
Deletion of TATA 2, 3, 4, and 5	SHP102C	0
Deletion of TATA3	SHP103C	90
Deletion of TATA2 and TATA3	SHP110C	3
Mutations in TATA3		
A to C mutation in TATA3	SHP104C	23
T to C in TATA1 and A to C in TATA3	SHP105C	30
T to C in TATA1, T to G in TATA2, and A to C in TATA3	SHP106C	21
TAT to GCA mutation in TATA3	SHP107C	15
ATA to CCG in TATA1 and TAT to GCA in TATA3	SHP108C	19
TAT to CAG in TATA5, TAT to GCA in TATA3	SHP114C	11
Mutations in other TATA boxes		
TAT to CAG mutation in TATA2	SHP111C	93
TAT to CAG mutation in TATA4	SHP112C	97
TAT to CAG mutation in TATA5	SHP113C	89
Point mutation to eliminate upORF start codon	SHP120C	105
Point mutation to insert stop codon just upstream of +1 transcription start site	SHP121C	92
Synthetic promoters		
Deletion of all 5 TATA + element I ^a	SHP101	3
Deletion of TATA 2, 3, 4, and 5 + element I	SHP102	4
Deletion of TATA3 + element I	SHP103	310
T to C in TATA1, T to G in TATA2, and A to C in TATA3 + element I	SHP106	377
TAT to GCA mutation in TATA3 + element I	SHP107	129
Deletion of all 5 TATA + element II ^b	SHP201	23
Deletion of TATA3 + element II	SHP203	272
T to C in TATA1, T to G in TATA2, and A to C in TATA3 + element II	SHP206	195

^a Element I is a synthetic core promoter sequence derived from Sequence 1 in Bowen et al., 2000

^b Element II consists of Element 1 plus a transcription enhancer region called Rsyn7 derived from Sequence 2 in Bowen et al., 2000.

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A second method to characterize the role of the predicted TATA sequences was mutagenesis of the specific sequences, changing either 1 or 3 nucleotides. In all cases, if TATA3 was mutated, no matter what other mutations were present, promoter activity was reduced by 70-90%. A triplet nucleotide change reduced the activity more than a single nucleotide change. Triplet nucleotide mutations in either TATA2, TATA4, or TATA5 showed little or no effect on accumulation when DsRed2 replaced the short protein open reading frame. Upstream ORFs have been shown to reduce both transcription and translation in plant genes (Saul et al., 2009). A mutation to eliminate the ATG start codon of the HPPD upORF did not have any affect as DsRed2 was within 5% of that measured with the unaltered promoter construct. Both deletion and mutation analysis provide evidence that TATA3 is important in maintaining full activity of the soy HPPD promoter but, because deletion of the TATA3 sequence altogether still leaves 90% promoter activity, other elements are also functional.

Example 7. Derivations of Soybean HPPD Promoter for Transgene Expression

To create transgene expression cassettes for HPPD inhibitor herbicide tolerance in soybean, cassettes were constructed with the intention of maintaining the HPPD regulatory information while modestly increasing the expression level. In one case, the inactive fragment with deletion of TATA3, 2 and 5 was fused with a synthetic core promoter sequence (Element I in Table 4, Bowen et al., 2000; sequence 1) followed by the 45bp 5'UTR sequence including the predicted transcription start site from the soybean native HPPD gene comprising the nucleotides between TATA3 and ATG. In other cases either TATA3 or TATA5 were replaced with the synthetic core promoter to drive the short or long transcripts at an increased level. DsRed2 fusion constructs with these promoters showed that inclusion of the synthetic TATA region resulted in two- to three-fold increased DsRed2 accumulation (Table 4). The 35S enhancer region (Ow et al., 1987) at the 5' end of the altered promoter fragments or an enhancing region called Rsyn7 containing OCS transcription factor motif sequences (Element II in Table 4, Bowen et al., 2000; sequence 2) near the core TATA box were added to increase expression. Plant transformation constructs were designed either with full-length insensitive maize HPPD in combination with promoters giving rise to only one transcript or with the N-terminal 86 amino acids of the long chloroplast targeting sequence of *G. max* followed by the conserved mature maize HPPD protein starting at amino acid 50 (FIG. 4F). The latter was designed to provide dual targeting of a highly insensitive HPPD enzyme. Final functional validation of the promoter/CTP/variant HPPD cassettes was by herbicide tolerance efficacy testing of transgenic soybean plants.

Example 8. Generating and identifying beneficial novel amino acid diversity

Saturation mutagenesis of maize HPPD

The backbone gene for saturation mutagenesis was Round 7, variant 9070 (SEQ ID NO:2), which had the highest fitness parameter with inhibitors such as mesotrione and tembotrione among the variants generated previously (US20120042413(A1)). Libraries were created for each position in the Round 7, variant 9070 (SEQ ID NO:2) using NNK (where N represents a 25% mix each of adenine, thymine, guanine, and cytosine nucleotides; and K represents a 50% mix each of thymine and guanine nucleotides) as the degenerate codon for the position to be mutagenized. PCR reaction mixtures contained a mutagenic forward primer (NNK codon flanked by 28 nucleotides matching with template at each side of the NNK) and a reverse primer that was the complement of the sequence preceding the forward primer, 28 nucleotides in length. To eliminate the mis-matched bubble, a second PCR reaction was performed with a forward, resolution primer that matched the 28 nucleotides of the template upstream of the NNK site, and the same reverse primer as in the first reaction. The template for the second PCR reaction was the product of the first, diluted 10-fold. To make circular double stranded DNA plasmids from the blunt ended PCR products, the products were digested with T4 polynucleotide kinase, T4 DNA ligase, and DpnI (to disrupt the parental DNA template). After desalting by ultrafiltration, the ligation products were ready for transformation and downstream applications.

The transformation mixture for each position mutagenized was plated on LB agar containing kanamycin. 47-94 colonies from each plate were picked into LB liquid, in 96-well format. The cells were sub-cultured into 2xYT medium unmodified, or containing 50 μ M tembotrione or 100 μ M mesotrione and evaluated by their ability to turn the medium brown, as described in Example 1. Wells in which the medium turned as brown as or browner than the Round 7, variant 9070 (SEQ ID NO:2) controls were selected as hits. The amino acid substitutions in Round 7, variant 9070 (SEQ ID NO:2) of the 554 variants selected as hits are listed in Megatable 1. Sequencing of entire plates selected at random indicated that on average, 17 amino acid substitutions were accessed per position.

The variants selected in the medium browning screen were expressed in *E. coli* and purified as described in Example 1, then evaluated for affinity for tembotrione by obtaining the ON and OFF rate ratios described in Example 1. The results for insensitivity evaluation (ON and OFF rate ratio) of 119 single substitutions (relative to Round 7, variant 9070 (SEQ ID NO:2)) selected from medium browning in the presence of 100 μ M tembotrione are shown in Table 5.

Table 5.

Substitution	k_{cat} min ⁻¹	Parameters with tembotrione			ON x OFF, fold vs wt	ON x OFF x k_{cat}	
		ON ratio	OFF ratio	ON x OFF		Value	Fold vs wt
331N	40.4	0.75	0.43	0.32	63.0	12.99	12.14

Substitution	k_{cat} min ⁻¹	Parameters with tembotrione			ON x OFF, fold vs wt	ON x OFF x k_{cat}	
		ON ratio	OFF ratio	ON x OFF		Value	Fold vs wt
438G	33.8	0.74	0.42	0.31	60.3	10.41	9.73
331R	30.3	0.73	0.38	0.28	54.6	8.44	7.89
437K	35.7	0.76	0.36	0.27	52.9	9.63	9.00
438R	29.2	0.75	0.33	0.25	48.5	7.23	6.76
385V	21.4	0.75	0.33	0.25	48.2	5.26	4.92
376W	23.1	0.68	0.34	0.23	46.0	5.42	5.07
417S	18.5	0.82	0.28	0.23	45.2	4.26	3.98
161E	17.2	0.78	0.29	0.23	44.8	3.92	3.67
161Q	27.7	0.77	0.30	0.23	44.8	6.33	5.91
439R	24.7	0.73	0.30	0.22	43.3	5.45	5.09
161S	24.5	0.76	0.29	0.22	43.2	5.41	5.06
182M	26.0	0.79	0.28	0.22	42.5	5.63	5.26
171F	16.6	0.75	0.28	0.21	41.8	3.54	3.31
215G	24.4	0.79	0.27	0.21	41.8	5.19	4.85
191S	12.4	0.75	0.28	0.21	40.8	2.59	2.42
158K	50.4	0.77	0.27	0.21	40.6	10.44	9.76
332A	41.5	0.75	0.28	0.21	40.5	8.58	8.02
161Y	21.6	0.78	0.26	0.20	40.0	4.40	4.11
385I	27.5	0.75	0.27	0.20	39.5	5.55	5.18
331G	29.0	0.73	0.27	0.20	39.1	5.78	5.40
161N	23.5	0.78	0.25	0.20	38.6	4.63	4.33
417Q	20.6	0.74	0.27	0.20	38.4	4.04	3.77
161L	14.0	0.77	0.25	0.19	37.8	2.71	2.53
159C	21.0	0.79	0.24	0.19	37.6	4.03	3.77
166P	11.4	0.77	0.25	0.19	37.2	2.17	2.03
166A	23.1	0.77	0.24	0.19	36.4	4.29	4.01
331D	32.4	0.75	0.25	0.18	36.2	5.98	5.59
169H	13.8	0.75	0.25	0.18	36.0	2.54	2.38
366E	20.1	0.75	0.24	0.18	34.9	3.58	3.35
382S	29.6	0.71	0.24	0.17	33.0	4.97	4.65
360L	26.5	0.77	0.22	0.17	32.6	4.41	4.12
390N	30.3	0.75	0.22	0.17	32.4	5.00	4.67
189P	26.2	0.76	0.22	0.16	32.3	4.31	4.02
358E	15.5	0.69	0.23	0.16	31.5	2.49	2.33
356K	38.8	0.75	0.21	0.16	31.2	6.18	5.78
206Q	35.4	0.74	0.21	0.16	30.8	5.57	5.20
191L	21.5	0.77	0.20	0.16	30.5	3.35	3.13
432R	30.6	0.78	0.20	0.16	30.4	4.75	4.44
356R	39.1	0.75	0.21	0.15	30.0	5.97	5.58
205T	25.1	0.74	0.20	0.15	29.0	3.72	3.48
340K	44.7	0.75	0.20	0.15	28.7	6.56	6.13
376V	31.0	0.71	0.21	0.15	28.7	4.53	4.23
206S	35.5	0.75	0.20	0.15	28.5	5.15	4.82

Substitution	k_{cat} min ⁻¹	Parameters with tembotrione			ON x OFF, fold vs wt	ON x OFF x k_{cat}	
		ON ratio	OFF ratio	ON x OFF		Value	Fold vs wt
437F	12.7	0.76	0.19	0.14	28.1	1.82	1.70
214N	35.4	0.76	0.19	0.14	27.9	5.04	4.71
399Y	27.8	0.71	0.20	0.14	27.9	3.95	3.70
357Y	28.7	0.72	0.20	0.14	27.5	4.03	3.76
399H	28.8	0.72	0.19	0.14	27.4	4.02	3.75
387L	33.3	0.75	0.19	0.14	27.0	4.58	4.28
352K	32.2	0.70	0.19	0.13	26.2	4.30	4.02
221V	9.8	0.75	0.18	0.13	26.1	1.30	1.21
437V	19.9	0.67	0.20	0.13	26.0	2.63	2.46
432K	31.5	0.76	0.17	0.13	25.7	4.13	3.86
399N	37.4	0.75	0.17	0.13	25.2	4.81	4.50
396V	29.0	0.72	0.18	0.13	25.2	3.73	3.49
211L	36.9	0.77	0.17	0.13	25.0	4.72	4.41
217Y	20.7	0.76	0.17	0.13	24.9	2.63	2.45
431F	28.9	0.80	0.16	0.13	24.5	3.61	3.37
195E	16.6	0.69	0.18	0.12	24.4	2.07	1.93
231S	20.9	0.68	0.18	0.12	23.8	2.54	2.37
399R	34.8	0.74	0.17	0.12	23.6	4.18	3.91
200C	21.4	0.73	0.16	0.12	23.6	2.57	2.40
396T	37.7	0.71	0.17	0.12	23.5	4.51	4.22
187M	23.5	0.70	0.17	0.12	23.2	2.78	2.60
227G	34.6	0.79	0.15	0.12	23.2	4.09	3.83
432V	N.A.	0.67	0.17	0.12	23.1	0.00	0.00
190M	17.7	0.71	0.16	0.12	22.9	2.07	1.93
375L	36.1	0.71	0.16	0.12	22.8	4.20	3.92
352R	39.4	0.75	0.15	0.11	21.9	4.40	4.11
202T	13.1	0.70	0.16	0.11	21.9	1.46	1.36
399Q	36.1	0.72	0.15	0.11	21.5	3.96	3.70
202A	12.0	0.69	0.16	0.11	21.5	1.32	1.23
396M	35.4	0.73	0.15	0.11	21.4	3.86	3.61
399S	35.8	0.70	0.15	0.11	21.3	3.90	3.64
434R	52.1	0.73	0.15	0.11	21.2	5.63	5.26
221A	42.2	0.62	0.17	0.10	20.3	4.37	4.09
195V	20.1	0.73	0.14	0.10	20.2	2.07	1.93
214T	39.2	0.74	0.14	0.10	20.2	4.03	3.76
191G	42.6	0.77	0.13	0.10	20.1	4.36	4.08
206K	21.9	0.74	0.14	0.10	19.9	2.23	2.08
342V	40.6	0.78	0.13	0.10	19.7	4.08	3.82
398D	38.7	0.72	0.14	0.10	19.6	3.87	3.62
434H	51.9	0.78	0.13	0.10	19.5	5.16	4.82
193Q	23.9	0.77	0.13	0.10	19.3	2.35	2.20
189E	21.6	0.72	0.14	0.10	19.2	2.11	1.98
211S	18.9	0.79	0.12	0.10	19.1	1.83	1.71

Substitution	k_{cat} min ⁻¹	Parameters with tembotrione			ON x OFF, fold vs wt	ON x OFF x k_{cat}	
		ON ratio	OFF ratio	ON x OFF		Value	Fold vs wt
190E	27.2	0.72	0.13	0.10	18.7	2.59	2.42
190T	28.0	0.75	0.13	0.10	18.6	2.66	2.48
396L	40.5	0.57	0.17	0.09	18.6	3.85	3.60
432S	34.6	0.72	0.13	0.09	18.6	3.28	3.06
056S	41.9	0.78	0.12	0.09	18.5	3.97	3.71
423W	36.7	0.48	0.19	0.09	18.0	3.37	3.15
383M	45.9	0.70	0.13	0.09	17.5	4.11	3.84
206L	18.3	0.76	0.12	0.09	17.3	1.62	1.51
383L	47.2	0.66	0.13	0.08	16.3	3.92	3.67
192V	17.6	0.73	0.11	0.08	16.2	1.45	1.36
342M	52.3	0.73	0.11	0.08	16.0	4.28	4.00
125L	37.3	0.76	0.11	0.08	16.0	3.04	2.84
432L	30.7	0.67	0.12	0.08	15.7	2.47	2.30
413A	39.0	0.59	0.13	0.08	15.5	3.09	2.88
214R	25.4	0.75	0.10	0.08	14.9	1.94	1.81
434L	55.5	0.71	0.11	0.08	14.8	4.20	3.93
053F	47.0	0.75	0.10	0.08	14.8	3.54	3.31
349Q	42.6	0.76	0.10	0.07	14.7	3.19	2.98
434K	58.5	0.70	0.11	0.07	14.5	4.33	4.05
398A	42.8	0.72	0.10	0.07	14.4	3.14	2.93
342C	71.5	0.71	0.10	0.07	14.0	5.09	4.76
381N	55.8	0.74	0.10	0.07	13.7	3.91	3.66
227E	27.5	0.72	0.10	0.07	13.7	1.93	1.80
375R	71.8	0.60	0.10	0.06	12.1	4.43	4.14
373V	49.2	0.76	0.08	0.06	12.0	3.01	2.81
349E	47.9	0.76	0.08	0.06	11.6	2.84	2.65
429G	50.2	0.74	0.08	0.06	11.6	2.97	2.78
234K	33.8	0.65	0.09	0.06	11.6	2.00	1.87
429T	53.7	0.72	0.08	0.06	11.5	3.16	2.95
202K	9.2	0.61	0.09	0.06	11.3	0.53	0.50
214M	22.6	0.74	0.07	0.06	10.9	1.25	1.17
418E	83.8	0.69	0.05	0.04	7.1	3.03	2.83
Zm wt	210	0.17	0.03	0.0051	1.00	1.07	1.00
9070	34.2	0.74	0.21	0.15	30.0	5.25	4.90

Thirty nine variants were desensitized to tembotrione compared with the backbone enzyme, Round 7, variant 9070 (SEQ ID NO:2) and all were desensitized compared with maize wild type HPPD (SEQ ID NO:1). Because the assay for ON rate ratio includes an initial rate measurement at saturating hydroxyphenylpyruvate without inhibitor and with known enzyme concentration, an estimate of k_{cat} was available. Taking into account k_{cat} and insensitivity parameters, 30 variants were

selected for measurement of substrate saturation kinetic parameters and re-evaluation of insensitivity to tembotrione. The kinetic and insensitivity parameters were determined as described in Example 1, and were multiplied to generate the “trait fitness” parameter. The complete list of variants, *i.e.*, of selected single substitutions in SEQ ID NO:2, analyzed with multiple inhibitors and the results
5 obtained are shown in Table 6. The same set of 30 variants were evaluated for insensitivity to mesotrione, sulcotrione, isoxaflutole (its active diketonitrile form, abbreviated DKN in Table 6) and topramezone.

All variants desensitized to tembotrione were desensitized to all other inhibitors, but not to the same magnitude or proportion. Fold improvement with tembotrione and diketonitrile (Trait
10 fitness for the mutant / Trait fitness for maize wild type) was generally greater than with mesotrione or sulcotrione because the native maize HPPD was already less sensitive to inhibition by the latter two inhibitors. Further, each variant was not proportionately desensitized to all inhibitors. When trait fitness for tembotrione is plotted versus trait fitness for the other inhibitors, the correlation coefficients (R^2 value) obtained are 0.74 with mesotrione, 0.64 with sulcotrione, 0.86 with
15 diketonitrile and 0.94 with topramezone (data not shown). These results show that although beneficial substitutions identified by selection with one inhibitor accrues benefits with other inhibitors, though selection with inhibitors outside of those used here may result in a different set of preferred substitutions.

Table 6.

Substitution	Kinetic parameters			Insensitivity parameter, ON x OFF					Trait fitness, ON x OFF x k_{cat}/K_M				
	k_{cat} , min ⁻¹	K_M (uM)	k_{cat}/K_M	Tembo-trione	Meso-trione	Sulco-trione	DKN	Topra-mezon	Tembo-trione	Meso-trione	Sulco-trione	DKN	Topra-mezon
366E	38.8	1.61	24.1	0.33	0.78	0.74	0.44	0.48	8.0	18.7	17.8	10.6	11.6
417Q	33.5	1.56	21.5	0.33	0.73	0.74	0.39	0.39	7.0	15.8	15.8	8.4	8.4
432R	56.7	1.97	28.8	0.23	0.53	0.59	0.27	0.31	6.7	15.2	17.0	7.8	8.8
432K	67.5	2.08	32.4	0.21	0.52	0.59	0.26	0.29	6.7	17.0	19.2	8.5	9.4
417S	26.7	1.41	18.9	0.33	0.67	0.77	0.41	0.41	6.2	12.6	14.5	7.7	7.8
214N	84.6	3.62	23.4	0.25	0.48	0.59	0.33	0.32	5.8	11.2	13.8	7.6	7.5
385I	46.1	2.29	20.1	0.28	0.56	0.70	0.41	0.38	5.6	11.4	14.2	8.2	7.6
158K	47.1	2.49	18.9	0.29	0.54	0.63	0.38	0.37	5.5	10.3	12.0	7.1	7.1
385V	30.3	1.91	15.9	0.34	0.65	0.78	0.33	0.49	5.4	10.3	12.4	5.2	7.8
191G	71.7	2.46	29.1	0.19	0.40	0.51	0.25	0.24	5.4	11.7	14.9	7.2	6.9
211L	77.6	3.2	24.2	0.22	0.37	0.47	0.25	0.27	5.4	9.1	11.5	6.1	6.5
340K	73.5	2.41	30.5	0.16	0.44	0.54	0.23	0.22	5.0	13.3	16.4	7.0	6.7
206Q	66.6	2.63	25.3	0.19	0.46	0.53	0.26	0.27	4.9	11.6	13.4	6.7	6.7
214T	84.2	2.94	28.6	0.17	0.41	0.50	0.21	0.23	4.8	11.7	14.3	6.1	6.5
360L	32.6	1.71	19.0	0.25	0.51	0.63	0.34	0.32	4.8	9.7	12.0	6.5	6.0
434H	84.5	2.77	30.5	0.15	0.46	0.55	0.21	0.22	4.7	14.1	16.7	6.5	6.7
387L	74.3	2.46	30.2	0.15	0.46	0.56	0.26	0.21	4.7	14.0	17.0	7.8	6.5
356K	65.8	2.6	25.3	0.18	0.48	0.55	0.25	0.23	4.5	12.2	13.9	6.3	5.8
356R	79.0	2.89	27.3	0.16	0.44	0.53	0.21	0.24	4.5	11.9	14.4	5.7	6.5
182M	56.1	2.88	19.5	0.23	0.44	0.60	0.30	0.29	4.5	8.7	11.7	5.9	5.7
434R	80.9	2.74	29.5	0.15	0.39	0.47	0.18	0.22	4.3	11.4	13.9	5.4	6.5
206S	67.9	3.36	20.2	0.20	0.46	0.53	0.29	0.29	4.1	9.4	10.7	5.9	6.0
191L	32.9	2.74	12.0	0.33	0.45	0.56	0.38	0.37	3.9	5.4	6.7	4.5	4.4
227G	45.3	2.61	17.4	0.22	0.52	0.63	0.26	0.28	3.7	9.0	10.9	4.5	4.9
390N	50.2	2.86	17.5	0.20	0.45	0.58	0.29	0.27	3.5	7.9	10.1	5.2	4.7
342V	54.2	2.59	20.9	0.17	0.42	0.51	0.22	0.23	3.5	8.7	10.6	4.6	4.7
431F	30.8	1.77	17.4	0.19	0.53	0.59	0.26	0.27	3.4	9.2	10.3	4.6	4.7

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Substi- tution	Kinetic parameters			Insensitivity parameter, ON x OFF					Trait fitness, ON x OFF x k_{cat}/K_M				
	$k_{cat},$ $\min^{-1}$	K_M (μM)	k_{cat}/K_M	Tembo- trione	Meso- trione	Sulco- trione	DKN	Topra- mezon	Tembo- trione	Meso- trione	Sulco- trione	DKN	Topra- mezon
189P	41.8	3.35	12.5	0.25	0.51	0.62	0.32	0.31	3.1	6.3	7.7	4.0	3.9
205T	34.4	2.72	12.6	0.22	0.47	0.60	0.30	0.29	2.8	6.0	7.6	3.8	3.7
376W	36.1	5.51	6.5	0.31	0.39	0.16	0.37	0.32	2.1	2.5	1.1	2.4	2.1
9070	55.1	2.35	23.4	0.19	0.45	0.56	0.24	0.26	4.4	10.5	13.0	5.6	6.2
Zm wt	219	6.4	34.2	0.0051	0.0437	0.0872	0.0096	0.0154	0.174	1.494	2.981	0.328	0.525

Example 9. Combining beneficial diversity to optimize fitness

To extend the benefits of the single substitutions identified in Example 2, twenty of the most preferred substitutions distributed among 14 positions in were selected for construction of a combinatorial library. The best of these, Q366E, was selected as the backbone for a library in which the remaining 19 substitutions were allowed to combine randomly. To construct the libraries, the technique of semi-synthetic shuffling was used, as described earlier (Ness JE et al., Nature Biotech 20, 1251-1255, 2002). Two libraries were made in which the frequency of mutations averaged 2.7 or 4.1 per gene and screened for insensitivity to tembotrione, as described in Example 2. Insensitivity parameters for tembotrione and k_{cat} of 77 variants obtained from saturation mutagenesis followed by combinatorial shuffling and selected from the medium browning screen are shown in Table 7.

Table 7.

Seq ID NO.	Variant name	ON rate ratio	OFF rate ratio	ON x OFF	kcat	ON x OFF x kcat
1	Mz wt	0.213	0.033	0.007	97.4	0.68
2	9070	0.744	0.116	0.086	40.0	3.45
3	9070Q366E	0.760	0.273	0.208	20.1	4.18
4	P056028-A-01	0.697	0.249	0.173	39.1	6.78
5	P056028-A-02	0.680	0.387	0.263	38.6	10.15
6	P056028-F-05	0.700	0.395	0.276	27.4	7.57
7	P056028-G-01	0.742	0.320	0.238	32.2	7.65
8	P056028-G-01R	0.719	0.280	0.201	70.8	14.24
9	P056028-B-02	0.800	0.469	0.375	28.2	10.58
10	P056028-F-01	0.718	0.304	0.218	31.3	6.82
11	P056028-D-08	0.681	0.361	0.246	27.5	6.77
12	P056028-E-04	0.740	0.360	0.267	19.2	5.13
13	P056028-A-06	0.760	0.281	0.214	27.0	5.77
14	P056028-F-02	0.730	0.430	0.314	28.2	8.84
15	P056028-C-01	0.742	0.310	0.230	25.9	5.95
16	P056028-E-02	0.761	0.326	0.248	23.6	5.86
17	P056028-C-07	0.714	0.382	0.273	21.2	5.80
18	P056028-A-03	0.748	0.243	0.181	24.0	4.36
19	P056028-C-05	0.715	0.375	0.268	21.7	5.80
20	P056028-A-08	0.669	0.324	0.217	26.2	5.68
21	P056028-D-03	0.770	0.453	0.349	19.6	6.83
22	P056028-B-09	0.648	0.187	0.121	34.5	4.19
23	P056028-A-10	0.707	0.367	0.260	16.8	4.35
24	P056028-A-11	0.706	0.366	0.258	8.6	2.21
25	P056028-A-04	0.724	0.133	0.096	11.1	1.06
26	P056028-A-05	0.707	0.280	0.198	17.7	3.50
27	P056028-A-07	0.726	0.329	0.239	19.5	4.65
28	P056028-A-09	0.692	0.339	0.235	13.9	3.26

Seq ID NO.	Variant name	ON rate ratio	OFF rate ratio	ON x OFF	kcat	ON x OFF x kcat
29	P056028-B-01	0.745	0.309	0.230	18.6	4.28
30	P056028-B-10	0.703	0.327	0.230	17.9	4.12
31	P056028-B-03	0.732	0.353	0.259	17.9	4.63
32	P056028-B-04	0.718	0.273	0.196	16.5	3.24
33	P056028-B-05	0.734	0.287	0.211	18.3	3.85
34	P056028-B-06	0.713	0.353	0.252	20.0	5.03
35	P056028-B-07	0.788	0.389	0.307	9.2	2.83
36	P056028-B-08	0.681	0.325	0.221	15.5	3.44
37	P056028-C-10	0.699	0.356	0.249	14.6	3.63
38	P056028-C-11	0.731	0.293	0.214	12.3	2.64
39	P056028-C-02	0.717	0.379	0.272	18.8	5.11
40	P056028-C-03	0.739	0.356	0.263	13.7	3.62
41	P056028-C-04	0.727	0.322	0.234	22.7	5.32
42	P056028-C-06	0.742	0.351	0.261	10.6	2.77
43	P056028-C-08	0.712	0.381	0.271	13.5	3.65
44	P056028-C-09	0.706	0.314	0.222	17.0	3.76
45	P056028-D-01	0.728	0.348	0.254	21.3	5.39
46	P056028-D-10	0.670	0.253	0.170	18.1	3.07
47	P056028-D-11	0.719	0.261	0.188	17.1	3.21
48	P056028-D-02	0.730	0.299	0.218	24.3	5.31
49	P056028-D-04	0.696	0.197	0.137	12.4	1.71
50	P056028-D-05	0.730	0.508	0.371	12.6	4.66
51	P056028-D-06	0.731	0.424	0.310	9.9	3.07
52	P056028-D-07	0.775	0.370	0.286	9.1	2.60
53	P056028-D-09	0.652	0.371	0.242	13.0	3.16
54	P056028-E-10	0.702	0.339	0.238	11.5	2.73
55	P056028-E-11	0.721	0.304	0.219	16.9	3.70
56	P056028-E-03	0.735	0.412	0.303	16.9	5.10
57	P056028-E-05	0.704	0.325	0.229	15.2	3.48
58	P056028-E-06	0.346	0.186	0.064	28.2	1.76
59	P056028-E-07	0.585	0.262	0.153	20.6	3.08
60	P056028-E-08	0.685	0.410	0.281	12.6	3.55
61	P056028-E-09	0.676	0.308	0.208	14.9	3.10
62	P056028-F-10	0.726	0.291	0.211	13.7	2.89
63	P056028-F-11	0.706	0.322	0.228	22.1	5.04
64	P056028-F-03	0.738	0.491	0.363	13.0	4.73
65	P056028-F-04	0.660	0.245	0.161	16.7	2.70
66	P056028-F-06	0.739	0.305	0.225	14.5	3.25
67	P056028-F-07	0.735	0.322	0.236	21.4	5.04
68	P056028-F-08	0.705	0.377	0.266	12.1	3.23
69	P056028-F-09	0.700	0.360	0.252	17.1	4.30
70	P056028-G-10	0.607	0.259	0.157	29.3	4.61
71	P056028-G-02	0.761	0.304	0.231	23.0	5.30

Seq ID NO.	Variant name	ON rate ratio	OFF rate ratio	ON x OFF	k _{cat}	ON x OFF x k _{cat}
72	P056028-G-04	0.752	0.248	0.186	13.6	2.54
73	P056028-G-05	0.739	0.361	0.267	16.7	4.47
74	P056028-G-06	0.725	0.382	0.277	12.0	3.30
75	P056028-G-07	0.702	0.304	0.213	15.3	3.23
76	P056028-G-08	0.737	0.379	0.279	11.8	3.29
77	P056028-G-09	0.716	0.385	0.275	15.8	4.37

Combinatorial variants exhibiting insensitivity and k_{cat} near those of the backbone sequence were further analyzed for kinetic parameters (k_{cat} and K_M obtained by substrate saturation kinetic analysis) and insensitivity parameters for mesotrione, sulcotrione, topramezone and the diketonitrile form of isoxaflutole. Trait fitness of combinatorial variants of HPPD were compared with wild type and Round 7, variant 9070 (SEQ ID NO:2; see Table 8). The data show that when variants composed of random combinations of single substitutions were selected based on fitness with tembotrione, fitness for the other inhibitors was also improved.

Table 8.

SEQ ID NO	Variant Name	Kinetic parameters			Insensitivity, tembotrione				Insensitivity, mesotrione			
		k_{cat}	K_M	k_{cat}/K_M	Rate ratio			Trait fitness	Rate ratio		Trait fitness	OFF
					ON	OFF	ONxOFF		ON	OFF		ONxOFF
22	P056028-B-09	93.2	3.00	31.6	0.75	0.35	0.26	8.28	0.67	1.00	0.67	21.2
4	P056028-A-01	72.4	2.49	29.1	0.84	0.43	0.36	10.43	0.67	1.00	0.67	19.6
18	P056028-A-03	62.8	2.98	21.0	0.80	0.40	0.32	6.81	0.70	1.00	0.70	14.8
21	P056028-D-03	44.2	2.08	21.3	0.77	0.45	0.35	7.44	0.68	1.00	0.68	14.5
9	P056028-B-02	58.7	3.00	19.6	0.80	0.47	0.37	7.35	0.63	1.00	0.63	12.3
7	P056028-G-01	41.4	2.06	20.4	0.77	0.47	0.36	7.32	0.71	1.00	0.71	14.6
8	P056028-G-01R	70.8	2.02	35.3	0.72	0.28	0.20	7.09	0.73	1.00	0.73	25.6
12	P056028-E-04	29.0	2.11	13.8	0.74	0.36	0.27	3.68	0.67	1.00	0.67	9.2
14	P056028-F-02	45.5	2.22	20.5	0.73	0.43	0.31	6.43	0.67	1.00	0.67	13.7
5	P056028-A-02	31.4	1.76	17.8	0.68	0.39	0.26	4.69	0.59	1.00	0.59	10.5
13	P056028-A-06	74.6	4.05	18.4	0.76	0.28	0.21	3.94	0.63	0.74	0.47	8.6
6	P056028-F-05	32.3	2.39	13.5	0.70	0.39	0.28	3.74	0.64	1.00	0.64	8.7
2	9070	88.9	2.91	30.7	0.80	0.22	0.17	5.36	0.66	0.89	0.59	18.1
3	9070Q366E	30.8	1.57	19.5	0.82	0.33	0.27	5.25	0.72	1.00	0.72	14.0
1	Zm wt	219	6.41	34.2	0.20	0.04	0.007	0.26	0.20	0.31	0.062	2.1

Table 8, continued

SEQ ID NO	Variant Name	Insensitivity, sulcotrione				Insensitivity, isoxaflutole (DKN)				Insensitivity, topramezone			
		Rate ratio		Trait fitness	Rate ratio	Rate ratio		Trait fitness	Rate ratio	Rate ratio		Trait Fitness	
		ON	OFF			ON	OFF			ON	OFF		
22	P056028-B-09	0.78	0.88	0.68		0.77	0.33	0.25		0.80	0.48	0.38	12.12
4	P056028-A-01	0.80	1.22	0.97		0.82	0.44	0.36		0.85	0.60	0.51	14.86
18	P056028-A-03	0.80	0.93	0.75		0.81	0.43	0.34		0.81	0.47	0.38	7.97
21	P056028-D-03	0.76	1.11	0.84		0.76	0.40	0.31		0.80	0.58	0.46	9.88
9	P056028-B-02	0.81	1.00	0.81		0.80	0.43	0.35		0.79	0.53	0.42	8.17
7	P056028-G-01	0.78	1.14	0.89		0.77	0.42	0.32		0.80	0.57	0.46	9.31
12	P056028-G-01R	0.75	1.11	0.83		0.77	0.38	0.29		0.81	0.53	0.43	5.91
14	P056028-E-04	0.72	1.09	0.79		0.75	0.42	0.31		0.80	0.60	0.48	9.85
5	P056028-F-02	0.69	1.28	0.88		0.70	0.43	0.30		0.69	0.64	0.44	7.87
13	P056028-A-02	0.77	0.76	0.58		0.77	0.28	0.22		0.76	0.32	0.24	4.52
6	P056028-A-06	0.73	1.13	0.82		0.73	0.42	0.31		0.76	0.57	0.44	5.91
2	9070	0.81	0.71	0.38		0.82	0.20	0.11		0.79	0.25	0.13	3.99
3	9070Q366E	0.80	1.13	0.50		0.83	0.39	0.17		0.81	0.57	0.26	5.04
1	Zm wt	0.30	0.28	0.042		0.08	0.01	0.0005		0.100	0.045	0.002	0.077

Example 10. Tolerance to multiple HPPD-inhibiting herbicides in transformed soybean

A 1228 bp genomic sequence at the 3' end of the G. max HPPD gene was isolated, sequenced and analyzed for promoter elements as described earlier (US20120042414(A1)). Elements were modified so as to decrease or increase expression and various transformation cassettes, designed to direct a range of expression of shuffled maize HPPD, were constructed (see US Patent Publication US2012/0042414(A1)). Transgenic soybean events were created using particle bombardment of embryogenic callus cultures and selection with either hygromycin or chlorsulfuron. T0 regenerated plants were acclimated to greenhouse conditions in soil for two weeks and sprayed with two times labeled use rate of mesotrione. When sibling regenerants from the same event were available, the second plant was sprayed with 2x tembotrione. For each construct approximately 30 events were created with one or two copies of the HPPD insert at a single locus. To assess the expression cassettes, the number of events from each construct that showed 30% or less injury to 2X mesotrione and 2X tembotrione (on sibling plants) were counted. T0 plants were sampled for DNA analysis to verify single copy inserts and for protein analysis to measure HPPD accumulation in young leaves. Events with high injury routinely occurred due to the variability inherent in random genomic insertion methods. Plants with less than 30% visible injury were grown to maturity and the T1 seeds that were harvested were sown in the greenhouse or the field. The T1 plants were treated with 472 g/ha (4x field rate) mesotrione and screened by PCR to identify homozygotes. Tolerant, homozygous lines were grown and harvested for field trials.

A field trial performed in 2013 was designed to test: 1) various promoters known to drive a range of expression levels, described in US20120042414(A1), and 2) two N-terminal variations of the HPPD protein 9075 (SEQ ID NO:79), which is polypeptide sequence corresponding to Round 7, variant 9070 (SEQ ID NO:2) with R316 changed to Q. In one case, the N-terminus is that of the full-length enzyme. In the other case, the first 44 amino acids of SEQ ID NO:79 were replaced with the first 86 amino acids of the long form of soy HPPD (see US Patent Publication US2012/0042412(A1)). Both N-termini were previously shown to direct transgenically expressed HPPD to both chloroplasts and cytosol (see US Patent Publication US2012/0042412(A1)). Transformation events from several of the constructs were highly tolerant to mesotrione, tembotrione, and isoxaflutole at two to four times labeled field application rates.

For positive control comparisons, the strong synthetic viral-based promoters SUP and SCP1 (Bowen et al., 2000) were used to drive various HPPD variants. Repeated occurrences of wrinkled leaf and chlorotic leaf phenotypes along with occasional sterility in the SUP and SCP1 events indicated that these promoters were not suitable for commercial event production. Measurements of HPPD protein accumulation in young leaves of T0 plants as shown in Table 9 revealed that the

strong synthetic promoters accumulated HPPD protein near 1% of total soluble protein (10,000 ppm). The soybean derived HPPD promoters led to accumulation of HPPD protein in tolerant events at levels in the range of 0.02 – 0.1% total soluble protein (200-1000 ppm). In general, promoters with added synthetic core elements and enhancers produced more tolerant events and more protein than promoters with mutations and deletions. The lower expressing HPPD constructs provided higher recovery of phenotypically normal plants.

To look more closely at the role of the dual targeting of heterologous HPPD in soybean, constructs with N-terminal truncations (no targeting), truncated maize HPPD protein fused to known CTP elements (single transcript), and soybean N-terminal 1-86 fused to truncated maize HPPD for dual targeting were compared. All localization scenarios resulted in production of herbicide tolerant events, of varying efficacy (Table 9). Table 9 shows greenhouse characterization of T0 transgenic soybean plants expressing HPPD cassettes with varied promoters and an insensitive HPPD gene. Visual injury rating, 0 (no injury)-100 (dead) scale, average of 3 plots. DAT, days after treatment; All treatments were sprayed after two weeks acclimation from transformation(V2-V8) and included 0.25% v/v NIS and 8 lb/100 gal (NH₄)₂SO₄. Neither protein accumulation nor construct properties alone explain the spectrum of variability in green house T0 efficacy results. In all cases, however, tolerance to mesotrione at the maize field rate was more easily achieved than tolerance to tembotrione.

Table 9.

Promoter	N-terminus fusion	HPPD 9075 start	# Events meso screen	% Events ≤30% injury meso ^d	# Events tembo screen	% Events ≤30% injury tembo ^e	HPPD protein, ppm ave ≤30% injury ⁶
SHP210 ^a	GM-HPPD N1-86	50	15	100	12	100	326
SUP	none	24	29	79	8	50	12779
SHP110 ^b	GM-HPPD N1-86	50	13	100	11	27	266
SHP103C	none	1	26	80	26	42	594
SHP106	6H1 CTP	24	6	100	5	0	69
SHP206 ^c	none	1	22	86	na	na	679
SHP110e ^c	GM-HPPD N1-86	50	43	81	na	na	876
SHP111	GM-HPPD N1-86	50	37	70	13	7.7	318
SHP120C	GM-HPPD N1-86	50	57	61	31	3.2	165
SHP110	GM-HPPD N1-86	50	13	38	1	0	382

Promoter	N-terminus fusion	HPPD 9075 start	# Events meso screen	% Events $\leq 30\%$ injury meso ^d	# Events tembo screen	% Events $\leq 30\%$ injury tembo ^e	HPPD protein, ppm ave $\leq 30\%$ injury ^f
SHP103C	AT-RBCS CTP	24	28	17	25	0	397

^a Promoter SHP210 has deletion of TATA5, 2 and 3 with insertion of Element II at the 3' end of the promoter.

^b Promoter SHP110 has deletion of TATA5, 2 and 3 with insertion of Element I at the 3' end of the promoter.

^c Construct events tested in 2013 field tests.

^d 210 g ai/ha mesotrione, evaluated at 8 days after treatment

^e 93 g ai/ha tembotrione, evaluated at 8 days after treatment

^f Parts per million of total soluble protein.

Because T0 plants are heterozygous for the HPPD insert and because field conditions can be more stringent than greenhouse conditions, field testing of homozygous lines is essential to fully evaluate the efficacy of the transgene traits. Field tests in 2013 with a round 6 desensitized HPPD variant showed that several events created were highly tolerant to mesotrione, tembotrione, and isoxaflutole at 2X or 4X treatment levels. Some transient injury was apparent, but by 14 days after treatment, the plants were almost completely recovered as shown in FIG. 9. Tabulated results for 2 different events are shown in Table 10, which shows data obtained from transgenic soybean plants expressing insensitive HPPD variant constructs showing tolerance to three different HPPD-inhibitor herbicides in the field.

Seed were homozygous for single copy inserts in elite genetics. Visual injury rating, 0 (no injury)-100 (dead) scale, average of 3 plots. In the table, the following abbreviations are used: DAT, days after treatment; 2X and 4X, treatment levels. All treatments were sprayed at the V4 stage and included 0.25% v/v NIS and 8 lb/100 gal (NH₄)₂SO₄.

Table 10.

Event Cassette	DAT	Isoxaflutole		Mesotrione		Tembotrione
		2X	4X	2X	4X	2X
PRO206:FL9075	3	1	3	10	20	8
PRO206:FL9075	7	0.7	1.0	1.7	8.3	4.3
PRO206:FL9075	14	0.0	0.0	0.0	3.3	1.0
PRO110e:Soy N-term	3	0	2	13	20	13
PRO110e:Soy N-term	7	0.0	0.0	3.7	11.7	19.0
PRO110e:Soy N-term	14	0.0	0.0	0.0	0.7	4.0

PRO206: Promoter 206 described in US20120042414(A1)

PRO110e: Promoter SHP110, described in US20120042414(A1) with the addition of a CaMV enhancer

FL9075: Full-length shuffled maize variant 9075 (SEQ ID NO:79)

Soy N-term: 9075 (SEQ ID NO:79) in which the first 44 amino acids were replaced with the first 86 amino acids of the long form of soy HPPD (US20120042412(A1))

Mesotrione 1x = 0.105 lb ai/a = 118 g ai/ha

Tembotrione 1x = 0.08 lb ai/a = 90 g ai/ha

Isoxaflutole 1x = 0.06 lb ai/a = 67 g ai/ha

All treatments were at the V4 stage and included 0.25% v/v NIS and 8 lb/100 gal

NH₄2SO₄.

The data set from the field trial also illustrates the importance of attaining an expression level that is neither too low nor too high. FIG. 14 plots the efficacy of the transgenic trait, expressed as the visual rating of injury on a 100 scale as a function of the expression level, µg HPPD/g total protein. Excellent tolerance was obtained at a level of ~500-1000 µg/g, while lower expression results in more injury. However, perhaps because the HPPD reaction is practically irreversible and not inhibited by product, excessive catalytic capacity was associated with abnormal phenotypes such as chlorosis and leaf wrinkling (FIG. 14). The data show that symptoms are associated with high expression level.

The velocity of an enzyme catalyzed reaction is a function of k_{cat}/K_M , [E] and [S], where E is enzyme and S is substrate. The substrate concentration in soybean tissues is unknown but presumed to be constant, while enzyme concentration is determined by the expression level. In the presence of inhibitor, the fraction of the enzyme that is free to react with substrate is proportional to the K_i of the inhibitor. A common parameter used to quantify performance of an enzyme in the presence of an inhibitor is $k_{cat}/K_M \times K_i$, which takes into account catalytic efficiency and inhibitor affinity. An approximation of that expression is the “trait fitness” parameter, $k_{cat}/K_M \times (\text{ON} \times \text{OFF})$. Fitness times [E], or expression level, quantifies the expected performance *in vivo*, depending on substrate and inhibitor concentrations. Those are not known, but from the data presented, an empirical estimate of the range of values for the quantity “fitness parameter” x “expression level” is the following:

Fitness range, 2 to 200 min⁻¹uM⁻¹

Expression range, 200 to 2000 µg/g

Fitness x expression range, 400 to 400,000 min⁻¹uM⁻¹

Example 11. Identification of shuffled variants of maize HPPD for tolerance to as yet non-commercialized herbicidal inhibitors

The value of the substitutions identified as beneficial for desensitizing the enzyme to tembotrione is not limited to tembotrione, but extends to mesotrione, sulcotrione, topramezone and isoxaflutole diketonitrile. To discover a variant that confers tolerance to a new HPPD-inhibiting herbicide, the 554 single substitutions are screened by the same tiered screen as in Examples 2 and 3. It is anticipated that different sets of preferred substitutions will emerge depending on the structure of the inhibitor. The substitutions identified are then combined randomly and the resulting library screened as described, and novel recombinant variants selected for fitness to the novel compound are identified. Thus, any of the 554 substitutions identified, either singly or in combination, can be useful in developing a tolerance trait for future inhibitor classes.

For example, Round 6, variant 9070 (SEQ ID NO:2) and Round 8, variant 1973 (SEQ ID NO:8) can be compared with maize wild type HPPD (SEQ ID NO:1) for their ability to catalyze the HPPD reaction in the presence of additional HPPD inhibiting compounds, *e.g.*, compounds such as those depicted in FIGS. 1 and 16. Compounds such as those depicted in FIGS. 1 and 16, can be purchased or synthesized following published synthesis methods. Enzymes are purified and kinetic and insensitivity parameters are determined as described in herein above. It is anticipated that the catalytic efficiency as indicated by k_{cat}/K_M can be retained through multiple rounds of shuffling, while insensitivity to additional HPPD inhibitors can be increased, *e.g.*, increased insensitivity to HPPD inhibitors depicted in FIGS. 1 and 16. Example 10 above describes a field trial in which a Round 6 HPPD variant expressed in soybean plants provided a high degree of tolerance to mesotrione, tembotrione and isoxaflutole. The most stringent condition for tolerance was a spray rate of two times the labeled rate of tembotrione. Therefore, fitness of the Round 6 variant to tembotrione can be used as the minimal level needed for efficacy when expressed in a suitable expression construct, to which can be compared additional variants of HPPD obtained through additional rounds of shuffling. In addition, the insensitivity to the novel HPPD inhibitors can be determined and shuffled variants selected wherein the insensitivity to novel HPPD inhibitors is increased.

Megatable Legends

Megatable 1. The definitions of the column headings are as follows: “MUT ID,” a unique identifier for each substitutions; “Position,” amino acid position according to the numbering convention of SEQ ID NO:1, “Ref. A.A.,” the standard single letter code for the amino acid present in the SEQ ID NO:1 sequence at the indicated position; and “Substitution,” the standard single letter code for the amino acid present in the mutant sequence at the indicated position.

Megatable 2. The definitions of the column headings are as follows: “MUT ID,” a unique identifier for each substitutions; “Position,” amino acid position according to the numbering convention of SEQ ID NO:1, “Ref. A.A.,” the standard single letter code for the amino acid present in the SEQ ID NO:1 sequence at the indicated position; and “Substitution,” the standard single letter code for the amino acid present in the mutant sequence at the indicated position. The remaining column headings refer to the kinetic parameters described herein above, i.e. “k_{cat}” indicates the parameter k_{cat}; “ON rat.” Refers to the ON ratio parameter; “OFF rat.” refers to the OFF ratio parameter; “ON x OFF” refers to the calculated value that is the product of the ON ratio and OFF ratio parameters; “Fold vs. wt” is the calculated increase in “ON x OFF” value versus the wild-type sequence, SEQ ID NO:1; “ON x OFF x k_{cat}” refers to the calculated value that is the product of the ON ratio, OFF ratio, and k_{cat}; and the last column with “Fold vs. wt” refers to the fold-increase in calculated value that is the product of the ON ratio, OFF ratio, and k_{cat} of the indicated mutant versus the wild-type sequence, SEQ ID NO:1.

Megatable 3. The definitions of the column headings are as follows: “SEQ ID” is the SEQ ID NO for the sequences herein above; and “Variant name” is a trivial but unique name for the indicated sequence. The remaining column headings refer to the kinetic parameters described herein above, i.e. “ON ratio” Refers to the ON ratio parameter; “OFF ratio” refers to the OFF ratio parameter; “ON x OFF” refers to the calculated value that is the product of the ON ratio and OFF ratio parameters; “k_{cat}” indicates the parameter k_{cat}; and “ON x OFF x k_{cat}” refers to the calculated value that is the product of the ON ratio, OFF ratio, and k_{cat}.

Megatable 1

MUT ID	Position	Ref A.A.	Substitution	Activity 1st tier
1	52	E	D	+
2	52	E	G	+
3	52	E	H	+
4	52	E	R	+
5	52	E	S	+
6	52	E	T	+
7	53	L	F	+
8	53	L	I	+
9	53	L	M	+
10	54	W	C	+
11	54	W	L	+
12	54	W	Q	+
13	55	C	A	+
14	55	C	G	+
15	55	C	T	+
16	55	C	V	+
17	56	A	H	+
18	56	A	L	+
19	56	A	N	+
20	56	A	Q	+
21	56	A	S	+
22	56	A	T	+
23	58	A	G	+
24	58	A	T	+
25	125	R	L	+
26	147	A	S	+
27	148	F	W	+
28	148	F	Y	+
29	149	R	A	+
30	149	R	I	+
31	149	R	K	+
32	149	R	M	+
33	149	R	P	+
34	149	R	S	+
35	149	R	T	+
36	149	R	V	+
37	149	R	W	+
38	152	V	E	+
39	153	A	F	+

40	153	A	I	+
41	153	A	K	+
42	153	A	L	+
43	153	A	Q	+
44	153	A	R	+
45	153	A	T	+
46	153	A	V	+
47	154	A	C	+
48	154	A	G	+
49	154	A	N	+
50	154	A	R	+
51	154	A	T	+
52	157	R	H	+
53	157	R	N	+
54	157	R	T	+
55	157	R	V	+
56	158	P	A	+
57	158	P	E	+
58	158	P	G	+
59	158	P	K	+
60	159	A	C	+
61	159	A	M	+
62	159	A	S	+
63	159	A	T	+
64	159	A	V	+
65	160	F	L	+
66	160	F	M	+
67	160	F	Y	+
68	161	G	A	+
69	161	G	E	+
70	161	G	L	+
71	161	G	M	+
72	161	G	N	+
73	161	G	P	+
74	161	G	Q	+
75	161	G	R	+
76	161	G	S	+
77	161	G	T	+
78	161	G	W	+
79	161	G	Y	+
80	162	P	T	+
81	163	V	A	+
82	163	V	C	+
83	163	V	M	+

84	163	V	T	+
85	164	D	A	+
86	164	D	E	+
87	164	D	H	+
88	164	D	S	+
89	164	D	T	+
90	165	L	C	+
91	165	L	M	+
92	165	L	V	+
93	166	G	A	+
94	166	G	P	+
95	167	R	+	+
96	167	R	V	+
97	169	F	A	+
98	169	F	H	+
99	169	F	W	+
100	169	F	Y	+
101	170	R	C	+
102	170	R	G	+
103	170	R	I	+
104	170	R	K	+
105	170	R	L	+
106	170	R	M	+
107	170	R	P	+
108	170	R	Q	+
109	170	R	S	+
110	170	R	T	+
111	170	R	V	+
112	170	R	W	+
113	171	L	F	+
114	171	L	I	+
115	171	L	M	+
116	171	L	V	+
117	172	A	P	+
118	172	A	R	+
119	172	A	S	+
120	173	E	P	+
121	174	V	C	+
122	174	V	I	+
123	175	E	D	+
124	175	E	V	+
125	175	E	W	+
126	175	E	+	+
127	176	L	A	+

128	176	L	M	+
129	181	V	F	+
130	181	V	I	+
131	181	V	M	+
132	181	V	N	+
133	182	L	M	+
134	187	Y	C	+
135	187	Y	E	+
136	187	Y	G	+
137	187	Y	H	+
138	187	Y	M	+
139	187	Y	V	+
140	188	P	D	+
141	188	P	G	+
142	188	P	Q	+
143	189	D	E	+
144	189	D	P	+
145	189	D	S	+
146	189	D	T	+
147	190	G	A	+
148	190	G	C	+
149	190	G	D	+
150	190	G	E	+
151	190	G	L	+
152	190	G	M	+
153	190	G	N	+
154	190	G	P	+
155	190	G	Q	+
156	190	G	R	+
157	190	G	S	+
158	190	G	T	+
159	190	G	V	+
160	190	G	W	+
161	191	A	E	+
162	191	A	F	+
163	191	A	G	+
164	191	A	H	+
165	191	A	I	+
166	191	A	K	+
167	191	A	L	+
168	191	A	M	+
169	191	A	N	+
170	191	A	R	+
171	191	A	S	+

172	191	A	T	+
173	191	A	V	+
174	192	A	V	+
175	193	G	A	+
176	193	G	H	+
177	193	G	I	+
178	193	G	L	+
179	193	G	P	+
180	193	G	Q	+
181	193	G	R	+
182	193	G	S	+
183	193	G	T	+
184	193	G	V	+
185	194	E	D	+
186	194	E	L	+
187	195	P	A	+
188	195	P	C	+
189	195	P	D	+
190	195	P	E	+
191	195	P	Q	+
192	195	P	S	+
193	195	P	T	+
194	195	P	V	+
195	196	F	W	+
196	200	F	C	+
197	202	G	A	+
198	202	G	E	+
199	202	G	K	+
200	202	G	L	+
201	202	G	T	+
202	202	G	V	+
203	202	G	Y	+
204	204	A	C	+
205	204	A	D	+
206	204	A	E	+
207	204	A	G	+
208	204	A	H	+
209	204	A	K	+
210	204	A	L	+
211	204	A	M	+
212	204	A	N	+
213	204	A	Q	+
214	204	A	T	+
215	204	A	V	+

216	205	S	D	+
217	205	S	G	+
218	205	S	L	+
219	205	S	Q	+
220	205	S	T	+
221	205	S	V	+
222	206	P	C	+
223	206	P	D	+
224	206	P	K	+
225	206	P	L	+
226	206	P	Q	+
227	206	P	S	+
228	206	P	T	+
229	207	G	Q	+
230	207	G	S	+
231	207	G	V	+
232	208	A	G	+
233	208	A	H	+
234	208	A	Q	+
235	208	A	R	+
236	208	A	W	+
237	209	A	C	+
238	209	A	D	+
239	209	A	E	+
240	209	A	G	+
241	209	A	I	+
242	209	A	K	+
243	209	A	L	+
244	209	A	M	+
245	209	A	P	+
246	209	A	Q	+
247	209	A	S	+
248	209	A	T	+
249	209	A	W	+
250	210	D	E	+
251	210	D	G	+
252	210	D	S	+
253	210	D	T	+
254	211	Y	C	+
255	211	Y	F	+
256	211	Y	L	+
257	211	Y	S	+
258	211	Y	W	+
259	212	G	K	+

260	213	L	W	+
261	214	S	K	+
262	214	S	M	+
263	214	S	N	+
264	214	S	Q	+
265	214	S	R	+
266	214	S	T	+
267	215	R	A	+
268	215	R	G	+
269	217	D	Q	+
270	217	D	Y	+
271	220	V	T	+
272	221	G	A	+
273	221	G	F	+
274	221	G	H	+
275	221	G	I	+
276	221	G	L	+
277	221	G	M	+
278	221	G	T	+
279	221	G	V	+
280	222	N	G	+
281	222	N	V	+
282	227	A	D	+
283	227	A	E	+
284	227	A	G	+
285	227	A	I	+
286	227	A	K	+
287	227	A	L	+
288	227	A	M	+
289	227	A	N	+
290	227	A	Q	+
291	227	A	R	+
292	227	A	S	+
293	227	A	T	+
294	227	A	V	+
295	229	A	T	+
296	230	A	R	+
297	230	A	V	+
298	231	A	C	+
299	231	A	H	+
300	231	A	L	+
301	231	A	S	+
302	231	A	T	+
303	233	M	V	+

304	234	A	C	+
305	234	A	E	+
306	234	A	G	+
307	234	A	K	+
308	234	A	L	+
309	234	A	M	+
310	234	A	Q	+
311	234	A	S	+
312	234	A	V	+
313	238	G	S	+
314	240	H	R	+
315	241	E	A	+
316	241	E	D	+
317	241	E	G	+
318	241	E	N	+
319	241	E	P	+
320	241	E	R	+
321	241	E	S	+
322	241	E	T	+
323	242	F	A	+
324	242	F	D	+
325	242	F	S	+
326	273	L	V	+
327	292	H	N	+
328	331	L	D	+
329	331	L	G	+
330	331	L	N	+
331	331	L	R	+
332	332	S	A	+
333	339	R	K	+
334	340	R	E	+
335	340	R	K	+
336	340	R	N	+
337	342	A	C	+
338	342	A	L	+
339	342	A	M	+
340	342	A	N	+
341	342	A	R	+
342	342	A	V	+
343	343	G	R	+
344	345	V	I	+
345	347	T	S	+
346	348	E	Y	+
347	349	A	E	+

348	349	A	Q	+
349	349	A	R	+
350	349	A	S	+
351	351	I	C	+
352	351	I	V	+
353	352	N	E	+
354	352	N	K	+
355	352	N	L	+
356	352	N	Q	+
357	352	N	R	+
358	353	E	L	+
359	353	E	M	+
360	353	E	S	+
361	353	E	T	+
362	356	E	K	+
363	356	E	R	+
364	357	L	Y	+
365	358	G	E	+
366	358	G	R	+
367	360	M	L	+
368	360	M	T	+
369	364	D	G	+
370	364	D	S	+
371	364	D	V	+
372	365	D	A	+
373	366	Q	E	+
374	368	V	L	+
375	369	L	M	+
376	369	L	V	+
377	372	I	A	+
378	372	I	K	+
379	372	I	Q	+
380	372	I	S	+
381	372	I	T	+
382	373	F	G	+
383	373	F	L	+
384	373	F	R	+
385	373	F	V	+
386	375	K	L	+
387	375	K	R	+
388	376	P	C	+
389	376	P	G	+
390	376	P	S	+
391	376	P	V	+

392	376	P	W	+
393	377	V	E	+
394	377	V	G	+
395	377	V	L	+
396	379	D	K	+
397	381	P	N	+
398	382	T	A	+
399	382	T	F	+
400	382	T	S	+
401	383	F	L	+
402	383	F	M	+
403	384	F	W	+
404	384	F	Y	+
405	385	L	I	+
406	385	L	V	+
407	386	E	C	+
408	386	E	I	+
409	386	E	V	+
410	387	I	G	+
411	387	I	L	+
412	388	I	L	+
413	388	I	S	+
414	388	I	V	+
415	389	Q	G	+
416	389	Q	K	+
417	389	Q	T	+
418	390	R	N	+
419	391	I	L	+
420	391	I	V	+
421	392	G	R	+
422	392	G	V	+
423	394	M	I	+
424	394	M	K	+
425	394	M	L	+
426	394	M	Q	+
427	394	M	V	+
428	394	M	Y	+
429	395	E	K	+
430	395	E	Q	+
431	395	E	S	+
432	395	E	V	+
433	396	K	A	+
434	396	K	L	+
435	396	K	M	+

436	396	K	Q	+
437	396	K	R	+
438	396	K	T	+
439	396	K	V	+
440	397	D	G	+
441	397	D	S	+
442	398	E	A	+
443	398	E	D	+
444	398	E	G	+
445	398	E	P	+
446	398	E	S	+
447	399	K	A	+
448	399	K	D	+
449	399	K	G	+
450	399	K	H	+
451	399	K	I	+
452	399	K	M	+
453	399	K	N	+
454	399	K	Q	+
455	399	K	R	+
456	399	K	S	+
457	399	K	T	+
458	399	K	V	+
459	399	K	Y	+
460	400	G	E	+
461	400	G	K	+
462	400	G	S	+
463	401	Q	A	+
464	401	Q	E	+
465	401	Q	S	+
466	401	Q	V	+
467	406	G	C	+
468	407	G	A	+
469	407	G	K	+
470	407	G	L	+
471	407	G	R	+
472	407	G	S	+
473	407	G	T	+
474	408	C	G	+
475	408	C	R	+
476	408	C	T	+
477	408	C	V	+
478	408	C	W	+
479	411	F	A	+

480	411	F	L	+
481	413	K	A	+
482	413	K	P	+
483	413	K	R	+
484	413	K	S	+
485	415	N	A	+
486	416	F	R	+
487	416	F	V	+
488	417	G	Q	+
489	417	G	S	+
490	418	Q	A	+
491	418	Q	C	+
492	418	Q	E	+
493	418	Q	G	+
494	418	Q	L	+
495	418	Q	T	+
496	422	S	M	+
497	423	I	V	+
498	423	I	W	+
499	424	E	Q	+
500	425	D	A	+
501	425	D	E	+
502	425	D	G	+
503	425	D	M	+
504	425	D	S	+
505	425	D	T	+
506	426	Y	L	+
507	426	Y	W	+
508	427	E	A	+
509	427	E	L	+
510	427	E	M	+
511	428	K	R	+
512	428	K	R	+
513	428	K	V	+
514	429	S	C	+
515	429	S	D	+
516	429	S	G	+
517	429	S	T	+
518	430	L	M	+
519	430	L	V	+
520	431	E	A	+
521	431	E	F	+
522	431	E	G	+
523	431	E	L	+

524	431	E	N	+
525	432	A	D	+
526	432	A	G	+
527	432	A	K	+
528	432	A	L	+
529	432	A	R	+
530	432	A	S	+
531	432	A	V	+
532	433	K	E	+
533	433	K	H	+
534	433	K	L	+
535	433	K	P	+
536	433	K	R	+
537	433	K	S	+
538	433	K	V	+
539	434	Q	A	+
540	434	Q	C	+
541	434	Q	D	+
542	434	Q	F	+
543	434	Q	G	+
544	434	Q	H	+
545	434	Q	K	+
546	434	Q	L	+
547	434	Q	R	+
548	437	A	F	+
549	437	A	K	+
550	437	A	V	+
551	438	A	G	+
552	438	A	R	+
553	439	A	R	+

Megatable 2

MUT. NO.	Pos.	Ref. AA	Sub.	kcat	ON Rat.	OFF Rat.	ON x OFF	Fold vs. wt	ONxOFF kcat	Fold vs. wt
SEQ ID NO 1			210	0.17	0.03	0.0051	1.00	1.07	1.00	
SEQ ID NO 2			34.2	0.74	0.21	0.15	30.0	5.25	4.90	
330	331	L	N	40.4	0.75	0.43	0.32	63.0	12.99	12.14
551	438	A	G	33.8	0.74	0.42	0.31	60.3	10.41	9.73
331	331	L	R	30.3	0.73	0.38	0.28	54.6	8.44	7.89
549	437	A	K	35.7	0.76	0.36	0.27	52.9	9.63	9.00
552	438	A	R	29.2	0.75	0.33	0.25	48.5	7.23	6.76
406	385	L	V	21.4	0.75	0.33	0.25	48.2	5.26	4.92
392	376	P	W	23.1	0.68	0.34	0.23	46.0	5.42	5.07
489	417	G	S	18.5	0.82	0.28	0.23	45.2	4.26	3.98
69	161	G	E	17.2	0.78	0.29	0.23	44.8	3.92	3.67
74	161	G	Q	27.7	0.77	0.30	0.23	44.8	6.33	5.91
553	439	A	R	24.7	0.73	0.30	0.22	43.3	5.45	5.09
76	161	G	S	24.5	0.76	0.29	0.22	43.2	5.41	5.06
133	182	L	M	26.0	0.79	0.28	0.22	42.5	5.63	5.26
113	171	L	F	16.6	0.75	0.28	0.21	41.8	3.54	3.31
268	215	R	G	24.4	0.79	0.27	0.21	41.8	5.19	4.85
171	191	A	S	12.4	0.75	0.28	0.21	40.8	2.59	2.42
59	158	P	K	50.4	0.77	0.27	0.21	40.6	10.44	9.76
332	332	S	A	41.5	0.75	0.28	0.21	40.5	8.58	8.02
79	161	G	Y	21.6	0.78	0.26	0.20	40.0	4.40	4.11
405	385	L	I	27.5	0.75	0.27	0.20	39.5	5.55	5.18
329	331	L	G	29.0	0.73	0.27	0.20	39.1	5.78	5.40
72	161	G	N	23.5	0.78	0.25	0.20	38.6	4.63	4.33
488	417	G	Q	20.6	0.74	0.27	0.20	38.4	4.04	3.77
70	161	G	L	14.0	0.77	0.25	0.19	37.8	2.71	2.53
60	159	A	C	21.0	0.79	0.24	0.19	37.6	4.03	3.77
94	166	G	P	11.4	0.77	0.25	0.19	37.2	2.17	2.03
93	166	G	A	23.1	0.77	0.24	0.19	36.4	4.29	4.01
328	331	L	D	32.4	0.75	0.25	0.18	36.2	5.98	5.59
98	169	F	H	13.8	0.75	0.25	0.18	36.0	2.54	2.38
373	366	Q	E	20.1	0.75	0.24	0.18	34.9	3.58	3.35
400	382	T	S	29.6	0.71	0.24	0.17	33.0	4.97	4.65
367	360	M	L	26.5	0.77	0.22	0.17	32.6	4.41	4.12
418	390	R	N	30.3	0.75	0.22	0.17	32.4	5.00	4.67
144	189	D	P	26.2	0.76	0.22	0.16	32.3	4.31	4.02
365	358	G	E	15.5	0.69	0.23	0.16	31.5	2.49	2.33
362	356	E	K	38.8	0.75	0.21	0.16	31.2	6.18	5.78
226	206	P	Q	35.4	0.74	0.21	0.16	30.8	5.57	5.20

167	191	A	L	21.5	0.77	0.20	0.16	30.5	3.35	3.13
529	432	A	R	30.6	0.78	0.20	0.16	30.4	4.75	4.44
363	356	E	R	39.1	0.75	0.21	0.15	30.0	5.97	5.58
335	340	R	K	44.7	0.75	0.20	0.15	28.7	6.56	6.13
227	206	P	S	35.5	0.75	0.20	0.15	28.5	5.15	4.82
548	437	A	F	12.7	0.76	0.19	0.14	28.1	1.82	1.70
263	214	S	N	35.4	0.76	0.19	0.14	27.9	5.04	4.71
411	387	I	L	33.3	0.75	0.19	0.14	27.0	4.58	4.28
279	221	G	V	9.8	0.75	0.18	0.13	26.1	1.30	1.21
527	432	A	K	31.5	0.76	0.17	0.13	25.7	4.13	3.86
453	399	K	N	37.4	0.75	0.17	0.13	25.2	4.81	4.50
256	211	Y	L	36.9	0.77	0.17	0.13	25.0	4.72	4.41
270	217	D	Y	20.7	0.76	0.17	0.13	24.9	2.63	2.45
521	431	E	F	28.9	0.80	0.16	0.13	24.5	3.61	3.37
455	399	K	R	34.8	0.74	0.17	0.12	23.6	4.18	3.91
438	396	K	T	37.7	0.71	0.17	0.12	23.5	4.51	4.22
284	227	A	G	34.6	0.79	0.15	0.12	23.2	4.09	3.83
386	375	K	L	36.1	0.71	0.16	0.12	22.8	4.20	3.92
357	352	N	R	39.4	0.75	0.15	0.11	21.9	4.40	4.11
454	399	K	Q	36.1	0.72	0.15	0.11	21.5	3.96	3.70
435	396	K	M	35.4	0.73	0.15	0.11	21.4	3.86	3.61
456	399	K	S	35.8	0.70	0.15	0.11	21.3	3.90	3.64
547	434	Q	R	52.1	0.73	0.15	0.11	21.2	5.63	5.26
272	221	G	A	42.2	0.62	0.17	0.10	20.3	4.37	4.09
266	214	S	T	39.2	0.74	0.14	0.10	20.2	4.03	3.76
163	191	A	G	42.6	0.77	0.13	0.10	20.1	4.36	4.08
224	206	P	K	21.9	0.74	0.14	0.10	19.9	2.23	2.08
342	342	A	V	40.6	0.78	0.13	0.10	19.7	4.08	3.82
443	398	E	D	38.7	0.72	0.14	0.10	19.6	3.87	3.62
544	434	Q	H	51.9	0.78	0.13	0.10	19.5	5.16	4.82
180	193	G	Q	23.9	0.77	0.13	0.10	19.3	2.35	2.20
257	211	Y	S	18.9	0.79	0.12	0.10	19.1	1.83	1.71
158	190	G	T	28.0	0.75	0.13	0.10	18.6	2.66	2.48
434	396	K	L	40.5	0.57	0.17	0.09	18.6	3.85	3.60
530	432	A	S	34.6	0.72	0.13	0.09	18.6	3.28	3.06
21	56	A	S	41.9	0.78	0.12	0.09	18.5	3.97	3.71
498	423	I	W	36.7	0.48	0.19	0.09	18.0	3.37	3.15
402	383	F	M	45.9	0.70	0.13	0.09	17.5	4.11	3.84
225	206	P	L	18.3	0.76	0.12	0.09	17.3	1.62	1.51
401	383	F	L	47.2	0.66	0.13	0.08	16.3	3.92	3.67
339	342	A	M	52.3	0.73	0.11	0.08	16.0	4.28	4.00
25	125	R	L	37.3	0.76	0.11	0.08	16.0	3.04	2.84
481	413	K	A	39.0	0.59	0.13	0.08	15.5	3.09	2.88
265	214	S	R	25.4	0.75	0.10	0.08	14.9	1.94	1.81

546	434	Q	L	55.5	0.71	0.11	0.08	14.8	4.20	3.93
7	53	L	F	47.0	0.75	0.10	0.08	14.8	3.54	3.31
348	349	A	Q	42.6	0.76	0.10	0.07	14.7	3.19	2.98
545	434	Q	K	58.5	0.70	0.11	0.07	14.5	4.33	4.05
442	398	E	A	42.8	0.72	0.10	0.07	14.4	3.14	2.93
337	342	A	C	71.5	0.71	0.10	0.07	14.0	5.09	4.76
397	381	P	N	55.8	0.74	0.10	0.07	13.7	3.91	3.66
387	375	K	R	71.8	0.60	0.10	0.06	12.1	4.43	4.14
385	373	F	V	49.2	0.76	0.08	0.06	12.0	3.01	2.81
347	349	A	E	47.9	0.76	0.08	0.06	11.6	2.84	2.65
516	429	S	G	50.2	0.74	0.08	0.06	11.6	2.97	2.78
517	429	S	T	53.7	0.72	0.08	0.06	11.5	3.16	2.95
262	214	S	M	22.6	0.74	0.07	0.06	10.9	1.25	1.17
492	418	Q	E	83.8	0.69	0.05	0.04	7.1	3.03	2.83

Megatable 3

SEQ ID	Variant Name	ON ratio	OFF ratio	ON x OFF	kcat	ONxOFF x kcat
1	Mz wt	0.213	0.033	0.007	97.4	0.68
2	9070	0.744	0.116	0.086	40.0	3.45
3	9070Q366E	0.760	0.273	0.208	20.1	4.18
4	P056028-A-01	0.697	0.249	0.173	39.1	6.78
5	P056028-A-02	0.680	0.387	0.263	38.6	10.15
6	P056028-F-05	0.700	0.395	0.276	27.4	7.57
7	P056028-G-01	0.742	0.320	0.238	32.2	7.65
8	P056028-G-01R	0.719	0.280	0.201	70.8	14.24
9	P056028-B-02	0.800	0.469	0.375	28.2	10.58
10	P056028-F-01	0.718	0.304	0.218	31.3	6.82
11	P056028-D-08	0.681	0.361	0.246	27.5	6.77
12	P056028-E-04	0.740	0.360	0.267	19.2	5.13
13	P056028-A-06	0.760	0.281	0.214	27.0	5.77
14	P056028-F-02	0.730	0.430	0.314	28.2	8.84
15	P056028-C-01	0.742	0.310	0.230	25.9	5.95
16	P056028-E-02	0.761	0.326	0.248	23.6	5.86
17	P056028-C-07	0.714	0.382	0.273	21.2	5.80
18	P056028-A-03	0.748	0.243	0.181	24.0	4.36
19	P056028-C-05	0.715	0.375	0.268	21.7	5.80
20	P056028-A-08	0.669	0.324	0.217	26.2	5.68
21	P056028-D-03	0.770	0.453	0.349	19.6	6.83
22	P056028-B-09	0.648	0.187	0.121	34.5	4.19
23	P056028-A-10	0.707	0.367	0.260	16.8	4.35
24	P056028-A-11	0.706	0.366	0.258	8.6	2.21
25	P056028-A-04	0.724	0.133	0.096	11.1	1.06
26	P056028-A-05	0.707	0.280	0.198	17.7	3.50
27	P056028-A-07	0.726	0.329	0.239	19.5	4.65
28	P056028-A-09	0.692	0.339	0.235	13.9	3.26
29	P056028-B-01	0.745	0.309	0.230	18.6	4.28
30	P056028-B-10	0.703	0.327	0.230	17.9	4.12
31	P056028-B-03	0.732	0.353	0.259	17.9	4.63
32	P056028-B-04	0.718	0.273	0.196	16.5	3.24
33	P056028-B-05	0.734	0.287	0.211	18.3	3.85
34	P056028-B-06	0.713	0.353	0.252	20.0	5.03
35	P056028-B-07	0.788	0.389	0.307	9.2	2.83
36	P056028-B-08	0.681	0.325	0.221	15.5	3.44
37	P056028-C-10	0.699	0.356	0.249	14.6	3.63
38	P056028-C-11	0.731	0.293	0.214	12.3	2.64
39	P056028-C-02	0.717	0.379	0.272	18.8	5.11

40	P056028-C-03	0.739	0.356	0.263	13.7	3.62
41	P056028-C-04	0.727	0.322	0.234	22.7	5.32
42	P056028-C-06	0.742	0.351	0.261	10.6	2.77
43	P056028-C-08	0.712	0.381	0.271	13.5	3.65
44	P056028-C-09	0.706	0.314	0.222	17.0	3.76
45	P056028-D-01	0.728	0.348	0.254	21.3	5.39
46	P056028-D-10	0.670	0.253	0.170	18.1	3.07
47	P056028-D-11	0.719	0.261	0.188	17.1	3.21
48	P056028-D-02	0.730	0.299	0.218	24.3	5.31
49	P056028-D-04	0.696	0.197	0.137	12.4	1.71
50	P056028-D-05	0.730	0.508	0.371	12.6	4.66
51	P056028-D-06	0.731	0.424	0.310	9.9	3.07
52	P056028-D-07	0.775	0.370	0.286	9.1	2.60
53	P056028-D-09	0.652	0.371	0.242	13.0	3.16
54	P056028-E-10	0.702	0.339	0.238	11.5	2.73
55	P056028-E-11	0.721	0.304	0.219	16.9	3.70
56	P056028-E-03	0.735	0.412	0.303	16.9	5.10
57	P056028-E-05	0.704	0.325	0.229	15.2	3.48
58	P056028-E-06	0.346	0.186	0.064	28.2	1.76
59	P056028-E-07	0.585	0.262	0.153	20.6	3.08
60	P056028-E-08	0.685	0.410	0.281	12.6	3.55
61	P056028-E-09	0.676	0.308	0.208	14.9	3.10
62	P056028-F-10	0.726	0.291	0.211	13.7	2.89
63	P056028-F-11	0.706	0.322	0.228	22.1	5.04
64	P056028-F-03	0.738	0.491	0.363	13.0	4.73
65	P056028-F-04	0.660	0.245	0.161	16.7	2.70
66	P056028-F-06	0.739	0.305	0.225	14.5	3.25
67	P056028-F-07	0.735	0.322	0.236	21.4	5.04
68	P056028-F-08	0.705	0.377	0.266	12.1	3.23
69	P056028-F-09	0.700	0.360	0.252	17.1	4.30
70	P056028-G-10	0.607	0.259	0.157	29.3	4.61
71	P056028-G-02	0.761	0.304	0.231	23.0	5.30
72	P056028-G-04	0.752	0.248	0.186	13.6	2.54
73	P056028-G-05	0.739	0.361	0.267	16.7	4.47
74	P056028-G-06	0.725	0.382	0.277	12.0	3.30
75	P056028-G-07	0.702	0.304	0.213	15.3	3.23
76	P056028-G-08	0.737	0.379	0.279	11.8	3.29
77	P056028-G-09	0.716	0.385	0.275	15.8	4.37

CLAIMS**THAT WHICH IS CLAIMED:**

1. A recombinant polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity comprises:

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

Xaa	Xaa	Xaa	Xaa	Xaa	Phe	Xaa	His	Ile	Xaa	Xaa	Xaa	Val	Pro	Glu
				230					235					240
Leu	Xaa	Pro	Xaa	Xaa	Xaa	Tyr	Xaa	Xaa	Gly	Phe	Thr	Xaa	Phe	Xaa
				245					250					255
Xaa	Xaa	Ala	Glu	Phe	Thr	Thr	Glu	Asp	Val	Gly	Thr	Thr	Glu	Ser
				260					265					270
Gly	Leu	Asn	Ser	Met	Ala	Leu	Ala	Asn	Asn	Ser	Glu	Asn	Val	Leu
				275					280					285
Leu	Pro	Xaa	Asn	Glu	Pro	Val	His	Gly	Thr	Lys	Arg	Arg	Ser	Gln
				290					295					300
Ile	Gln	Thr	Phe	Leu	Asp	Xaa	His	Gly	Gly	Pro	Gly	Val	Gln	His
				305					310					315
Ile	Ala	Leu	Ala	Ser	Asp	Asp	Val	Leu	Arg	Thr	Leu	Arg	Glu	Met
				320					325					330
Arg	Ala	Arg	Ser	Ala	Met	Gly	Gly	Phe	Glu	Phe	Leu	Pro	Pro	Pro
				335					340					345
Xaa	Xaa	Asp	Tyr	Tyr	Asp	Gly	Val	Xaa	Xaa	Cys	Xaa	Xaa	Asp	Xaa
				350					355					360
Leu	Xaa	Xaa	Xaa	Gln	Xaa	Xaa	Xaa	Cys	Gln	Xaa	Xaa	Xaa	Val	Xaa
				365					370					375
Val	Asp	Arg	Xaa	Xaa	Xaa	Gly	Xaa	Xaa	Leu	Gln	Xaa	Xaa	Thr	Xaa
				380					385					390
Xaa	Xaa	Gly	Xaa	Arg	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
				395					400					405
Xaa	Xaa	Cys	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Glu	Tyr	Gln	Lys
				410					415					420
Xaa	Xaa	Xaa	Gly	Gly	Xaa	Gly	Xaa	Gly	Xaa	Xaa	Xaa	Xaa	Leu	Phe
				425					430					435
Lys	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Ala
				440										

Ala Xaa Xaa Xaa Thr Ala Gln Gly Ser (SEQ ID NO:80);

wherein

Xaa at position 52 is Glu, Asp, Gly, His, Arg, Ser or Thr;

Xaa at position 53 is Leu, Phe, Ile or Met;

Xaa at position 54 is Trp, Cys, Leu or Gln;
Xaa at position 55 is Cys, Ala, Gly, Thr or Val;
Xaa at position 56 is Ala, His, Leu, Asn, Gln, Ser or Thr;
Xaa at position 58 is Ala, Gly or Thr;
Xaa at position 125 is Arg or Leu;
Xaa at position 147 is Ala or Ser;
Xaa at position 148 is Phe, Trp or Tyr;
Xaa at position 149 is Arg, Ala, Ile, Lys, Met, Pro, Ser, Thr, Val or Trp;
Xaa at position 152 is Val or Glu;
Xaa at position 153 is Ala, Phe, Ile, Lys, Leu, Gln, Arg, Thr or Val;
Xaa at position 154 is Ala, Cys, Gly, Asn, Arg or Thr;
Xaa at position 157 is Arg, His, Asn, Thr or Val;
Xaa at position 158 is Pro, Ala, Glu, Gly or Lys;
Xaa at position 159 is Ala, Cys, Met, Ser, Thr or Val;
Xaa at position 160 is Phe, Leu, Met or Tyr;
Xaa at position 161 is Gly, Ala, Glu, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Trp or Tyr;
Xaa at position 162 is Pro or Thr;
Xaa at position 163 is Val, Ala, Cys, Met or Thr;
Xaa at position 164 is Asp, Ala, Glu, His, Ser or Thr;
Xaa at position 165 is Leu, Cys, Met or Val;
Xaa at position 166 is Gly, Ala or Pro;
Xaa at position 167 is Arg or Val;
Xaa at position 169 is Phe, Ala, His, Trp or Tyr;
Xaa at position 170 is Arg, Cys, Gly, Ile, Lys, Leu, Met, Pro, Gln, Ser, Thr, Val or Trp;
Xaa at position 171 is Leu, Phe, Ile, Met or Val;
Xaa at position 172 is Ala, Pro, Arg or Ser;
Xaa at position 173 is Glu or Pro;
Xaa at position 174 is Val, Cys or Ile;
Xaa at position 175 is Glu, Asp, Val or Trp;
Xaa at position 176 is Leu, Ala or Met;
Xaa at position 181 is Val, Phe, Ile, Met or Asn;
Xaa at position 182 is Leu or Met;

Xaa at position 187 is Tyr, Cys, Glu, Gly, His, Met or Val;
Xaa at position 188 is Pro, Asp, Gly or Gln;
Xaa at position 189 is Asp, Glu, Pro, Ser or Thr;
Xaa at position 190 is Gly, Ala, Cys, Asp, Glu, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val or Trp;
Xaa at position 191 is Ala, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Arg, Ser, Thr or Val;
Xaa at position 192 is Ala or Val;
Xaa at position 193 is Gly, Ala, His, Ile, Leu, Pro, Gln, Arg, Ser, Thr or Val;
Xaa at position 194 is Glu, Asp or Leu;
Xaa at position 195 is Pro, Ala, Cys, Asp, Glu, Gln, Ser, Thr or Val;
Xaa at position 196 is Phe or Trp;
Xaa at position 200 is Phe or Cys;
Xaa at position 202 is Gly, Ala, Glu, Lys, Leu, Thr, Val or Tyr;
Xaa at position 204 is Ala, Cys, Asp, Glu, Gly, His, Lys, Leu, Met, Asn, Gln, Thr or Val;
Xaa at position 205 is Ser, Asp, Gly, Leu, Gln, Thr or Val;
Xaa at position 206 is Pro, Cys, Asp, Lys, Leu, Gln, Ser or Thr;
Xaa at position 207 is Gly, Gln, Ser or Val;
Xaa at position 208 is Ala, Gly, His, Gln, Arg or Trp;
Xaa at position 209 is Ala, Cys, Asp, Glu, Gly, Ile, Lys, Leu, Met, Pro, Gln, Ser, Thr or Trp;
Xaa at position 210 is Asp, Glu, Gly, Ser or Thr;
Xaa at position 211 is Tyr, Cys, Phe, Leu, Ser or Trp;
Xaa at position 212 is Gly or Lys;
Xaa at position 213 is Leu or Trp;
Xaa at position 214 is Ser, Lys, Met, Asn, Gln, Arg or Thr;
Xaa at position 215 is Arg, Ala or Gly;
Xaa at position 217 is Asp, Gln or Tyr;
Xaa at position 220 is Val or Thr;
Xaa at position 221 is Gly, Ala, Phe, His, Ile, Leu, Met, Thr or Val;
Xaa at position 222 is Asn, Gly or Val;
Xaa at position 227 is Ala, Asp, Glu, Gly, Ile, Lys, Leu, Met, Asn, Gln, Arg, Ser, Thr or Val;

Xaa at position 229 is Ala or Thr;
Xaa at position 230 is Ala, Arg or Val;
Xaa at position 231 is Ala, Cys, His, Leu, Ser or Thr;
Xaa at position 233 is Met or Val;
Xaa at position 234 is Ala, Cys, Glu, Gly, Lys, Leu, Met, Gln, Ser or Val;
Xaa at position 238 is Gly or Ser;
Xaa at position 240 is His or Arg;
Xaa at position 241 is Glu, Ala, Asp, Gly, Asn, Pro, Arg, Ser or Thr;
Xaa at position 242 is Phe, Ala, Asp or Ser;
Xaa at position 273 is Leu or Val;
Xaa at position 292 is His or Asn;
Xaa at position 331 is Leu, Asp, Gly, Asn or Arg;
Xaa at position 332 is Ser or Ala;
Xaa at position 339 is Arg or Lys;
Xaa at position 340 is Arg, Glu, Lys or Asn;
Xaa at position 342 is Ala, Cys, Leu, Met, Asn, Arg or Val;
Xaa at position 343 is Gly or Arg;
Xaa at position 345 is Val or Ile;
Xaa at position 347 is Thr or Ser;
Xaa at position 348 is Glu or Tyr;
Xaa at position 349 is Ala, Glu, Gln, Arg or Ser;
Xaa at position 351 is Ile, Cys or Val;
Xaa at position 352 is Asn, Glu, Lys, Leu, Gln or Arg;
Xaa at position 353 is Glu, Leu, Met, Ser or Thr;
Xaa at position 356 is Glu, Lys or Arg;
Xaa at position 357 is Leu or Tyr;
Xaa at position 358 is Gly, Glu or Arg;
Xaa at position 360 is Met, Leu or Thr;
Xaa at position 364 is Asp, Gly, Ser or Val;
Xaa at position 365 is Asp or Ala;
Xaa at position 366 is Gln or Glu;
Xaa at position 368 is Val or Leu;
Xaa at position 369 is Leu, Met or Val;
Xaa at position 372 is Ile, Ala, Lys, Gln, Ser or Thr;

Xaa at position 373 is Phe, Gly, Leu, Arg or Val;
Xaa at position 375 is Lys, Leu or Arg;
Xaa at position 376 is Pro, Cys, Gly, Ser, Val or Trp;
Xaa at position 377 is Val, Glu, Gly or Leu;
Xaa at position 379 is Asp or Lys;
Xaa at position 381 is Pro or Asn;
Xaa at position 382 is Thr, Ala, Phe or Ser;
Xaa at position 383 is Phe, Leu or Met;
Xaa at position 384 is Phe, Trp or Tyr;
Xaa at position 385 is Leu, Ile or Val;
Xaa at position 386 is Glu, Cys, Ile or Val;
Xaa at position 387 is Ile, Gly or Leu;
Xaa at position 388 is Ile, Leu, Ser or Val;
Xaa at position 389 is Gln, Gly, Lys or Thr;
Xaa at position 390 is Arg or Asn;
Xaa at position 391 is Ile, Leu or Val;
Xaa at position 392 is Gly, Arg or Val;
Xaa at position 394 is Met, Ile, Lys, Leu, Gln, Val or Tyr;
Xaa at position 395 is Glu, Lys, Gln, Ser or Val;
Xaa at position 396 is Lys, Ala, Leu, Met, Gln, Arg, Thr or Val;
Xaa at position 397 is Asp, Gly or Ser;
Xaa at position 398 is Glu, Ala, Asp, Gly, Pro or Ser;
Xaa at position 399 is Lys, Ala, Asp, Gly, His, Ile, Met, Asn, Gln, Arg, Ser, Thr, Val or Tyr;
Xaa at position 400 is Gly, Glu, Lys or Ser;
Xaa at position 401 is Gln, Ala, Glu, Ser or Val;
Xaa at position 406 is Gly or Cys;
Xaa at position 407 is Gly, Ala, Lys, Leu, Arg, Ser or Thr;
Xaa at position 408 is Cys, Gly, Arg, Thr, Val or Trp;
Xaa at position 411 is Phe, Ala or Leu;
Xaa at position 413 is Lys, Ala, Pro, Arg or Ser;
Xaa at position 415 is Asn or Ala;
Xaa at position 416 is Phe, Arg or Val;
Xaa at position 417 is Gly, Gln or Ser;

Xaa at position 418 is Gln, Ala, Cys, Glu, Gly, Leu or Thr;
Xaa at position 422 is Ser or Met;
Xaa at position 423 is Ile, Val or Trp;
Xaa at position 424 is Glu or Gln;
Xaa at position 425 is Asp, Ala, Glu, Gly, Met, Ser or Thr;
Xaa at position 426 is Tyr, Leu or Trp;
Xaa at position 427 is Glu, Ala, Leu or Met;
Xaa at position 428 is Lys, Arg or Val;
Xaa at position 429 is Ser, Cys, Asp, Gly or Thr;
Xaa at position 430 is Leu, Met or Val;
Xaa at position 431 is Glu, Ala, Phe, Gly, Leu or Asn;
Xaa at position 432 is Ala, Asp, Gly, Lys, Leu, Arg, Ser or Val;
Xaa at position 433 is Lys, Glu, His, Leu, Pro, Arg, Ser or Val;
Xaa at position 434 is Gln, Ala, Cys, Asp, Phe, Gly, His, Lys, Leu or Arg;
Xaa at position 437 is Ala, Phe, Lys or Val;
Xaa at position 438 is Ala, Gly or Arg;
Xaa at position 439 is Ala or Arg;

wherein one or more amino acid(s) designated by Xaa in SEQ ID NO:80 is an amino acid different from the corresponding amino acid of SEQ ID NO:1; and wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity has improved insensitivity to an HPPD inhibitor compared to the polypeptide of SEQ ID NO:1.

2. The polypeptide of claim 1, wherein the polypeptide further comprises substitution of one or more conservative amino acids, insertion of one or more amino acids, deletion of one or more amino acids, and combinations thereof.
3. The polypeptide of claim 1, wherein the polypeptide has an ON rate ratio of at least about 0.5; wherein the ON rate ratio is the ratio of the reaction rate with herbicidal inhibitor to the reaction rate without herbicidal inhibitor; and wherein the reaction rates are determined in an *in vitro* assay.
4. The polypeptide of claim 3, wherein the herbicidal inhibitor is mesotrione or tembrione.

5. The polypeptide of claim 4, wherein *in vitro* assay is carried out in the presence of 60 or 120 nM of the 4-hydroxyphenylpyruvate dioxygenase protein; and 100 μ M 4-hydroxyphenylpyruvate.
6. The polypeptide of claim 3, wherein the polypeptide has an OFF rate ratio of at least about 0.3; wherein the OFF rate ratio is the ratio of the steady state rate in the presence of inhibitor to the initial reaction rate in the absence of inhibitor; and wherein the reaction rates are determined in an *in vitro* assay.
7. The polypeptide of claim 1, wherein the polypeptide has a fitness parameter of about 2 to about 200 $\text{min}^{-1}\mu\text{M}^{-1}$; and wherein

$$\text{Fitness Parameter} = \frac{k_{cat}}{K_m} \times (\text{ON rate ratio} \times \text{OFF rate ratio}).$$

8. A nucleic acid construct comprising a polynucleotide sequence encoding a polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide encoded is the polypeptide of claim 1.
9. The nucleic acid construct of claim 8, further comprising a promoter operably linked to the polynucleotide sequence.
10. A plant cell comprising a nucleic acid construct comprising a polynucleotide sequence encoding a polypeptide having 4-hydroxyphenylpyruvate dioxygenase (HPPD) activity; wherein the polypeptide encoded is the polypeptide of claim 1.
11. The plant cell of claim 10, wherein the nucleic acid construct further comprises a promoter operably linked to the polynucleotide sequence.
12. The plant cell of claim 10, wherein the plant cell exhibits has an improved insensitivity to an HPPD inhibitor compared to a wild type plant cell of the same species, strain or cultivar.
13. The plant cell of claim 10, wherein the polypeptide having 4-hydroxyphenylpyruvate dioxygenase activity is present in an amount from about 1000 ppm to about 5000 ppm.

14. The plant cell of claim 10, wherein the plant cell further comprises at least one additional polypeptide imparting tolerance to an additional herbicide.
15. The plant cell of claim 14, wherein the at least one polypeptide imparting tolerance to an additional herbicide comprises: (a) a sulfonylurea-tolerant acetolactate synthase; (b) an imidazolinone-tolerant acetolactate synthase; (c) a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase; (d) a glyphosate-tolerant glyphosate oxidoreductase; (e) a glyphosate-N-acetyltransferase; (f) a phosphinothricin acetyl transferase; (g) a protoporphyrinogen oxidase; (h) an auxin enzyme; (i) a P450 polypeptide; or, (j) an acetyl coenzyme A carboxylase (ACCase).
16. The plant cell of claim 14, wherein the at least one polypeptide imparting tolerance to an additional herbicide comprises a high resistance allele of acetolactate synthase (HRA) and/or a glyphosate-N-acetyltransferase polypeptide.
17. The plant cell of claim 14, wherein the plant cell further comprises at least one additional polypeptide imparting tolerance to an HPPD herbicide.
18. A plant comprising the plant cell of any of claim 10.
19. A transgenic seed produced by the plant of claim 18.
20. A method of producing a 4-hydroxyphenylpyruvate dioxygenase (HPPD) herbicide tolerant plant cell comprising transforming a plant cell with a polynucleotide encoding the polypeptide of any of claim 1 or the nucleic acid construct of claim 8.

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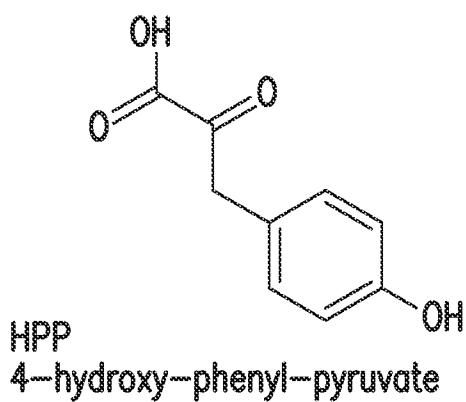
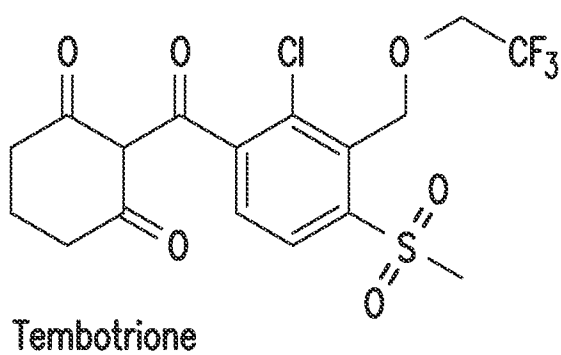
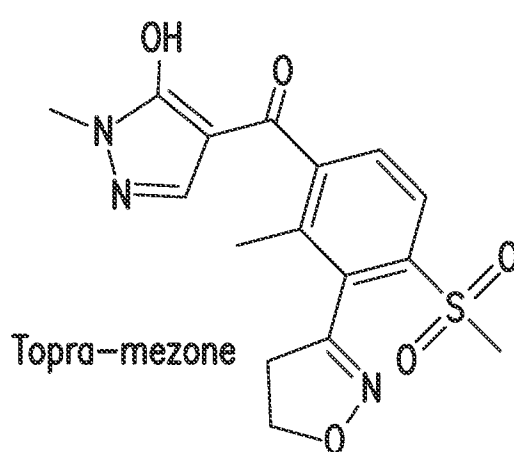
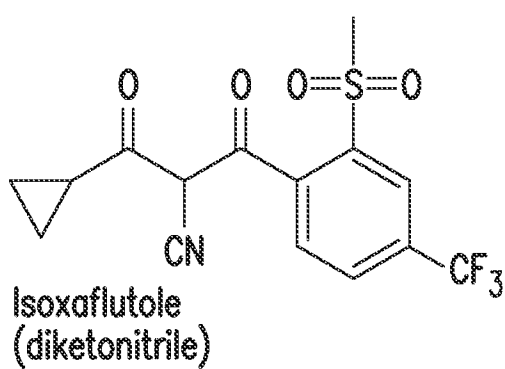
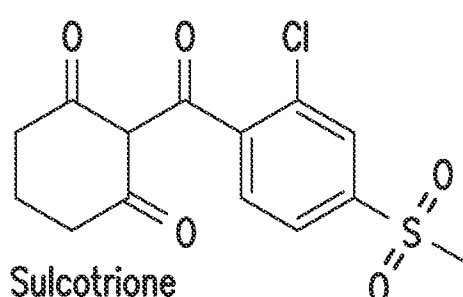
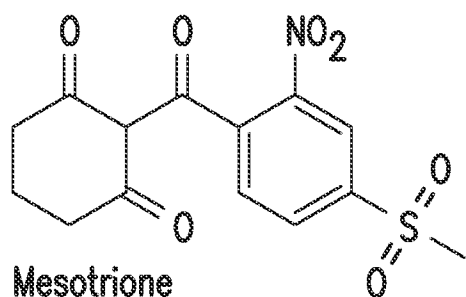


FIG. 1

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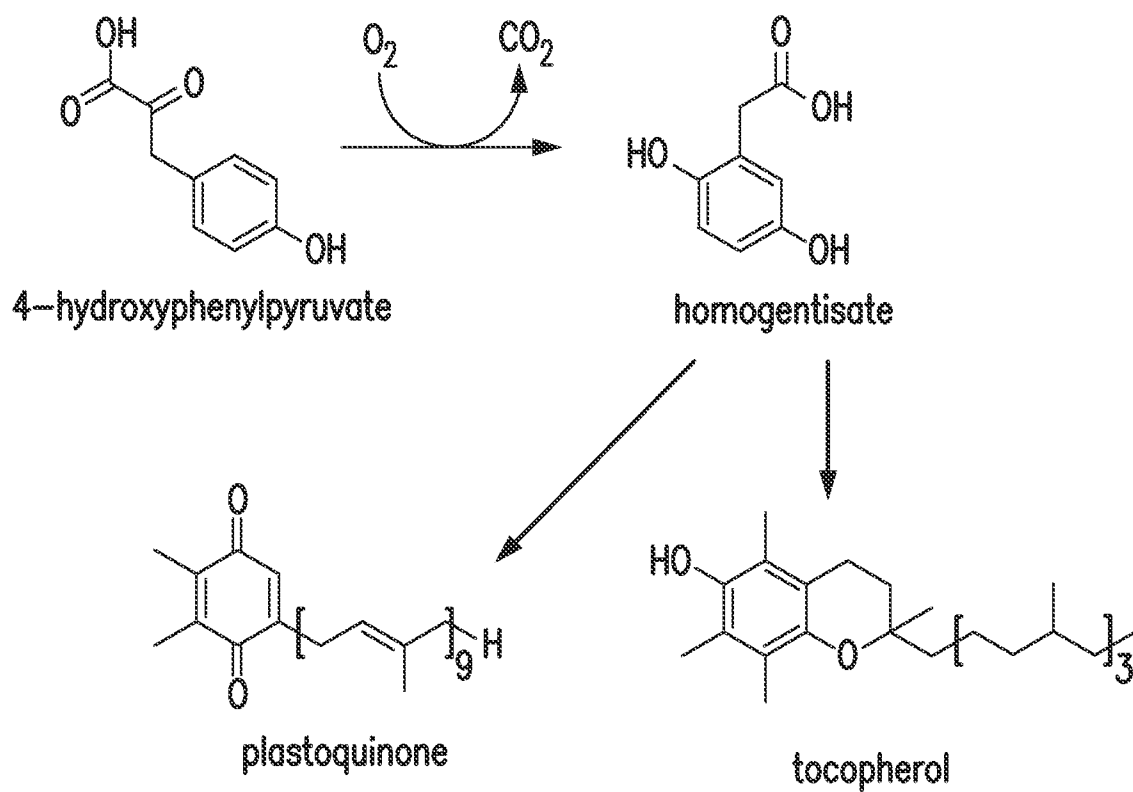


FIG. 2A



FIG. 2B

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MGPTPTAAAAGAAVAAASAAEQAAFRLVGHRNFVRFNPRSDR
FHTLAFHHVELWCADAASAAGRFSFGLGAPLAARSDLSTGNS
AHASLLLRSGSLSFLFTAPYAHGADAATAALPSFSAAAARRF
AADHGLAVRAVALRVADAEDAFRASVAAGARPAFGPVDLGRG
FRLAEVELYGDVVLRYVSYPDGAAGEPFLPGFEGVASPGAAD
YGLSRFDHIVGNVPELAPAAAYFAGFTGFHEFAEFTTEDVGT
AESGLNSMVLANNSENVLLPLNEPVHGTKRRSQIQTFLDHHG
GPGVQHMALASDDVLRRTLREMQARSAMGGFEFMAPPTSDDYYD
GVERRRAGDVLTEAQIKECQELGVLVDRDDQGVLLQIFTKPVG
DRPTLFLEIIQRIGCMEKDEKQGEYQKGGCGGFGKGNFSQLF
KSIEDYEKSLEAKQAAAAAAAAAQS (SEQ ID NO: 1)

FIG. 3A

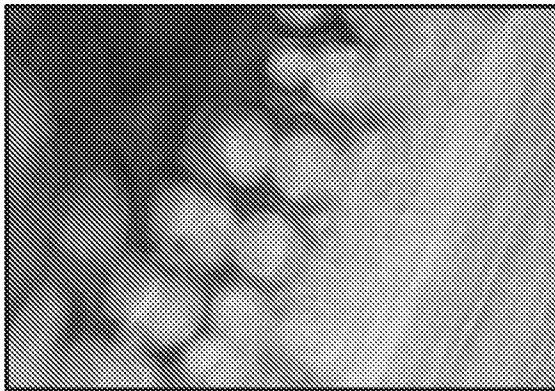


FIG. 3B

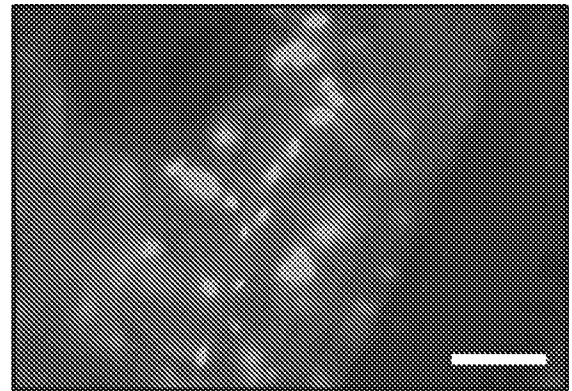


FIG. 3C

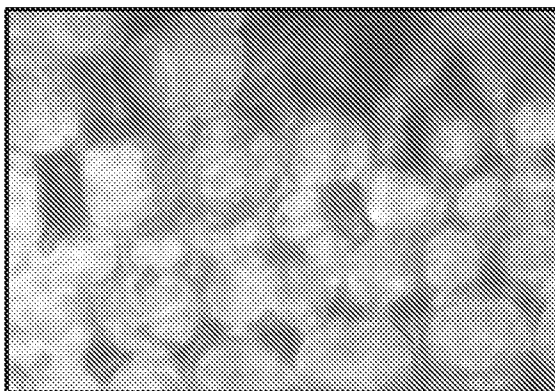


FIG. 3D

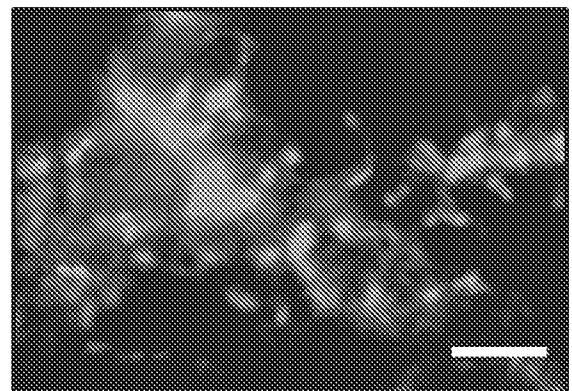


FIG. 3E

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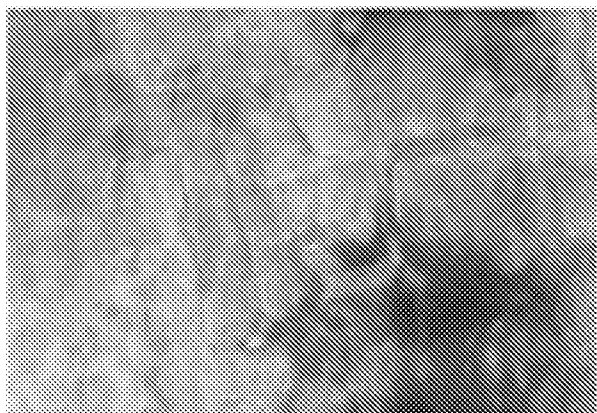


FIG. 3F

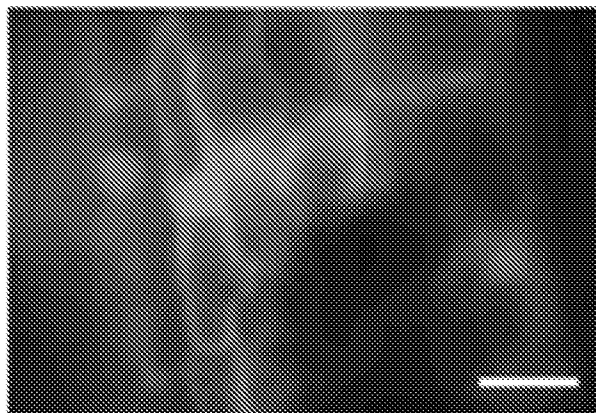


FIG. 3G

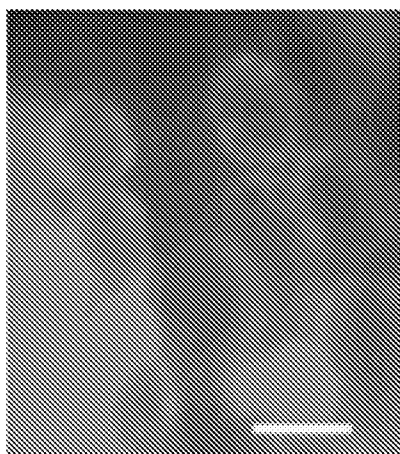


FIG. 3H

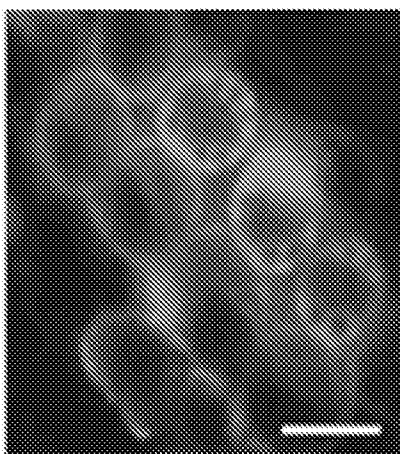


FIG. 3I

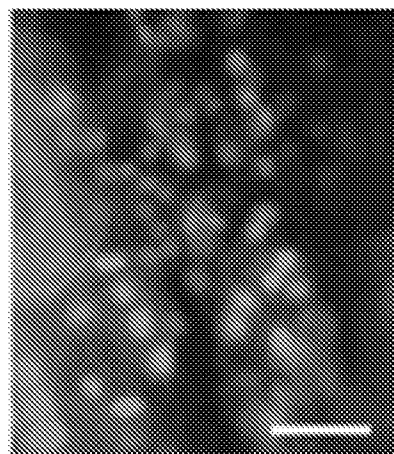


FIG. 3J

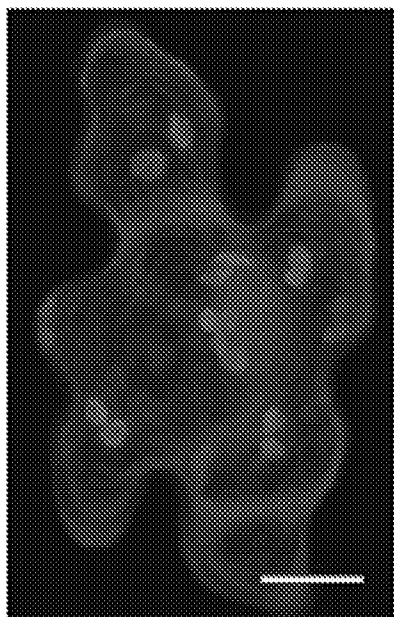


FIG. 3K

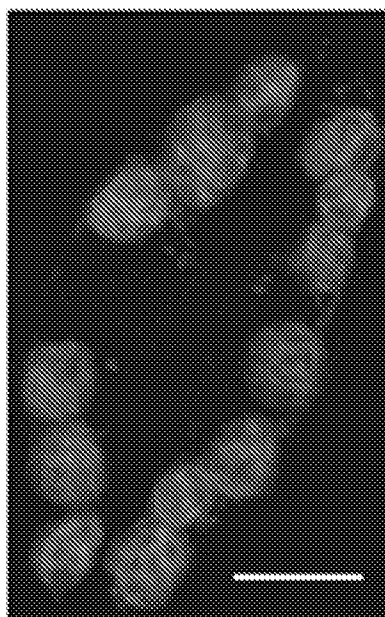


FIG. 3L

	1	10	20	30	40	53	
Avena sativa HPPD WO0246387 (1)	MPPTPATA	TGA--	AAAAVTPEHA	AR--	SFPPRVVRVNPRSDRFPVLS	FHHVE	
Hordeum vulgare HPPD 048604.1 (1)	MPPTPTT	PAA--	GAAAAVTPEHA	RP--	HRMVRFNPRSDRFHTLS	FHHVE	
Triticum aestivum HPPD AAZ67144.1 (1)	MPPTPTT	PAA	TGAGAAA	VTPEHA	RP--	RRMVRFNPRSDRFHTLS	FHHVE
Oryza sativa HPPD BAD26248.1 (1)	MPPTPTPT	AT	TGAVSAAA	AAAGENAG	FRLVGHRRFVRANPRSDRFQAL	LA	FHHVE
Sorghum bicolor HPPD XP_002453359 (1)	MPPTPTT	AAA	TGAAVAAA	SA--	EQAA	FRLVGHRRFVRVNPRSDRFHTLA	FHHVE
Zea mays_WO1997049816Seq#11 (1)	MPPTPT	AAA	GAAVAAA	SAA--	EQAA	FRLVGHRRFVRFNPRSDRFHTLA	FHHVE
	(1)	MPPTPTT	AAA	TGAGAAAA	VTPEHA	ARFRLVGHRRFVRFNPRSDRFHTLA	FHHVE

FIG. 3M

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1 MPMYTPSLSA PSSNHIQPSV TLPLYITTTK LNLKQQHHTT PMPIPMCNEI
51 QAQAQAQAQP GFKLVGFKNF VRTNPKSDRF QVNRFHHEF WCTDATNASR
101 RFSWGLGMPI VAKSDLSTGN QIHASYLLRS GDLSFLFSAP YSPSLSAGSS
151 AASSASIPSF DAATCLAFAA KHGFGVRAIA LEVADAEAAF SASVAKGAEP
201 ASPPVLVDDR TGFAEVRLYG DVVLRYVSYK DAAPQAPHAD PSRWFLPGFE
251 AAASSSSFPE LDYGIRRLDH AVGNVPELAP AVRYLKGFSG FHEFAEFTAE
301 DVGTSSEGLN SVVLANNSET VLLPLNEPVY GTKRKSQIET YLEHNEGAGV
351 QHLALVTHDI FTTLREMRKR SFLGGFEFMP SPPPTYANL HNRAADVLT
401 DQIKQCEELG ILVDRDDQGT LLQIFTKPVG DRPTIFIEII QRIGCMVEDE
451 EGKVYQKGAC GGFGKGNFSE LFKSIEEYEK TLEAKRTA

FIG. 4A

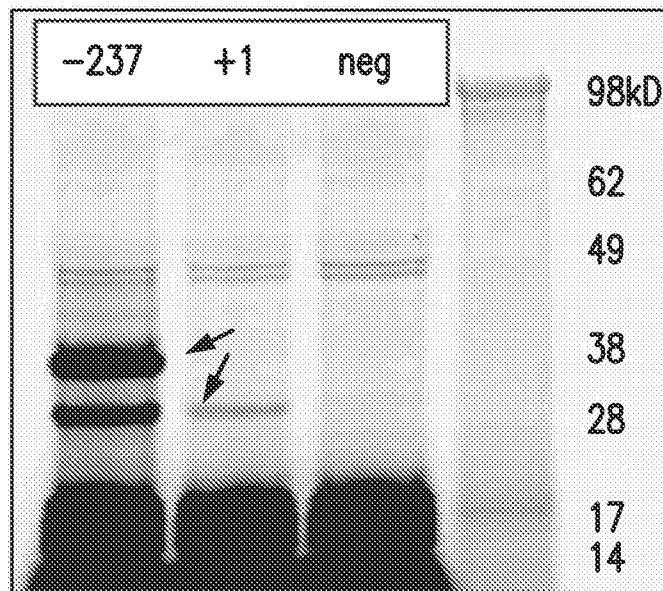


FIG. 4B

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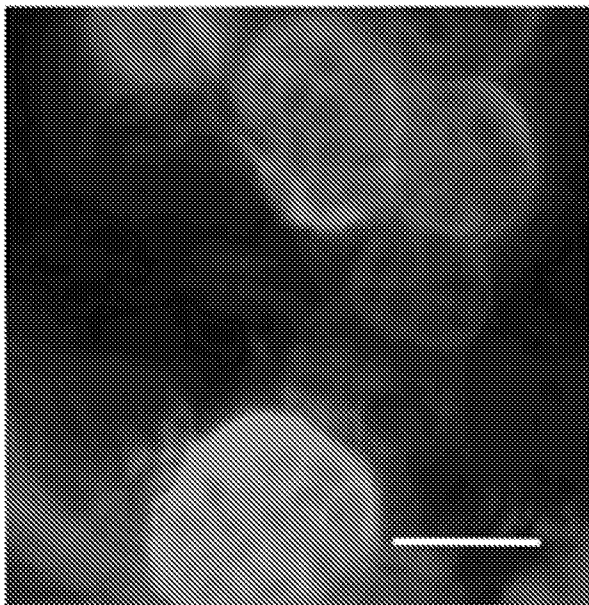


FIG. 4C

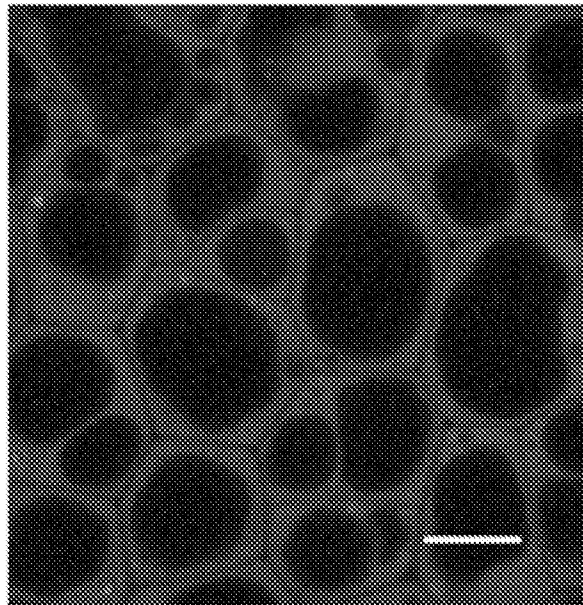


FIG. 4D

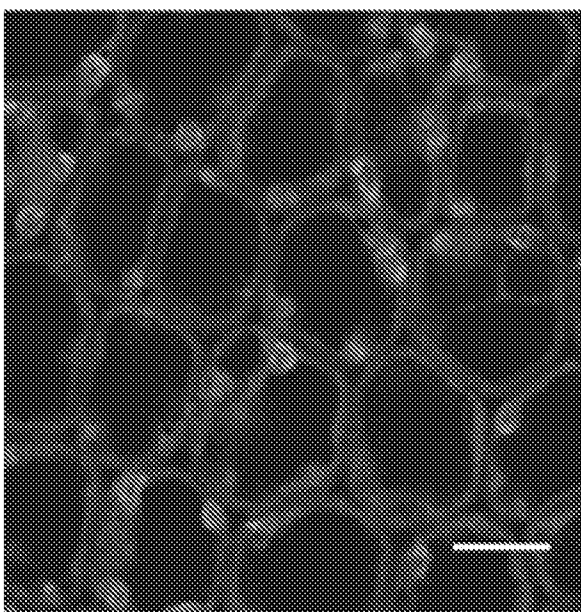


FIG. 4E

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Gm MPMYTPSLSAPSSNHIQPSVTLPLYITTTKLNLKQQHHTTPMPIPMCNEIQAQAQAQAQP
Zm -----MPPTPTAAAAGAAVAASAAEQ
* * *

Gm GFKLVGFKNFVRTNPKSDRFQVNRFFHHIEFWCTDATNASRRFSWGLGMPIVAKSDLSTGN
Zm AFRLVGHHRNFVRFNPRSDRFHTLAFHHVELWCADAASAAGRFSFGLGAPLAARSDLSTGN
* * * * * : * * * * *

Gm QIHASYLLRSGDLSFLFSAPYSPSLSAGSSAASSASIPSFDAATCLAFAAKHGFGVRAIA
Zm SAHASLLLRSGSLSFLFTAPY-----AHGADAATAALPSFSAAAARRFAADHGLAVRAVA
* * * * * * * * * *

Gm LEVADAEAAFSASVAKGAEPASPPVLVDDRTGFAEVRLYGDVVLRYVSYKDAAPQAPHAD
Zm LRVADAEDA FRASVAAGARPAFGPVDLGRGFRLAEVELYGDVVLRYVSYPDGAAGEP---
* * * * * * * * * *

Gm PSRWFLPGFEAAASSSSFPELDYGIRRLDHAVGNVPELAPAVRYLKGFSGFHEFAEFTAE
Zm ----FLPGFEGVASPG---AADYGLSRFDHIVGNVPELAPAAAYFAGFTGFHEFAEFTTE
* * * * * * * * * *

Gm DVGTSESGLNSVVLANNSETVLLPLNEPVYGTKRKSQIETYLEHNEGAGVQHLALVTHDI
Zm DVGTAESGLNSMVLANNSENVLLPLNEPVHGTRRSQIQTFLDHHGGPGVQHMALASDDV
* * * * * * * * * *

Gm FTTLREMRKRSFLGGFEFMPSPPTYYANLHNRAADVLTVDQIKQCEELGILVDRDDQGT
Zm LRTLREMQARSAMGGFEFMAPPTSDYDGVRRRAGDVLTEAQIKECQELGVLVDRDDQGV
* * * * * * * * * *

Gm LLQIFTKPVGDRPTIFIEIIQRIGCMVEDEEGKVYQKGACGGFGKGNFSELFKSIEEYK
LLQIFTKPVGDRPTLFLEIIQRIGCMKDEKQEQYQKGGCGGFGKGNFSQLFKSIEDYEK
* * * * * * * * * *

Gm TLEAKRTA-----
Zm SLEAKQAAAAAAQGS
* * *

FIG. 4F

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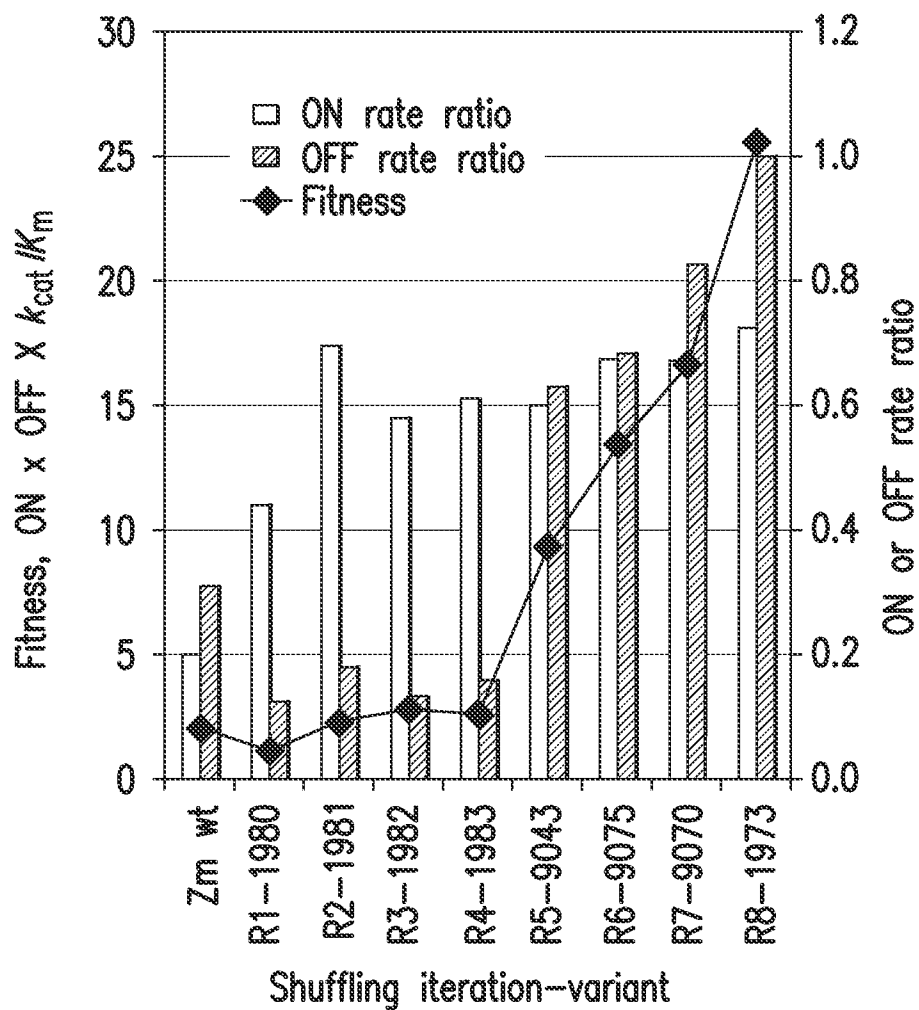


FIG. 5

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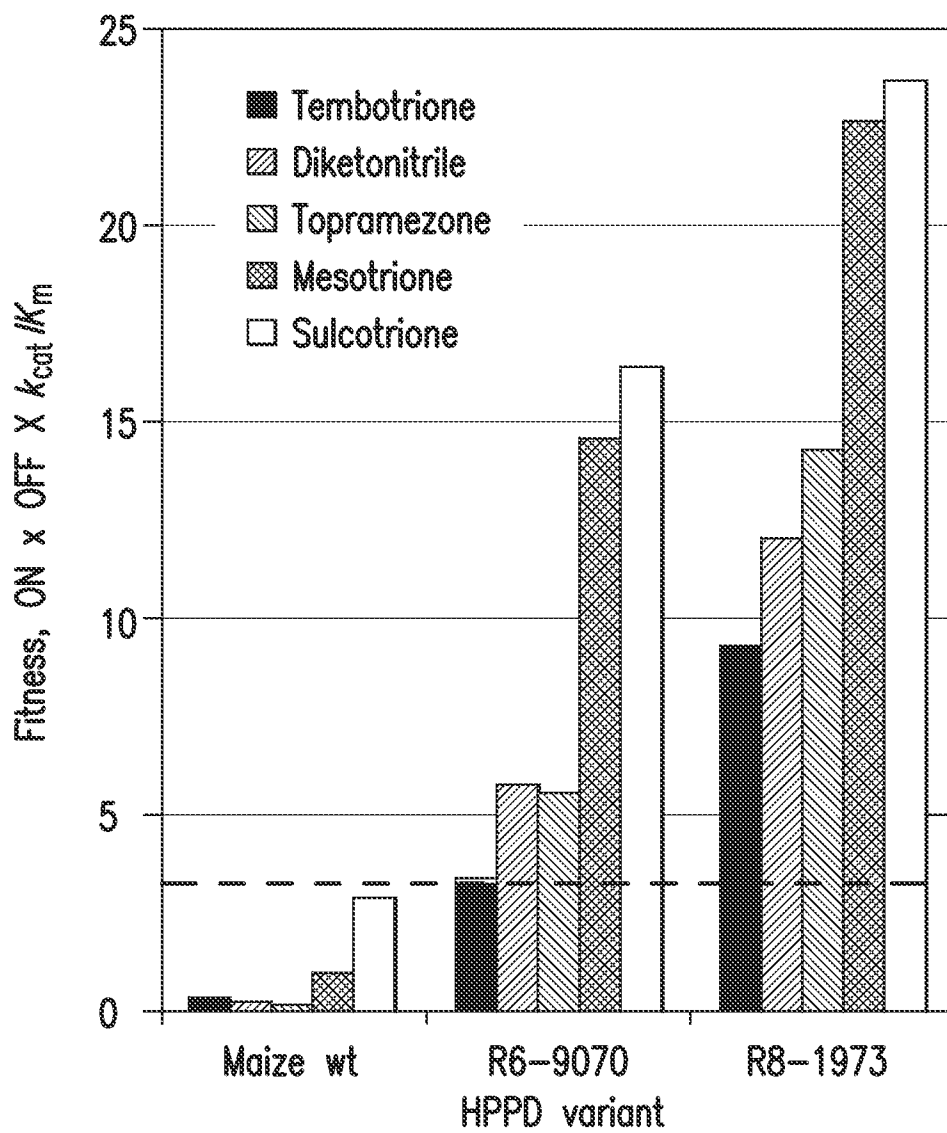


FIG. 6

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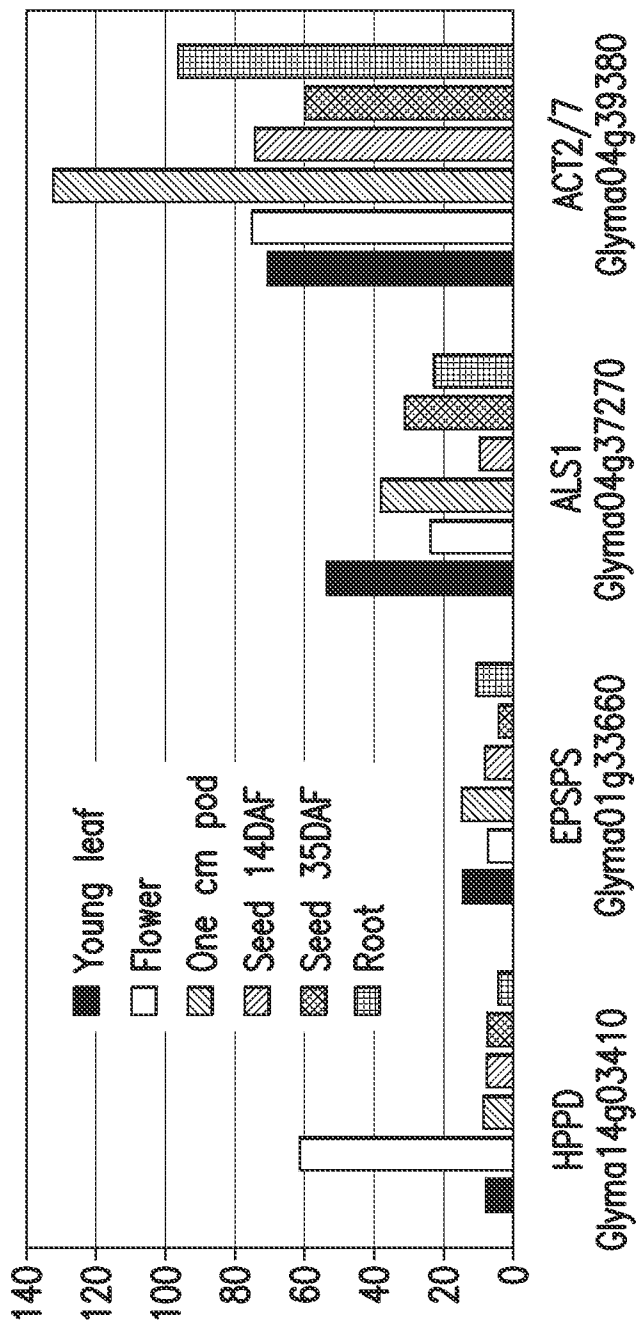


FIG. 7

TCAAGATGAGGATGATCCTTGTAGTGTGTTTGTATTGTTCTTTATAGTTTATACCTAATTT
TATCTATAAGCTTATTAAATTATGTGCAATAGTGACCCCTGATCTTCTGTAATTAT
CATTCAAATAGCTGATGATTTGTTTCCAAATTGTAACCGTAGCCAAAGATGTACGGTGCCATAA
ACCTTGGAGATATTGTTCTCTCTTCCCTTCATAGAGGACAAACCTTCATGTAATGGACATACT
AACGACAAATTAAATTATTTATCATTTTAAAGATTAAATATTTTCTTAAATTATTCCTGTGC
TTTAAAAATTCTTAACAGAAAATTAAATTAGACATTTGTACCATTAGAGAAAACCTGTGGGAC
TCATTTGTTTATTAGATTATTCAGCTAGCAACTGACTCTCTTGTACATTTTCATTTTACATTC
CTTTAAATTATGCATCATTAACAGTAGTAGATTGCATCTCTTAAAAAAAAATTAGATTGCAGTA
TTGCCCTTGGAAATATGGAATTACAAATGTCAAAATATTTTAACGAAATAACGATGCCGTGCTAAA
GTTCAAGACACAAATTTTAACGTTATATAGTGCAATCAATGTTTGAAATTTTAGTGTATATAATTAAC
GTATTTTGTATAATAATTTTACACAAACATCCTCTTAAATTTTCTTATCTTATTTCAATTTAAC
CGTTCTCTTAAATTGTCTTATCTTTTACACACAAATGAATCCCAATAAACATGGTTGGGATT
TATTTGAGTCTTAACTTTAGGAACCAAATATATAATAATTTTAAATAATTTTAAATAATTTTAA
ATAAATATAGAGAAAAGGATGTGATAAAGGCAAGAGAGCGTGTGAACAAGAGAGAGACGAAT
CTAGGTGGATTGACGTACGTGAATGAATGTTGAATATAAGTAAATTAACGCTGAGGCTGTAGGT
GTGGGTAAATAAAAAAGAGAGAGAGCGCATCAACATCATCCAAATATATGGACCGTTAAAGAGCGG
TCGTAAATCCATTTCATTTCTCATCTATCTTCACTTCCCTCGTCCCTCATCTCATCCACCTATTC
M P M Y T P S L S A P S S N H I
TCAACCCAGACGCAATGCCCATGTACACTCCATCCACTCTCCGACCCCTCCTCCAATCACATTCA
Q P S V T L P L Y I T T K L N L K Q Q H H
ACCAAGTGCACACTCCCTTATATATCACAAACCACCAAGCTCAATCTCAAGCAGCAGCATCAC
T T P M P I P M C N E I Q A Q A Q A Q
ACCACACCAATGCCAATACCCATGTGCAACGAAATTCAAGCCCAAGCCCAAGCCCAAGC

FIG. 8A

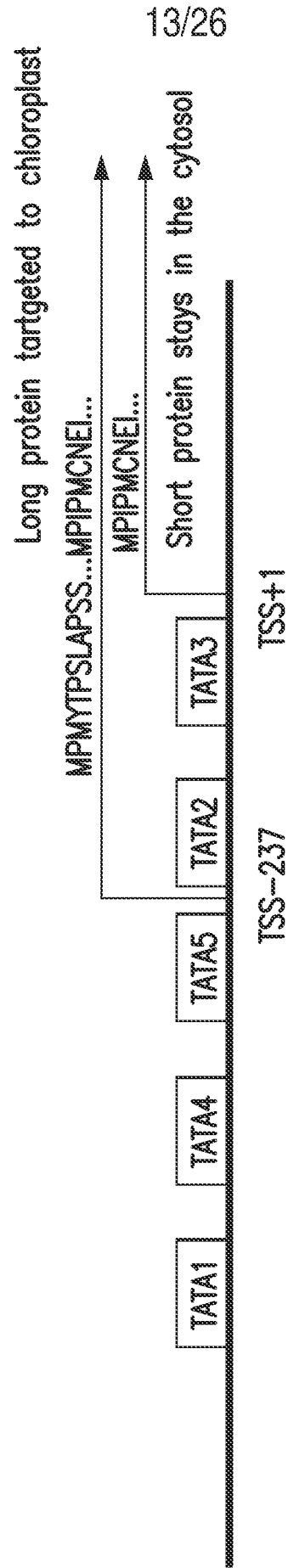


FIG. 8B

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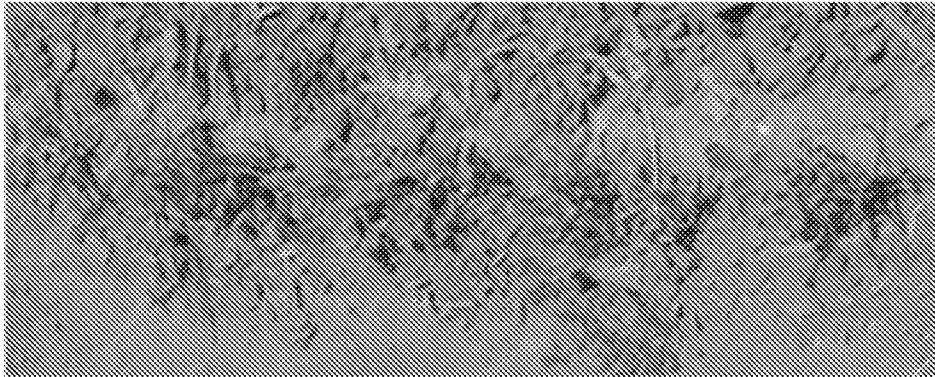


FIG. 9D

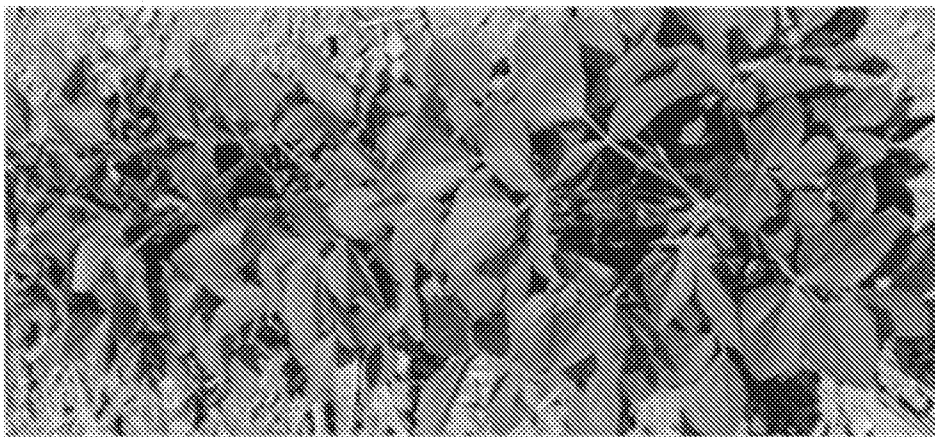


FIG. 9C



FIG. 9B



FIG. 9A

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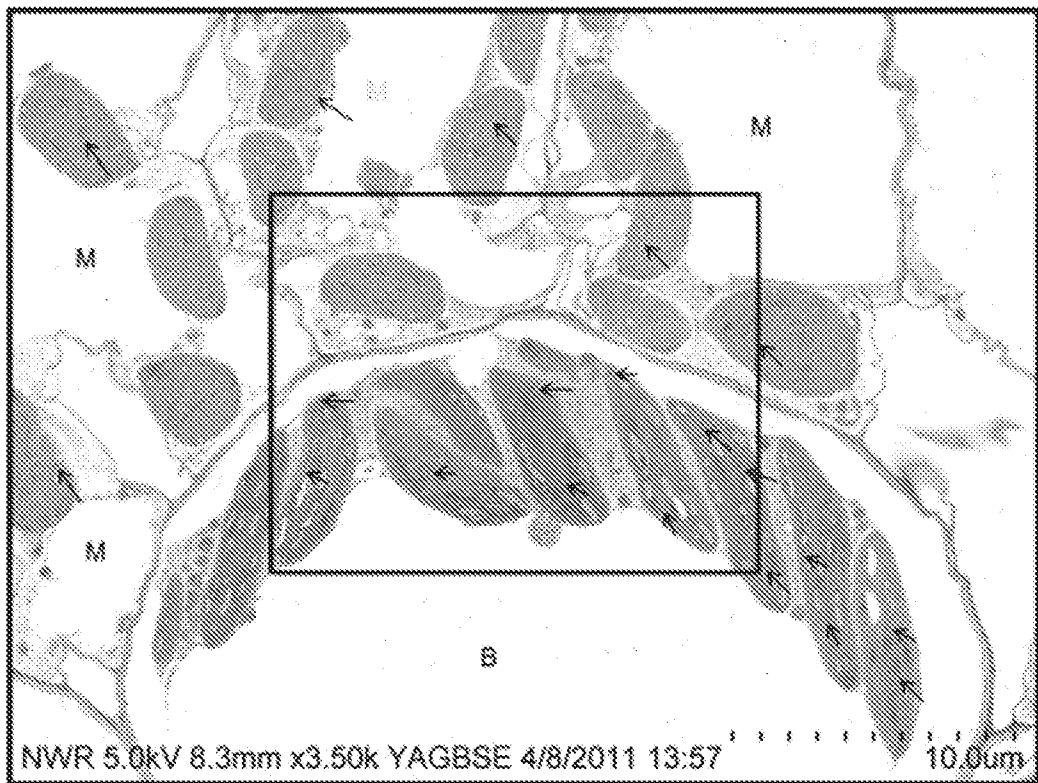


FIG. 10A

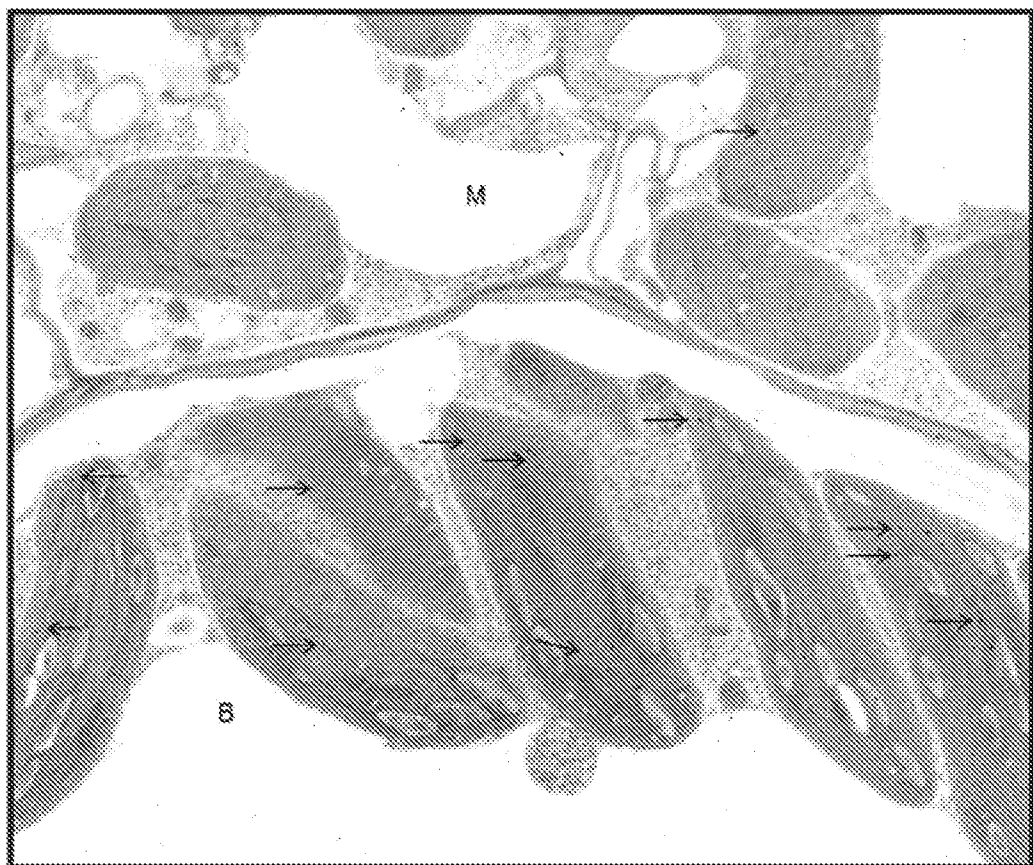


FIG. 10B

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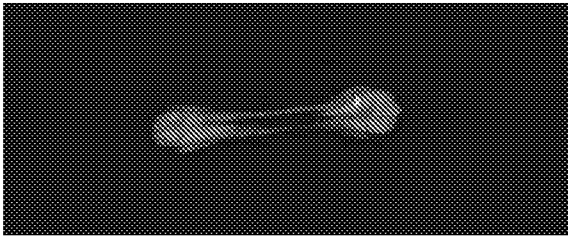


FIG. 11A

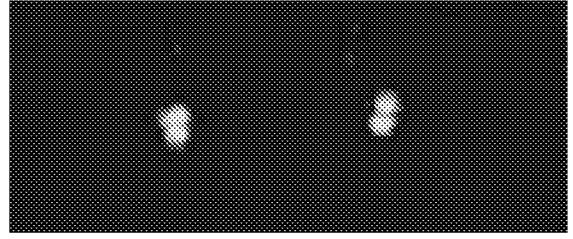


FIG. 11C

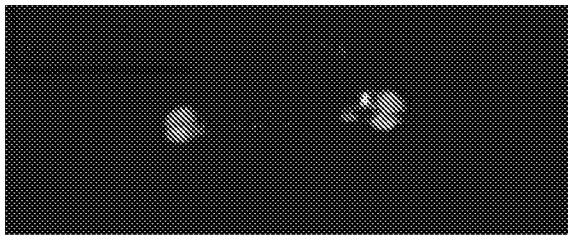


FIG. 11B

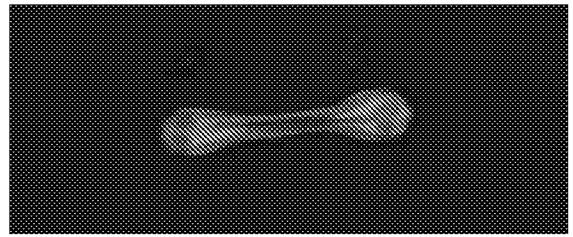


FIG. 11D

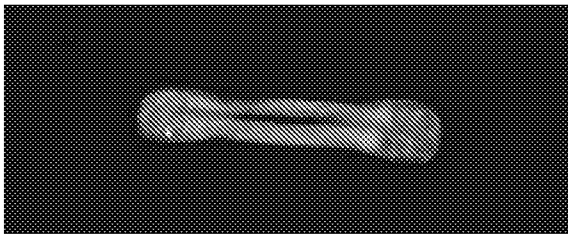


FIG. 11E

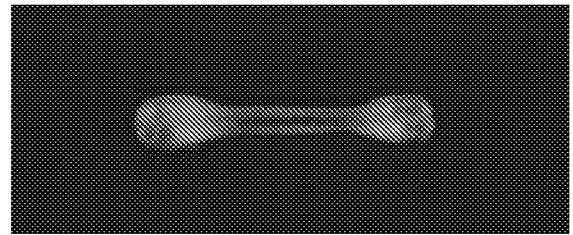


FIG. 11H

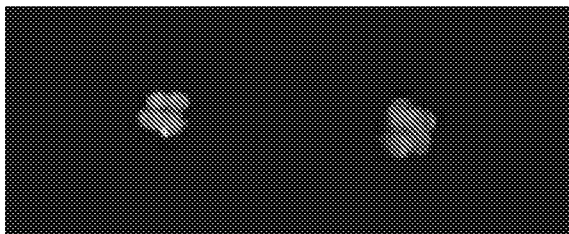


FIG. 11F

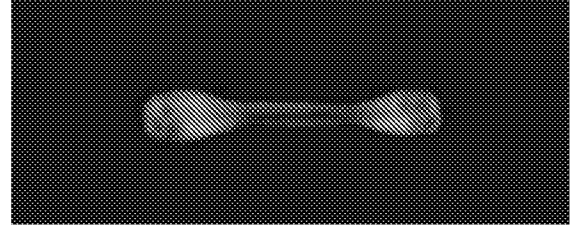


FIG. 11I

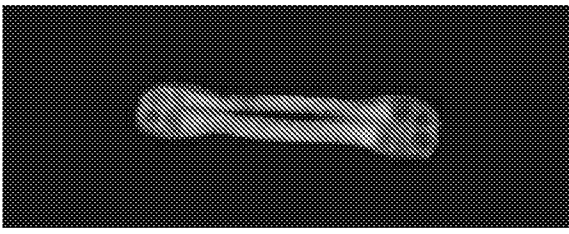


FIG. 11G

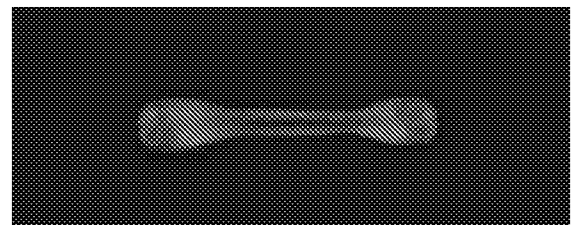


FIG. 11J

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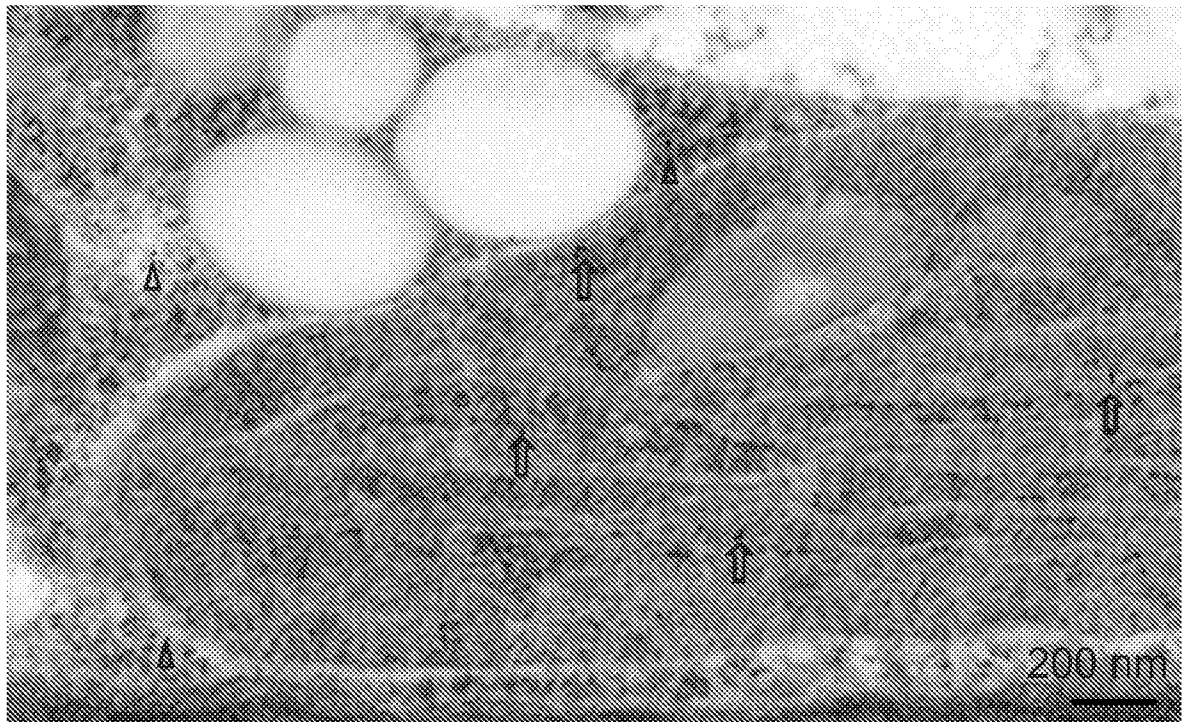


FIG. 12A

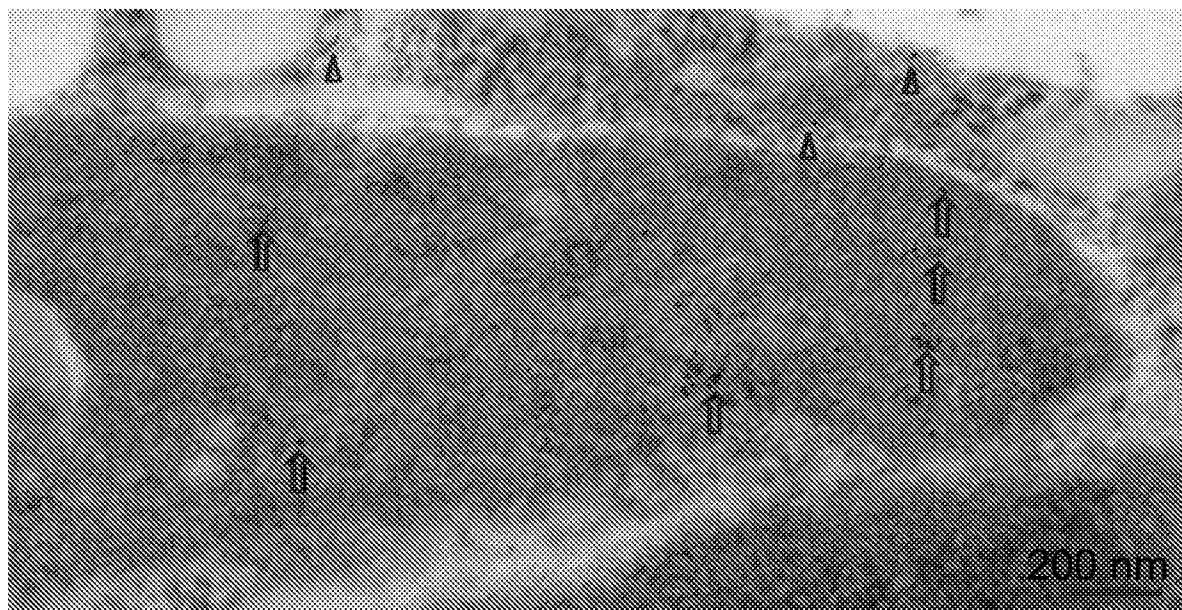


FIG. 12B

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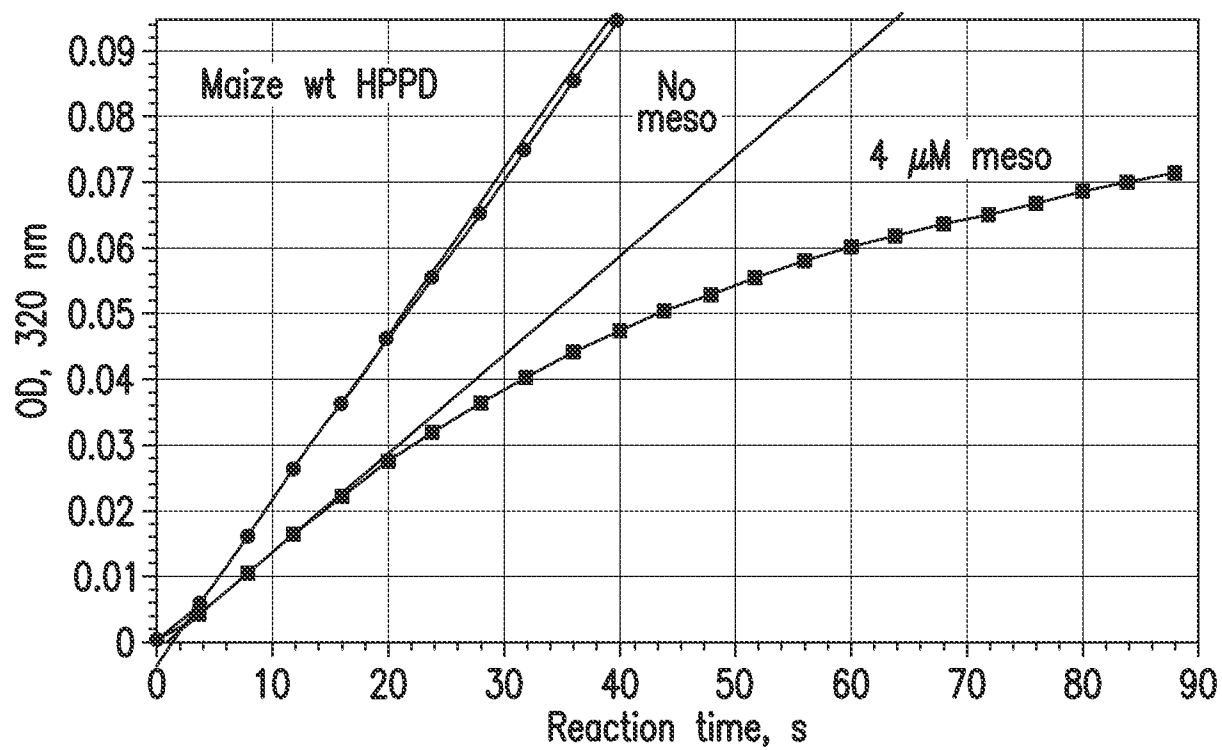


FIG. 13A

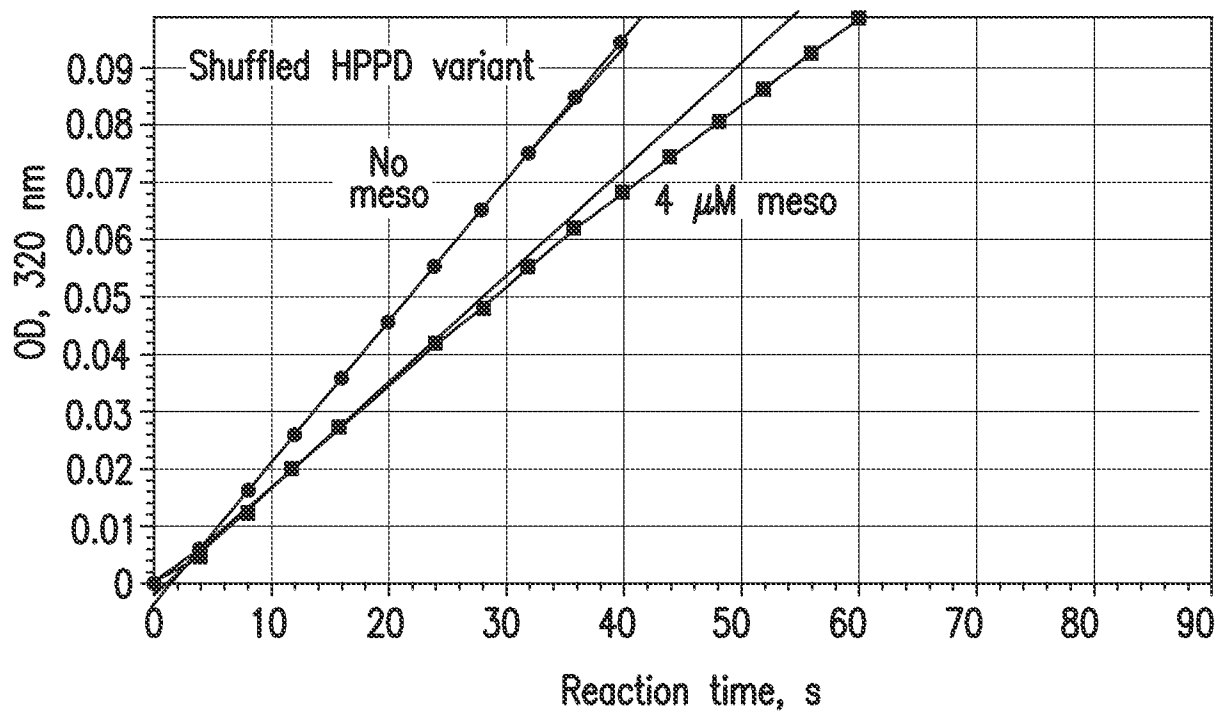


FIG. 13B

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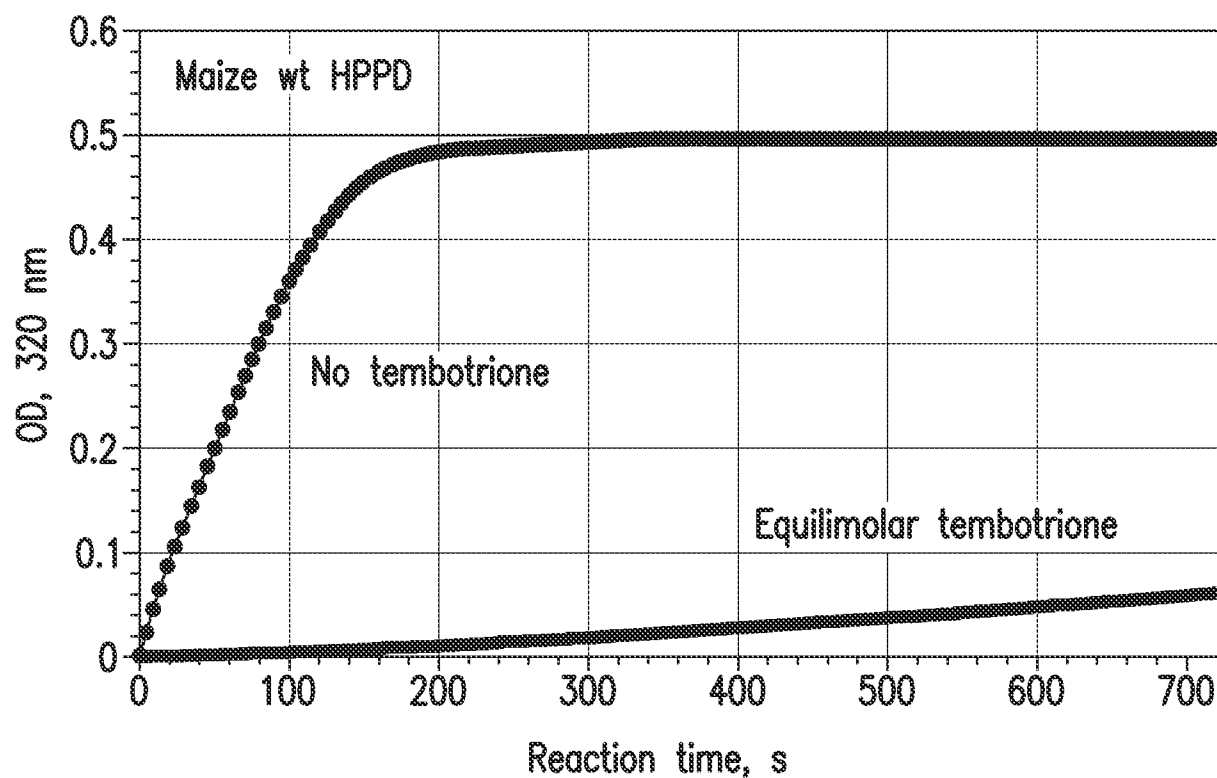


FIG. 13C

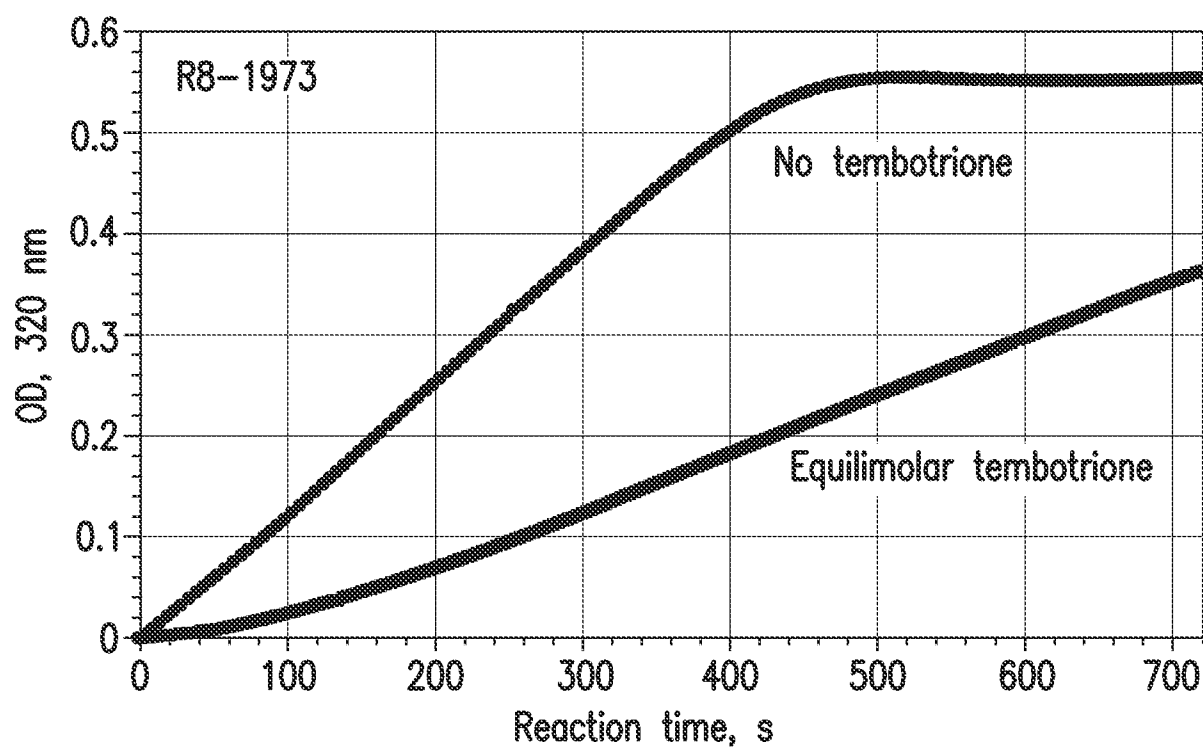


FIG. 13D

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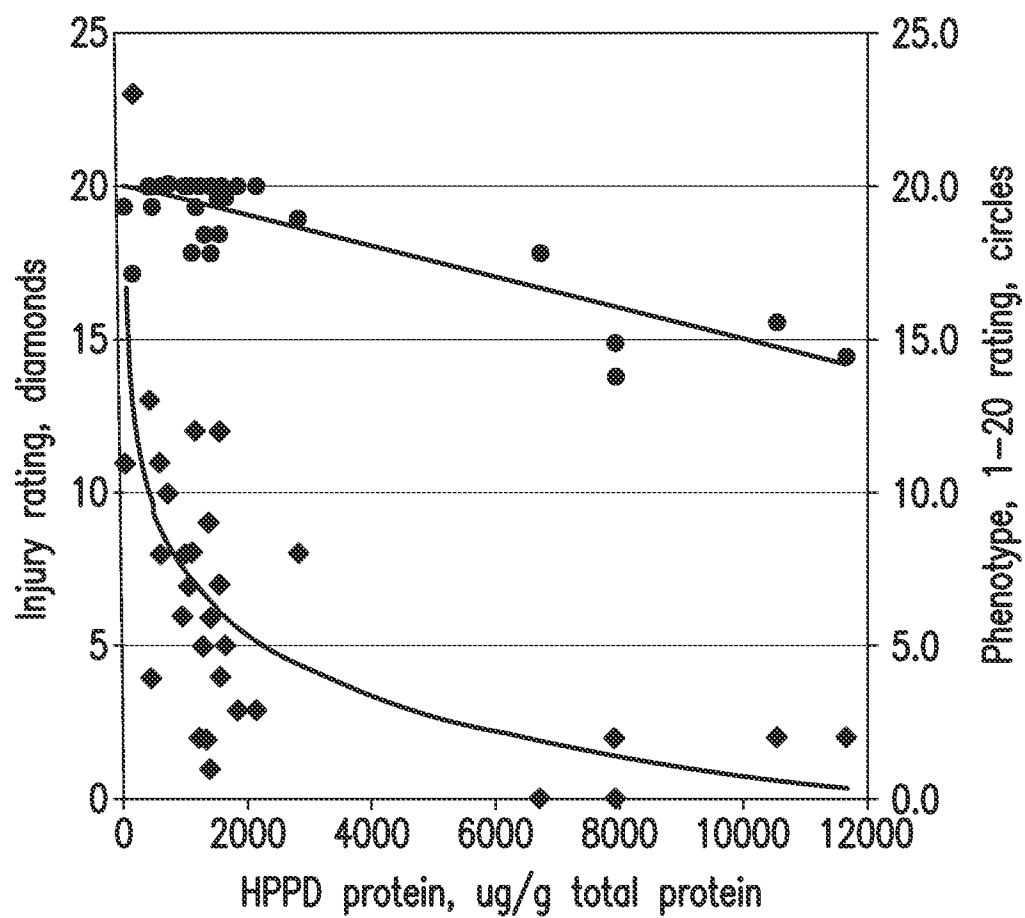


FIG. 14

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MUT ID	MUTA-	Kinetic parameters			Insensitivity parameter, ON x OFF				
NO.	TION	k_{cat}, min^{-1}	$K_M (\mu\text{M})$	k_{cat}/K_M	Tembo	Meso	Sulco	Isox(DKN)	Topra
386	366E	38.8	1.61	24.1	0.33	0.78	0.74	0.44	0.48
506	417Q	33.5	1.56	21.5	0.33	0.73	0.74	0.39	0.39
549	432R	56.7	1.97	28.8	0.23	0.53	0.59	0.27	0.31
505	432K	67.5	2.08	32.4	0.21	0.52	0.59	0.26	0.29
421	417S	26.7	1.41	18.9	0.33	0.67	0.77	0.41	0.41
230	214N	84.6	3.62	23.4	0.25	0.48	0.59	0.33	0.32
547	385I	46.1	2.29	20.1	0.28	0.56	0.70	0.41	0.38
270	158K	47.1	2.49	18.9	0.29	0.54	0.63	0.38	0.37
420	385V	30.3	1.91	15.9	0.34	0.65	0.78	0.33	0.49
61	191G	71.7	2.46	29.1	0.19	0.40	0.51	0.25	0.24
375	211L	77.6	3.2	24.2	0.22	0.37	0.47	0.25	0.27
374	340K	73.5	2.41	30.5	0.16	0.44	0.54	0.23	0.22
345	206Q	66.6	2.63	25.3	0.19	0.46	0.53	0.26	0.27
426	214T	84.2	2.94	28.6	0.17	0.41	0.50	0.21	0.23
263	360L	32.6	1.71	19.0	0.25	0.51	0.63	0.34	0.32
567	434H	84.5	2.77	30.5	0.15	0.46	0.55	0.21	0.22

FIG. 15

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231	387L	74.3	2.46	30.2	0.15	0.46	0.56	0.26	0.21
564	356K	65.8	2.6	25.3	0.18	0.48	0.55	0.25	0.23
170	356R	79.0	2.89	27.3	0.16	0.44	0.53	0.21	0.24
166	182M	56.1	2.88	19.5	0.23	0.44	0.60	0.30	0.29
135	434R	80.9	2.74	29.5	0.15	0.39	0.47	0.18	0.22
434	206S	67.9	3.36	20.2	0.20	0.46	0.53	0.29	0.29
353	191L	32.9	2.74	12.0	0.33	0.45	0.56	0.38	0.37
273	227G	45.3	2.61	17.4	0.22	0.52	0.63	0.26	0.28
541	390N	50.2	2.86	17.5	0.20	0.45	0.58	0.29	0.27
147	342V	54.2	2.59	20.9	0.17	0.42	0.51	0.22	0.23
292	431F	30.8	1.77	17.4	0.19	0.53	0.59	0.26	0.27
379	189P	41.8	3.35	12.5	0.25	0.51	0.62	0.32	0.31
224	205T	34.4	2.72	12.6	0.22	0.47	0.60	0.30	0.29
407	376W	36.1	5.51	6.5	0.31	0.39	0.16	0.37	0.32
2	9070	55.1	2.35	23.4	0.19	0.45	0.56	0.24	0.26
1	Mz wt	219	6.4	34.2	0.0051	0.0437	0.0872	0.0096	0.0154

FIG. 15 (continued)

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MUT ID	MUTA-	Trait fitness, ON x OFF x k_{cat}/K_M					Trail fitness, fold vs maize wt				
NO.	TION	Tembo	Meso	Sulco	Isox(DKN)	Topra	Tembo	Meso	Sulco	ISOX (DKN)	Topra
386	366E	8.0	18.7	17.8	10.6	11.6	46.1	12.6	6.0	32.5	22.1
506	417Q	7.0	15.8	15.8	8.4	8.4	40.2	10.6	5.3	25.7	16.0
549	432R	6.7	15.2	17.0	7.8	8.8	38.6	10.2	5.7	23.7	16.8
505	432K	6.7	17.0	19.2	8.5	9.4	38.5	11.4	6.4	25.8	17.9
421	417S	6.2	12.6	14.5	7.7	7.8	35.6	8.5	4.9	23.4	14.9
230	214N	5.8	11.2	13.8	7.6	7.5	33.2	7.5	4.6	23.2	14.2
547	385I	5.6	11.4	14.2	8.2	7.6	32.3	7.6	4.8	24.9	14.5
270	158K	5.5	10.3	12.0	7.1	7.1	31.3	6.9	4.0	21.7	13.5
420	385V	5.4	10.3	12.4	5.2	7.8	31.1	6.9	4.2	15.9	14.8
61	191G	5.4	11.7	14.9	7.2	6.9	31.0	7.9	5.0	22.0	13.1
375	211L	5.4	9.1	11.5	6.1	6.5	30.9	6.1	3.8	18.6	12.4
374	340K	5.0	13.3	16.4	7.0	6.7	28.5	8.9	5.5	21.3	12.7
345	206Q	4.9	11.6	13.4	6.7	6.7	28.3	7.8	4.5	20.4	12.8
426	214T	4.8	11.7	14.3	6.1	6.5	27.5	7.8	4.8	18.5	12.4

FIG. 15 (continued)

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263	360L	4.8	9.7	12.0	6.5	6.0	27.4	6.5	4.0	19.8	11.5
567	434H	4.7	14.1	16.7	6.5	6.7	27.0	9.4	5.6	19.9	12.8
231	387L	4.7	14.0	17.0	7.8	6.5	26.8	9.4	5.7	23.7	12.3
564	356K	4.5	12.2	13.9	6.3	5.8	25.9	8.2	4.7	19.1	11.0
170	356R	4.5	11.9	14.4	5.7	6.5	25.8	8.0	4.8	17.5	12.4
166	182M	4.5	8.7	11.7	5.9	5.7	25.8	5.8	3.9	18.1	10.8
135	434R	4.3	11.4	13.9	5.4	6.5	24.9	7.6	4.7	16.6	12.4
434	206S	4.1	9.4	10.7	5.9	6.0	23.7	6.3	3.6	18.1	11.3
353	191L	3.9	5.4	6.7	4.5	4.4	22.6	3.6	2.3	13.8	8.4
273	227G	3.7	9.0	10.9	4.5	4.9	21.5	6.0	3.7	13.9	9.3
541	390N	3.5	7.9	10.1	5.2	4.7	20.2	5.3	3.4	15.8	8.9
147	342V	3.5	8.7	10.6	4.6	4.7	20.1	5.9	3.5	14.0	9.0
292	431F	3.4	9.2	10.3	4.6	4.7	19.4	6.2	3.5	13.9	8.9
379	189P	3.1	6.3	7.7	4.0	3.9	17.8	4.3	2.6	12.1	7.4
224	205T	2.8	6.0	7.6	3.8	3.7	15.9	4.0	2.5	11.5	7.0
407	376W	2.1	2.5	1.1	2.4	2.1	11.9	1.7	0.4	7.5	3.9
2	9070	4.4	10.5	13.0	5.6	6.2	25.5	7.0	4.4	17.2	11.7
1	Mz wt	0.174	1.494	2.981	0.328	0.525	1.0	1.0	1.0	1.0	1.0

FIG. 15 (continued)

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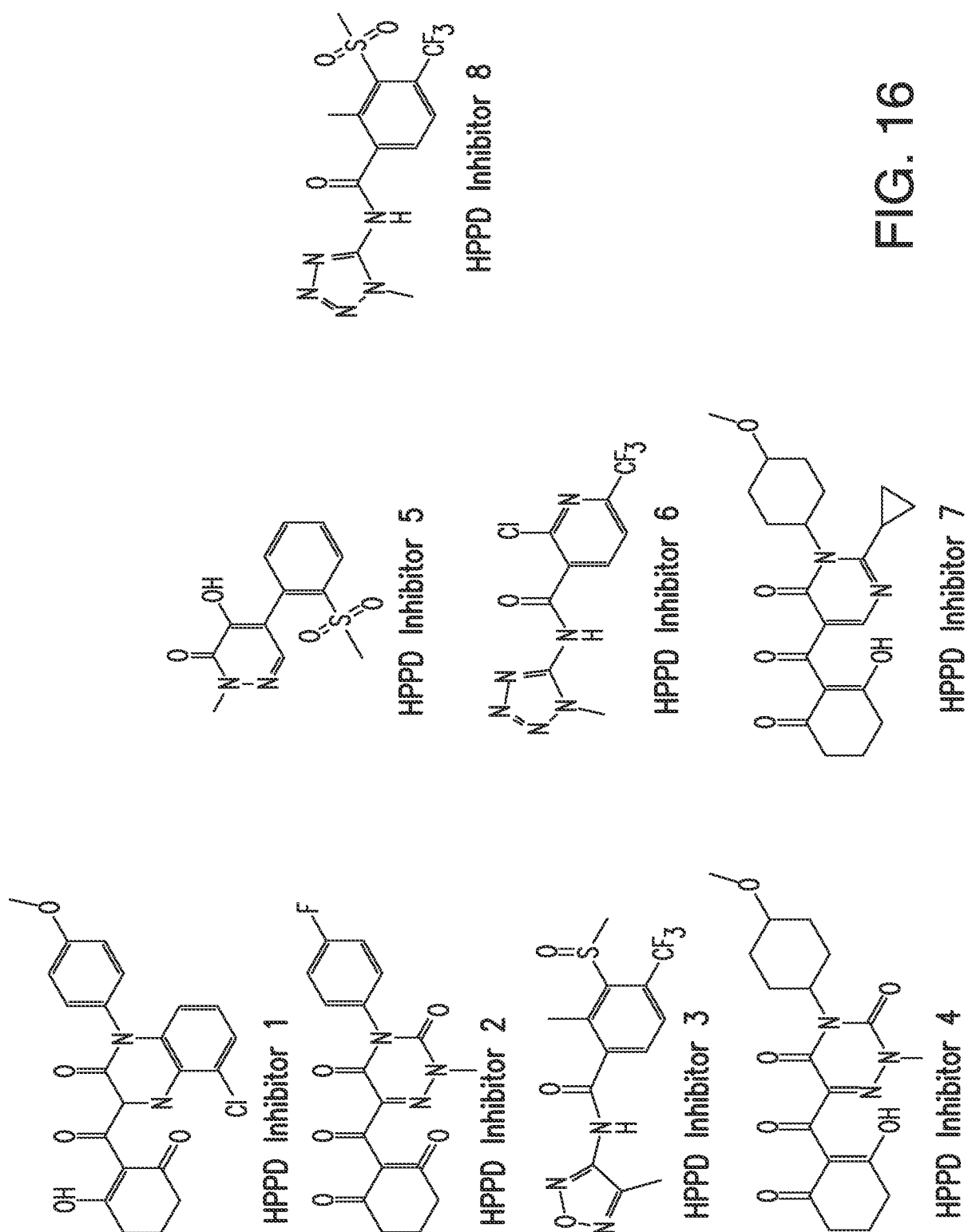


FIG. 16

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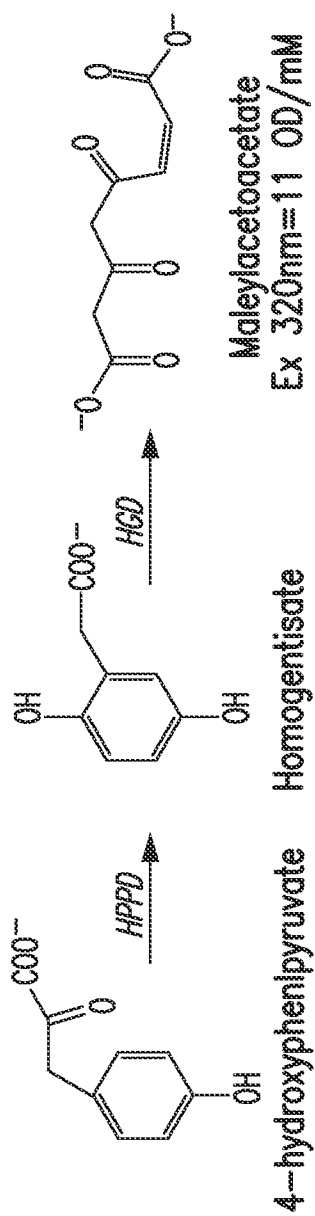


FIG. 17