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 (54) Title: CHIMERIC PROMOTERS AND METHODS OF USE

(57) **Abrégé/Abstract:**

Compositions and methods comprising promoters from the 4- hydroxyphenylpyruvate dioxygenase (HPPD) gene and active variants and fragments thereof, as well as chimeric promoters employing regulatory regions of the HPPD promoters are provided. Further provided are expression cassettes and plants comprising the various promoters disclosed herein operably linked to a polynucleotide of interest. Methods employing the various promoters described herein to modulate the expression of polynucleotides of interest are further provided.

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(57) Abstract: Compositions and methods comprising promoters from the 4- hydroxyphenylpyruvate dioxygenase (HPPD) gene and active variants and fragments thereof, as well as chimeric promoters employing regulatory regions of the HPPD promoters are provided. Further provided are expression cassettes and plants comprising the various promoters disclosed herein operably linked to a polynucleotide of interest. Methods employing the various promoters described herein to modulate the expression of polynucleotides of interest are further provided.



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CHIMERIC PROMOTERS AND METHODS OF USE

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FIELD OF THE INVENTION

The invention relates to the field of genetic manipulation of plants, particularly the modulation of gene activity in plants.

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REFERENCE TO A SEQUENCE LISTING SUBMITTED ON COMPACT DISK

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 408356SEQLIST.txt, created on August 12, 2011, and having a size of 139 KB 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

Expression of heterologous DNA sequences in a plant host is dependent upon the presence of an operably linked promoter that is functional within the plant host. Choice of the promoter sequence will determine when and where within the organism the heterologous DNA sequence is expressed. Modifications of the promoter sequences or additional regulatory sequences upstream and/or downstream from the promoter sequence may be included in expression constructs to bring about varying levels of expression of heterologous nucleotide sequences of interest in a transgenic plant.

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Frequently it is desirable to modulate the level of expression of a nucleotide sequence of interest along with the temporal and spatial expression of the nucleotide sequence of interest in a plant. For example, increased resistance of a plant to infection by soil- and air-borne pathogens might be accomplished by genetic manipulation of the plant's genome to comprise a tissue-preferred promoter operably linked to a heterologous herbicide-resistance gene or heterologous pathogen-resistance gene. Alternatively, it might be desirable to inhibit expression of a native DNA sequence within a plant's tissues to achieve a desired phenotype. In this case, such inhibition might be accomplished with transformation of the plant to comprise a tissue-preferred

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promoter operably linked to an antisense nucleotide sequence, such that expression of the antisense sequence produces an RNA transcript that interferes with translation of the mRNA of the native DNA sequence.

Thus, isolation and characterization of promoter sequences that allow varying
5 levels of expression, locations of expression, and inducible expression conditions of heterologous nucleotide sequences of interest in a transgenic plant are needed for genetic manipulation of plants.

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BRIEF SUMMARY OF THE INVENTION

Compositions and methods comprising promoters from the 4-hydroxyphenylpyruvate dioxygenase (HPPD) gene and active variants and fragments thereof, as well as chimeric promoters employing regulatory regions of the HPPD promoters are provided. Further provided are expression cassettes and plants
15 comprising the various promoters disclosed herein operably linked to a polynucleotide of interest. Methods employing the various promoters described herein to modulate the expression of polynucleotides of interest are further provided.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the conserved regions of the *Glycine max* HPPD gene promoter sequence (~1.2Kbp shown upstream of predicted start of translation). Shaded motifs indicate some level of conservation among sequenced genomes, with bold having highest weight, and italic medium. The predicted TATA boxes are shown in boxes.
25 Predicted transcription start site (TSS) "A" is shown bold and underlined. Experimentally determined transcription start sites "G" and "A" at position -231 and +7 relative to predicted TSS and the predicted translation start codons are shown underlined.

Figure 2 shows a protein gel of *in vitro* translation products of the two *G. max*
30 HPPD transcripts, demonstrating that the predicted polypeptide products from the upstream and downstream in-frame ATG start codons are both made. Linked transcription-translation was performed in wheat germ extracts using 3' truncated Gm HPPD transcription vectors. Predicted protein mass from -231 mRNA: 30.6kD; +7 mRNA: 26.0 kD.

Figure 3 provides a depiction of nested deletion mutants in the *G. max* HPPD promoter region. TATA1 (GTATAAATAA; SEQ ID NO: 44), TATA2 (CCAATATATG; SEQ ID NO: 45), TATA3 (CCTTATATATC; SEQ ID NO: 46), TATA4 (TATATAATAA; SEQ ID NO: 47), and TATA5 (GAATATAAG; SEQ ID NO: 48) are indicated by shaded regions.

Figure 4 shows fluorescence images from Agro-infiltrated leaf discs were captured as described in Example 3. Each plate contains 30 randomly picked leaf discs. A. dMMV (Dey and Matai, (1999) *Transgenics* 3:61-70) promoter driven GUS (negative control); B. dMMV promoter driven DsRed2; C. H2B promoter (US 6,177,611) driven DsRed2; ; D. Native HPPD promoter driven DsRed2; E. SHP101C (deletion of all 5 TATA boxes) driven DsRed2; F. SHP102C (Deletion of TATA 2, 3, 4, and 5) driven DsRed2; G. SHP103C (deletion of TATA3) driven DsRed2.

Figure 5 depicts the *Glycine max* HPPD promoter and separate transcripts.

Figure 6 shows schematics of the HPPD promoters described herein. TATA motifs are indicated by T1 (TATA1), T2 (TATA2), T3 (TATA3), T4 (TATA4), and T5 (TATA5).

Figure 7 shows schematics of the HPPD promoters having mutations within the TATA motif. Nucleotide point mutations are indicated by a "*" within the appropriate TATA motif.

Figure 8 shows schematics of the chimeric promoters described herein comprising a regulatory region of an HPPD promoter operably linked to synthetic element I (SEQ ID NO: 21). TATA motifs are indicated by T1 (TATA1), T2 (TATA2), T3 (TATA3), T4 (TATA4), and T5 (TATA5). Nucleotide point mutations are indicated by a "*" within the appropriate TATA motif.

Figure 9 shows schematics of the chimeric promoters described herein comprising a regulatory region of an HPPD promoter operably linked to synthetic element II (SEQ ID NO: 22). TATA motifs are indicated by T1 (TATA1), T2 (TATA2), T3 (TATA3), T4 (TATA4), and T5 (TATA5). Nucleotide point mutations are indicated by a "*" within the appropriate TATA motif.

Figure 10 shows transient expression of Gm HPPD-AcGFP fusion proteins in soy leaf cells. Epifluorescence micrographs of soy leaf sections infiltrated with both untargeted (cytoplasmic) DsRed2 and Gm-HPPD N terminus fusions to AcGFP. A and C. With both vectors red fluorescence is seen in the cytoplasm while plastids remain dark. B. When AcGFP is fused to Gm-HPPD amino acids 42-86 (from SEQ ID NO:

58), green fluorescence is seen in the cytoplasm and plastids remain dark. D. When AcGFP is fused to Gm HPPD amino acids 1-86 (from SEQ ID NO: 58), green fluorescence is clearly seen in plastids of infected cells.

Figure 11 depicts the pattern and relative level of DsRed2 expression detected by Typhoon variable mode imager from 24 day old stably transformed Arabidopsis plants indicating expression pattern and level of “wild-type” Col-0 (background fluorescence), H2B promoter, native GM-HPPD promoter and synthetic variants SHP106C, SHP205 and SHP206.

10 DETAILED DESCRIPTION OF THE INVENTION

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

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

25

I. Compositions

Compositions disclosed herein provide HPPD promoters and chimeric promoters comprising a regulatory region of an HPPD promoter operably linked to a core promoter. The various promoters and chimeric promoters disclosed herein have “transcription regulatory activity” or “promoter activity”. As used herein, “transcriptional regulatory activity” or “promoter activity” refers to the ability of a polynucleotide to direct the transcription of a polynucleotide of interest. Methods are available in the art for determining if a promoter sequence retains the ability to direct transcription. Such activity can be measured by Northern blot analysis. See, for

example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York), herein incorporated by reference. Alternatively, biological activity of the promoter can be measured using assays specifically designed for measuring the activity and or level of the polypeptide
5 being expressed from the promoter. Such assays are known in the art. For example, transcriptional regulatory activity of the HPPD promoter and/or chimeric promoters and/or regulatory regions of an HPPD promoter or active fragments and variants thereof can be determined by measuring the activity or level of a polynucleotide of interest expressed from the HPPD promoter and/or chimeric promoters and/or
10 regulatory regions of an HPPD promoter. Methods for measuring the activity and level of polynucleotides of interest are disclosed elsewhere herein.

By “modulating” the transcriptional regulatory activity is intended to mean the transcriptional regulatory activity of the promoter sequence is either increased or decreased when compared to an appropriate control. A decrease in transcriptional
15 regulatory activity is intended to mean the transcription regulatory activity of the promoter is statistically lower than the activity of an appropriate control. An increase in transcriptional regulatory activity is intended to mean the transcription regulatory activity of the promoter is statistically higher than the activity of an appropriate control.

In particular embodiments, modulating the transcriptional regulatory activity
20 results in at least a 95% decrease or increase, at least a 90% decrease or increase, at least a 80% decrease or increase, at least a 70% decrease or increase, at least a 60% decrease or increase, at least a 50% decrease or increase, at least a 40% decrease or increase, at least a 30% decrease or increase, at least a 20% decrease or increase, at least a 10% decrease or increase, or at least a 5% decrease or increase of the
25 transcriptional regulatory activity of the promoter or active variant or fragment thereof when compared to an appropriate control. Alternatively, modulating the transcriptional regulatory activity can include about a 0.5 fold, 1 fold, 2 fold, 4 fold, 8 fold, 16 fold, or 32 fold overall decrease or increase of the transcriptional regulatory activity of the promoter or active variant or fragment thereof when compared to an appropriate
30 control. In other embodiments, modulating the transcriptional regulatory activity of a promoter or active variant or fragment thereof results in a decrease or an increase in the transcription regulatory activity of about 3%-15%, 10%-25%, 20%-35%, 30%-45%, 40%-55%, 50%-65%, 60%-75%, 70%-90%, 70% to 80%, 70%-85%, 80%-95%, 90%-100% when compared to an appropriate control.

It is further recognized that the modulation of the transcriptional regulatory activity need not be an overall increase or decrease in activity but also includes a change in tissue distribution of the regulatory activity, a modification of the location within a cell of the product of the gene regulated by the promoter, or an alteration in response to specific inducing factors. If multiple transcripts are produced from a single polynucleotide sequence, modulation of the transcriptional regulatory activity could alter the native ratio of transcripts to increase one in relation to the other transcript or other transcripts.

10 *A. Hydroxyphenylpyruvate Dioxygenase (HPPD) Promoters*

Various promoters and active variants and fragments thereof from the hydroxyphenylpyruvate dioxygenase (HPPD) gene are provided herein. As used herein, an "HPPD promoter" encompasses the genomic region including and upstream of the transcription start site (TSS), including the furthest downstream TSS in the case of multiple TSS, and may include the untranslated region of the transcript up to the ATG, for a gene encoding HPPD. In specific embodiments, an HPPD promoter can comprise the genomic region found 5' to the translational start site. Such HPPD promoters include SEQ ID NO: 1, which comprises the native polynucleotide sequence of the *Glycine max* (soy) HPPD promoter, and active variants and fragments thereof. As used herein, a "native" or "wild type" polynucleotide comprises any naturally occurring nucleotide sequence. As discussed in further detail elsewhere herein, such HPPD promoters and active variants and fragments thereof find use in expression of polynucleotides of interest, and further when modified or constructed into a chimeric promoter, the expression profile (i.e. temporal expression, tissue specific expression, or expression level) can be modulated. Such alterations in expression find use when one desires to refine the level or pattern of expression of a polynucleotide of interest.

Various compositions are provided which employ HPPD promoters and active fragments and variants thereof. As used herein, a "promoter" is a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA syntheses at the appropriate transcription initiation site for a particular polynucleotide sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. The promoter sequences disclosed herein modulate transcription of an operably linked

polynucleotide of interest. In one embodiment, novel promoters set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, and 78 and variants and fragments thereof having transcriptional regulatory activity are provided.

5 The HPPD promoters and the active variants and fragments of the HPPD promoters disclosed herein have transcriptional regulatory activity. The transcriptional regulatory activity of the HPPD promoter or active variants and fragments thereof can reflect either an increase in transcriptional regulatory activity or a decrease in transcription regulatory activity when compared to a native HPPD promoter (such as
10 the native soy HPPD promoter as set forth in SEQ ID NO:1). For example, an HPPD promoter or active variant or fragment thereof can regulate transcription of an operably linked polynucleotide. In specific embodiments, the HPPD promoter or active variant or fragment thereof has an increase or a decrease in transcriptional regulatory activity of about 0.1%, 0.5%, 1%, 3% 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%,
15 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or greater when compared to the transcriptional regulatory activity of a native HPPD promoter or to the native soy HPPD promoter set forth in SEQ ID NO:1. Alternatively, the increase or decrease in transcriptional regulatory activity can include about a 0.5 fold, 1 fold, 2 fold, 4 fold, 8 fold, 16 fold, or 32 overall increase or decrease in transcription when compared to the
20 transcriptional regulatory activity of a native HPPD promoter or the native soy HPPD promoter set forth in SEQ ID NO:1.

 Various alterations can be made in the HPPD promoters disclosed herein. In some embodiments, the HPPD promoters or active variants and fragments thereof have been altered such that the transcriptional regulatory activity is decreased when
25 compared to the wild type HPPD promoter. In such embodiments, the HPPD promoter can comprise a deletion of at least one of the TATA motif polynucleotide segments in the HPPD promoter, including a deletion of the TATA1 motif (SEQ ID NO: 44), the TATA2 motif (SEQ ID NO: 45), the TATA3 motif (SEQ ID NO: 46), the TATA4 motif (SEQ ID NO: 47), and/or the TATA5 motif (SEQ ID NO: 48). See, also Figure 1
30 and Figure 3. Non-limiting examples of such active variants and fragments of an HPPD promoter are set forth in SEQ ID NO: 2, 3, 4, 11, 20, and 76.

 Alternatively, an HPPD promoter or an active variant or fragment thereof can comprise at least one nucleic acid mutation of at least one TATA motif, including, for example, at least one mutation in the TATA1 motif, at least one mutation in the

TATA2 motif, and/or at least one mutation in the TATA3 motif, or any combination thereof. As used herein, nucleic acid mutations encompass base additions, base deletions and base substitutions. In some embodiments an alteration of an HPPD promoter encompasses at least one or at least two alterations of at least one, at least two
5 or at least three TATA motifs. In some embodiments, alteration of an HPPD promoter comprises both a deletion of at least one TATA motif and an alteration of at least one TATA motif. See, also Figure 1 and Table 3. Non-limiting examples of such active variants and fragments of an HPPD promoter are set forth in SEQ ID NO: 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 74, or 75. It is further recognized that active variants
10 and fragments of the HPPD promoter may contain a deletion of any one or more TATA motif in combination with at least one or more alterations in the remaining TATA motifs.

In specific embodiments, active fragments of the HPPD promoter as set forth in SEQ ID NO:1 are provided. Such fragments can include various 5' deletions of SEQ
15 ID NO: 1 including but not limited to polynucleotides comprising nucleotides 100-2166 of SEQ ID NO: 1; 200-2166 of SEQ ID NO: 1; 300-2166 of SEQ ID NO: 1; 400-2166 of SEQ ID NO: 1; 500-2166 of SEQ ID NO: 1; 600-2166 of SEQ ID NO: 1; 700-2166 of SEQ ID NO: 1; 800-2166 of SEQ ID NO: 1; 900-2166 of SEQ ID NO: 1; 1000-2166 of SEQ ID NO: 1; 1200-2166 of SEQ ID NO: 1; 1400-2166 of SEQ ID NO: 1; 1600-
20 2166 of SEQ ID NO: 1; 1800-2166 of SEQ ID NO: 1; or 2000-2166 of SEQ ID NO: 1, wherein the various fragments continue to have transcriptional regulatory activity. Further provided are active variants of the 5' deletions of SEQ ID NO:1. Such active variants can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any one of the promoters comprising SEQ ID
25 NO: 1 or polynucleotides comprising nucleotides 100-2166 of SEQ ID NO: 1; 200-2166 of SEQ ID NO: 1; 300-2166 of SEQ ID NO: 1; 400-2166 of SEQ ID NO: 1; 500-2166 of SEQ ID NO: 1; 600-2166 of SEQ ID NO: 1; 700-2166 of SEQ ID NO: 1; 800-2166 of SEQ ID NO: 1; 900-2166 of SEQ ID NO: 1; 1000-2166 of SEQ ID NO: 1; 1200-2166 of SEQ ID NO: 1; 1400-2166 of SEQ ID NO: 1; 1600-2166 of SEQ ID NO:
30 1; 1800-2166 of SEQ ID NO: 1; or 2000-2166 of SEQ ID NO: 1.

In specific embodiments, active fragments of the HPPD promoter as set forth in SEQ ID NO:1 are provided. Such fragments can include various 3' deletions of SEQ ID NO: 1 including but not limited to polynucleotides comprising nucleotides 1-2066 of SEQ ID NO: 1; 1-1966 of SEQ ID NO: 1; 1-1866 of SEQ ID NO: 1; 1-1766 of SEQ

ID NO: 1; 1-1666 of SEQ ID NO: 1; 1-1566 of SEQ ID NO: 1; 1-1466 of SEQ ID NO: 1; 1-1366 of SEQ ID NO: 1; 1-1266 of SEQ ID NO: 1; 1-1166 of SEQ ID NO: 1; 1-966 of SEQ ID NO: 1; 1-766 of SEQ ID NO: 1; 1-566 of SEQ ID NO: 1; 1-366 of SEQ ID NO: 1; or 1-166 of SEQ ID NO: 1, wherein the various fragments continue to have
5 regulatory activity. Further provided are active variants of the 3' deletions of SEQ ID NO:1. Such active variants can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any one of the promoters comprising SEQ ID NO: 1 or polynucleotides comprising nucleotides 1-2066 of SEQ ID NO: 1; 1-1966 of SEQ ID NO: 1; 1-1866 of SEQ ID NO: 1; 1-1766 of
10 SEQ ID NO: 1; 1-1666 of SEQ ID NO: 1; 1-1566 of SEQ ID NO: 1; 1-1466 of SEQ ID NO: 1; 1-1366 of SEQ ID NO: 1; 1-1266 of SEQ ID NO: 1; 1-1166 of SEQ ID NO: 1; 1-966 of SEQ ID NO: 1; 1-766 of SEQ ID NO: 1; 1-566 of SEQ ID NO: 1; 1-366 of SEQ ID NO: 1; or 1-166 of SEQ ID NO: 1.

Nucleic acid molecules that are fragments of a HPPD promoter comprise at
15 least about 4, 6, 8, 10, 12, 16, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700 or 800 consecutive nucleotides of SEQ ID NO:1 or up to the total number of nucleotides present in a full-length HPPD promoter sequence disclosed herein (i.e. 2166 polynucleotides of SEQ ID NO:1). In certain embodiments, fragments of a HPPD promoter comprise at least 6-8 polynucleotides of SEQ ID NO: 1, or of any
20 length long enough to comprise transcriptional or regulatory features.

A structural gene or coding sequence is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

25 B. Chimeric Promoters

Further provided are chimeric promoter constructs or chimeric promoters which employ regulatory regions of the HPPD promoter operably linked to a core promoter region. As used herein a "chimeric promoter construct" refers to a first polynucleotide comprising a regulatory region operably linked to a second polynucleotide comprising
30 a heterologous core promoter. Depending on the regulatory region of the HPPD promoter and the core promoter region employed in a given chimeric promoter, one can influence the transcriptional regulatory activity of the chimeric promoter and thus provide means to refine the manner in which a polynucleotide of interest is expressed (i.e. influence the temporal expression, tissue-specific expression or level of

expression). In non-limiting embodiments, any one of the polynucleotides set forth in any one of SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or 77, or active variants or fragments thereof, can comprise a chimeric promoter.

Various structures within eukaryotic promoters are known. See, for example,
5 Kochetov, *Mol. Biol.*, 2002, 36:510-516; Priest, *Curr. Opin. in Plant Biol.*, 2009,
12:643-649; Fuda, *Nature*, 2009, 461: 186-192, and Saul, *The Plant Journal*, 2009,
60:1031-1042.

i. Regulatory Region of an HPPD Promoter

10 The chimeric promoters disclosed herein comprise a “regulatory region of an HPPD promoter” operably linked to a core promoter. As used herein, a “regulatory region of an HPPD promoter” comprises a fragment or variant of an HPPD promoter. Non-limiting examples of regulatory regions of the HPPD promoter can comprise the polynucleotide set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
15 17, 18, 19, 20, 74, 75, and 76 or active fragments and variants thereof.

In some embodiments, the regulatory region of the HPPD promoter or an active variant or fragment thereof has been modified to no longer retain regulatory activity in the absence of a core promoter. Such regulatory regions of the HPPD promoter when operably linked to a polynucleotide sequence of interest in the absence of a core
20 promoter, reduce the expression of the polynucleotide sequence of interest to that of background levels. The regulatory activity of these regulatory regions of the HPPD promoter is increased when the regulatory region of the HPPD promoter is operably linked to a core promoter. It is further recognized that the modulation of the regulatory activity need not be an overall increase or decrease in activity but also includes a
25 change in tissue distribution of the regulatory activity or an alteration in response to specific inducing factors. Thus, inactive fragments of the regulatory regions described herein can be made active by combination with a core promoter sequence or synthetic element, wherein the active regulatory region maintains the tissue or temporal specificity of the native HPPD promoter. Non-limiting examples of such regulatory
30 regions of the HPPD promoter are set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, and 76.

In other embodiments, the regulatory region of an HPPD promoter or an active variant or fragment thereof has regulatory activity equal to or less than 95%, 90%, 80%, 70%, 60%, 50%, 40%, 35%, 30%, 25%, 20%, 15%, 14%, 13%, 10%, 9%, 8%,

7%, 6%, 5%, 4%, 3%, 2% or 1% of the wild type HPPD promoter (such as that set forth in SEQ ID NO:1) in the absence of the core promoter. In some embodiments, the regulatory region of an HPPD promoter has regulatory activity between 10% and 20%, 20% and 30%, 23% and 36%, 30% and 40%, 40% and 50%, 50% and 60%, 60% and 70%, 70% and 80%, and 80% and 90% of the wild-type HPPD promoter (such as that set forth in SEQ ID NO:1) in the absence of the operably linked core promoter. Non-limiting examples of such regulatory regions of the HPPD promoter are set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, and 76.

10 In specific embodiments, active fragments of the regulatory region of an HPPD promoter as set forth in SEQ ID NO:1 are provided. Such fragments can include various 5' deletions of SEQ ID NO: 1 including but not limited to polynucleotides comprising nucleotides 100-2166 of SEQ ID NO: 1; 200-2166 of SEQ ID NO: 1; 300-2166 of SEQ ID NO: 1; 400-2166 of SEQ ID NO: 1; 500-2166 of SEQ ID NO: 1; 600-2166 of SEQ ID NO: 1; 700-2166 of SEQ ID NO: 1; 800-2166 of SEQ ID NO: 1; 900-2166 of SEQ ID NO: 1; 1000-2166 of SEQ ID NO: 1; 1200-2166 of SEQ ID NO: 1; 1400-2166 of SEQ ID NO: 1; 1600-2166 of SEQ ID NO: 1; 1800-2166 of SEQ ID NO: 1; or 2000-2166 of SEQ ID NO: 1, wherein the various fragments continue to act as regulatory regions of HPPD promoters as described above.

20 Further provided are regulatory regions of an HPPD promoter which are active variants of the 5' deletions of SEQ ID NO: 1. Such active variants can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any one of the polynucleotides comprising SEQ ID NO: 1 or comprising nucleotides 100-2166 of SEQ ID NO: 1; 200-2166 of SEQ ID NO: 1; 300-2166 of SEQ ID NO: 1; 400-2166 of SEQ ID NO: 1; 500-2166 of SEQ ID NO: 1; 600-2166 of SEQ ID NO: 1; 700-2166 of SEQ ID NO: 1; 800-2166 of SEQ ID NO: 1; 900-2166 of SEQ ID NO: 1; 1000-2166 of SEQ ID NO: 1; 1200-2166 of SEQ ID NO: 1; 1400-2166 of SEQ ID NO: 1; 1600-2166 of SEQ ID NO: 1; 1800-2166 of SEQ ID NO: 1; or 2000-2166 of SEQ ID NO: 1, wherein the various variants continue to act as regulatory regions of HPPD promoters as described above.

30 In specific embodiments, active fragments of the regulatory region of an HPPD promoter as set forth in SEQ ID NO:1 are provided. Such fragments can include various 3' deletions of SEQ ID NO: 1 including but not limited to polynucleotides comprising nucleotides 1-2066 of SEQ ID NO: 1; 1-1966 of SEQ ID NO: 1; 1-1866 of

SEQ ID NO: 1; 1-1766 of SEQ ID NO: 1; 1-1666 of SEQ ID NO: 1; 1-1566 of SEQ ID NO: 1; 1-1466 of SEQ ID NO: 1; 1-1366 of SEQ ID NO: 1; 1-1266 of SEQ ID NO: 1; 1-1166 of SEQ ID NO: 1; 1-966 of SEQ ID NO: 1; 1-766 of SEQ ID NO: 1; 1-566 of SEQ ID NO: 1; 1-366 of SEQ ID NO: 1; or 1-166 of SEQ ID NO: 1, wherein the
5 various fragments continue to act as regulatory regions of HPPD promoters as described above. Further provided are active variants of the 3' deletions of SEQ ID NO:1. Such active variants can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any one of the promoters comprising SEQ ID NO: 1 or polynucleotides comprising nucleotides 1-
10 2066 of SEQ ID NO: 1; 1-1966 of SEQ ID NO: 1; 1-1866 of SEQ ID NO: 1; 1-1766 of SEQ ID NO: 1; 1-1666 of SEQ ID NO: 1; 1-1566 of SEQ ID NO: 1; 1-1466 of SEQ ID NO: 1; 1-1366 of SEQ ID NO: 1; 1-1266 of SEQ ID NO: 1; 1-1166 of SEQ ID NO: 1; 1-966 of SEQ ID NO: 1; 1-766 of SEQ ID NO: 1; 1-566 of SEQ ID NO: 1; 1-366 of SEQ ID NO: 1; or 1-166 of SEQ ID NO: 1, wherein wherein the various fragments
15 continue to act as regulatory regions of HPPD promoters as described above.

Additional non-limiting examples of active variants and fragments of a regulatory region of the HPPD promoter can include a deletion of a at least one of the TATA motif polynucleotide segments in the HPPD promoter, including a deletion of the TATA1 motif (SEQ ID NO: 44), the TATA2 motif (SEQ ID NO: 45), the TATA3
20 motif (SEQ ID NO: 46), the TATA4 motif (SEQ ID NO: 47), and/or the TATA5 motif (SEQ ID NO: 48). Alternatively, variants and fragments of a regulatory region of an HPPD promoter can comprise at least one nucleic acid mutation of at least one TATA motif, including, for example, at least one mutation in the TATA1 motif, at least one mutation in the TATA2 motif, at least one mutation in the TATA3 motif, at least one
25 mutation in the TATA4 motif, and/or at least one mutation in the TATA5 motif, or any combination thereof. As used herein, nucleic acid mutations encompass base additions, base deletions and base substitutions. In some embodiments an alteration of the regulatory region of an HPPD promoter encompasses at least one or at least two alterations of at least one, at least two or at least three TATA motifs. In some
30 embodiments, alteration of the regulatory region comprises both a deletion of at least one TATA motif and an alteration of at least one TATA motif. See, for example, Table 3.

ii. Core Promoters

The chimeric promoter disclosed herein comprises a regulatory region of an HPPD promoter operably linked to a heterologous core promoter. As used herein, a “core promoter” refers to a polynucleotide comprising the essential nucleotide sequences for expression of an operably linked coding sequence, including, but not limited to, a TATA box and transcription start site. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity, such as the regulatory regions of the HPPD promoter. Such core promoter sequences are known. See for example, US Patent No. 6,072,050, US Patent No. 6,555,673, Vedel, *Plant Science*, 2011, 180:182-189, herein incorporated by reference in their entirety.

The core promoters described herein may also comprise heterologous or synthetic elements having core promoter activity. For example, core promoters may comprise the SynII core (SEQ ID NO: 70; US Patent No. 6,072,050, SEQ ID NO: 1), a 5' UTR, an enzyme recognition site, or other regions such as Rsyn7, or any combination thereof. Other elements that could be found in core promoters are identified in Smale, *Annu. Rev. Biochem.*, 2003, 72:449-79, herein incorporated by reference in its entirety

In specific embodiments, the core promoter can comprise synthetic elements as set forth in SEQ ID NO: 21 or 22 or active fragments or variants thereof. Synthetic element I (SEQ ID NO: 21) was derived from SynII core (US Patent No. 6,072,050 SEQ ID NO: 1) sequences followed by the 45bp putative 5' UTR sequence (SEQ ID NO: 56) including the predicted transcription start site from the soybean native HPPD gene. In other embodiments, the core promoter comprises synthetic element II (SEQ ID NO: 22) or active fragments or variants thereof. Synthetic element II contains the Rsyn7 region derived from US Patent No. 6,072,050 SEQ ID NO: 2 immediately upstream of synthetic element I (SEQ ID NO: 22). SEQ ID NO: 21 and 22 were derived from SEQ ID NO: 1 and 2 of US Patent No. 6,072,050 which is herein incorporated by reference in its entirety. In particular embodiments, the core promoter comprises synthetic element III (SEQ ID NO: 83) or active fragments or variants thereof. Synthetic element III contains the Rsyn7 and SynII core.

Fragments and variants of the core promoter maintain core promoter activity. As used herein, by “core promoter activity” is intended the ability of the core promoter to modulate the regulatory activity of an operably linked regulatory region of an HPPD promoter when compared to the regulatory activity of the regulatory region of the

HPPD promoter in the absence of the core promoter. Alternatively, "core promoter activity" can refer to the ability of a core promoter to direct transcription of a polynucleotide of interest in the absence of a regulatory region of an HPPD promoter. For example, a core promoter when operably linked to a regulatory region of an HPPD promoter can either decrease or increase the transcriptional regulatory activity of the regulatory region of the HPPD promoter when compared to the transcriptional regulatory activity of the regulatory region of an HPPD promoter in the absence of the core promoter. Such an increase or decrease in transcriptional regulatory activity can include about a 0.1%, 0.5%, 1%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or greater increase or decrease in transcriptional regulatory activity when compared to the regulatory region of the HPPD promoter in the absence of the core promoter. Alternatively, the modulated activity of the regulatory region can include about a 0.5 fold, 1 fold, 2 fold, 4 fold, 8 fold, 16 fold, or 32 fold overall increase or decrease in transcriptional regulatory activity of the regulatory region of the HPPD promoter in the absence of the core promoter.

It is further recognized that the modulation of the transcriptional regulatory activity by the core promoter need not be an overall increase or decrease in activity, but also includes a change in tissue distribution of the regulatory activity.

In some embodiments, a core promoter is operably linked to a regulatory region of an HPPD promoter thereby modulating the transcriptional regulatory activity of the regulatory region. Any one of the polynucleotides set forth in any one of SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 77, or 78 or active variants or fragments thereof, can comprise a chimeric promoter.

C. Fragments and Variants

Fragments and variants of the disclosed HPPD promoters, chimeric promoters, core promoters and/or regulatory regions of an HPPD promoter are also encompassed herein. By "fragment" is intended a portion of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter. A fragment of a HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter may encode a biologically active portion of a HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter, or it may be a fragment that can be used as a hybridization probe, a PCR primer using methods

disclosed below, or may be combined with another DNA fragment to create a new promoter. A biologically active portion of a HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter can be prepared by isolating a polynucleotide segment of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter disclosed herein, and assessing the activity of the portion of the promoter polynucleotide. Polynucleotides that are fragments of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter 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 1,450 contiguous nucleotides, or up to the number of nucleotides present in a full-length HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter disclosed herein. In some embodiments, a fragment comprises an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter where 1-500, 500-1000, 1000-1500 or 1500-2000 nucleotides have been deleted from the 5' end of the promoter polynucleotide.

A fragment of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter can be a fragment of a variant of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter. For example, a fragment of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter encompasses fragments of polynucleotides having 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the promoter polynucleotides of the invention.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises 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" or "wild type" polynucleotide comprises a naturally occurring nucleotide sequence. Variants of the HPPD promoters, chimeric promoters, core promoters and/or regulatory regions of an HPPD promoter disclosed herein may retain activity of the HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter as described in detail elsewhere herein. Naturally occurring variant polynucleotides 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. Generally, variants of a HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter disclosed
5 herein will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein. Variant polynucleotides disclosed herein can also have 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%,
10 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to fragments of the HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter disclosed herein, including fragments where 1-500, 500-1000, 1000-1500 or 1500-2000 nucleotides of the 5' end of the HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD
15 promoter have been deleted, or where 1-500, 500-1000, 1000-1500 or 1500-2000 nucleotides of the 3' end of the HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter have been deleted.

Variant polynucleotides also encompass sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or
20 more different HPPD promoters, chimeric promoters, core promoters and/or regulatory regions of an HPPD promoter can be manipulated to create a new HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides
25 comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. 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; Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.*
30 (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

D. Sequence Comparison

The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: (a) "reference sequence", (b)

5 "comparison window", (c) "sequence identity", and, (d) "percentage of 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 promoter
10 sequence, or the complete cDNA or promoter sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide 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)
15 for optimal alignment of the two polynucleotides. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally 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 polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

20 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
25 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.

30 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 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 of the invention. 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, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix. 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 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 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 integer
5 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
10 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
15 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
20 polynucleotide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window,
25 wherein the portion of the 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 occurs in both sequences to yield the number of
30 matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

E. DNA Constructs

The various HPPD promoters, chimeric promoters, core promoters and/or regulatory regions of an HPPD promoter of the invention can be provided in DNA constructs or expression cassettes for expression in plants of interest. The cassette will include the HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter disclosed herein operably linked to a polynucleotide of interest. "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 an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter is a functional link that allows for expression of the polynucleotide of interest. An operable linkage between a regulatory region of an HPPD promoter and a core promoter is a linkage that allows the core promoter to modulate the transcriptional regulatory activity of the regulatory region of the HPPD promoter. 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 polynucleotide of interest to be under the transcriptional regulation of an HPPD promoter, chimeric promoter, core promoter and/or regulatory region of an HPPD promoter of the invention. The expression cassette may additionally contain selectable marker genes.

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

and/or chimeric 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 substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

It is recognized that expression cassettes comprising the various HPPD promoters and chimeric HPPD promoters disclosed herein can be generated *in vivo*. For example, the native HPPD gene can be modified *in vivo* such that the structure of the native HPPD promoter is altered to reflect one of the HPPD promoters disclosed herein. Methods for targeted mutagenesis *in vivo* are known. For example, a DNA sequence having the desired sequence alteration can be flanked by sequences homologous to the genomic target. One can then select or screen for a successful homologous recombination event. See, US Patent Number 5,527,695. Generally, such a vector construct is designed having two regions of homology to the genomic target which flank a polynucleotide having the desired sequence. Introduction of the vector into a plant cell will allow homologous recombination to occur and to produce an exchange of sequences between the homologous regions at the target site.

Such methods of homologous recombination can further be combined with agents that induce site-specific genomic double-stranded breaks in plant cells. Such double strand break agents can be engineered to produce the break at a targeted site and thereby enhance the homologous recombination events. See, for example, Puchta, *et al.*, (1996) *Proc Natl Acad Sci USA* 93:5055-5060; US Patent Application Publication Number 2005/0172365A1; US Patent Application Publication Number 2006/0282914, WO 2005/028942; WO 2004/067736 published August 12, 2004; US Patent Number 5,792,632; US Patent Number 6,610,545; Chevalier *et al.*, (2002) *Mol Cell* 10:895-905; Chevalier *et al.*, (2001) *Nucleic Acids Res* 29:3757-3774; Seligman *et al.*, (2002) *Nucleic Acids Res* 30:3870-3879; US Application publication 2009-0133152; and, WO 2005/049842, each of which is herein incorporated by reference in their entirety.

The HPPD promoter or chimeric promoter used to express a polynucleotide of interest can be selected based on the desired outcome. For example, the selection of the HPPD promoter or chimeric promoter used in the expression cassettes described herein can determine the level of expression along with the spatial expression and/or temporal expression pattern of an operably linked polynucleotide of interest in plants, plant cells or plant explants. HPPD promoters and/or chimeric promoters disclosed herein can be

selected to express polynucleotides of interest in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves, seeds, or flowers, for example) and the selection reflects the desired location of accumulation of the gene product. Alternatively, the HPPD promoters and/or chimeric promoters disclosed herein can be selected to drive expression of the HPPD polynucleotide under various inducing conditions. The HPPD promoters and/or chimeric promoters also vary in their strength, i.e., ability to promote transcription. In one embodiment, the HPPD promoter and/or chimeric promoter described herein is selected to express an HPPD polypeptide, having insensitivity to an HPPD inhibitor, in sufficient levels in a plant to impart tolerance of the plant to the HPPD inhibitor.

The termination region may be native with the transcriptional initiation region of interest, may be native with the operably linked polynucleotide of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the polynucleotide of interest, 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 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.

The HPPD promoter and/or chimeric promoter described herein can further be operably linked to additional regulatory elements that influence transcription, including, but not limited to, introns, 5' untranslated regions and enhancer elements. As used herein, an "enhancer sequence," "enhancer domain," "enhancer element," or "enhancer," when operably linked to an appropriate promoter, will modulate the level of transcription of an operably linked polynucleotide of interest. For example, the HPPD promoter and/or chimeric promoter described herein could be operably linked to the 35S enhancer as described in US 2007-0061917, herein incorporated by reference in its entirety. In specific embodiments, the enhancer of the invention can alter normal promoter expression patterns. Thus, the HPPD promoter and/or chimeric promoter described herein can further comprise at least one, two, three, four or more copies of an enhancer domain or an active variant or fragment of the domain.

The HPPD promoters and/or chimeric promoters described herein can further comprise additional portions of other regulatory regions. Thus, the HPPD promoters and/or chimeric promoters described herein can comprise upstream regulatory elements such as those responsible for tissue and temporal expression of the coding
5 polynucleotide of interest. In the context of this disclosure, the term "regulatory element" also refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene or polynucleotide of interest, which includes sequences which modulate the expression of the polynucleotide of interest. It is to be understood that nucleotide sequences, located within introns, or 3' of the polynucleotide
10 of interest may also contribute to the regulation of expression of a polynucleotide of interest. Examples of suitable introns include, but are not limited to, the maize IVS6 intron, the maize actin intron, or maize *adh1*. A regulatory element may also include those elements located downstream (3') to the site of transcription initiation, or within transcribed regions, or both. In the context of the present invention a post-
15 transcriptional regulatory element may include elements that are active following transcription initiation, for example translational and transcriptional enhancers, translational and transcriptional repressors, and mRNA stability determinants.

The expression cassettes may additionally contain 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the
20 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
25 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,
30 Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968.

In some embodiments, the expression cassettes disclosed herein contain a polynucleotide in which the 5' untranslated region (UTR) of the promoter polynucleotide has been partially or completely deleted. As used herein, the 5' UTR, or leader sequence, refers to a particular section of mRNA and the encoding DNA,

beginning at the transcription start site and ending one nucleotide before the first nucleotide of the start codon. The 5' UTR can also refer to the region of a promoter polynucleotide of the invention downstream of the transcriptional start site. The 5' UTR of the promoter polynucleotides of the invention can comprise at least 10, 15, 20, 5 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 250 or 500 consecutive nucleotides.

In some embodiments, the expression cassettes disclosed herein include cis-elements, for example the transcription factor sequences such as found in Rsyn7, to alter the pattern or strength of the transcriptional regulatory activity of the promoter (see Priest *et al.* (2009) *Curr. Opin. Plant Bio.* 12:643-649 and references described elsewhere herein all of which are hereby incorporated by reference). 10

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 15 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, substitutions, e.g., transitions and transversions, may be involved.

The expression cassette can also comprise a selectable marker gene for the 20 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, sulfonyleureas, 25 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 30 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 invention, including for example, DsRed as described in Examples 3 and 4 and Table 2.

20 F. Polynucleotides of Interest

Any polynucleotide of interest may be operably linked to the HPPD promoters and/or chimeric promoters disclosed herein. Such polynucleotides of interest include, but are not limited to, herbicide-tolerance coding sequences, insecticidal coding sequences, nematocidal coding sequences, antimicrobial coding sequences, antifungal coding sequences, antiviral coding sequences, abiotic and biotic stress tolerance coding sequences, or sequences modifying plant traits such as yield, grain quality, nutrient content, starch quality and quantity, nitrogen fixation and/or utilization, and oil content and/or composition. More specific polynucleotides of interest for the present invention include, but are not limited to, genes that improve crop yield, polypeptides that improve desirability of crops, genes encoding proteins conferring resistance to abiotic stress, such as drought, temperature, salinity, toxic metals or trace elements, or those conferring resistance to toxins such as pesticides and herbicides, or to biotic stress, such as attacks by fungi, viruses, bacteria, insects, and nematodes, and development of diseases associated with these organisms. It is recognized that any polynucleotides of

interest can be operably linked to the HPPD promoters and/or chimeric promoters of the invention and expressed in a plant. The expression level of the polynucleotide or polypeptide of interest may be measured directly, for example, by assaying for the level of the polypeptide or polynucleotide in the organism, or indirectly, for example, by
5 measuring the activity of the polypeptide or polypeptide in the organism. These nucleotide sequences of interest may encode proteins involved in providing disease or pest resistance. By "disease resistance" or "pest resistance" is intended that the plants avoid the harmful symptoms that are the outcome of the plant-pathogen interactions. Disease resistance and insect resistance genes such as lysozymes or cecropins for
10 antibacterial protection, or proteins such as defensins, glucanases or chitinases for antifungal protection, or *Bacillus thuringiensis* endotoxins, protease inhibitors, collagenases, lectins, or glycosidases for controlling nematodes or insects are all examples of useful gene products.

As used herein, the term "pest" includes, but is not limited to, insects, fungi,
15 bacteria, viruses, nematodes, mites, ticks, and the like. Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera, Lepidoptera, and Diptera. Viruses include but are not limited to tobacco or cucumber mosaic virus, ringspot virus,
20 necrosis virus, maize dwarf mosaic virus, etc. Nematodes include but are not limited to parasitic nematodes such as root knot, cyst, and lesion nematodes, including *Heterodera* spp., *Meloidogyne* spp., and *Globodera* spp.; particularly members of the cyst nematodes, including, but not limited to, *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); *Heterodera avenae* (cereal cyst
25 nematode); and *Globodera rostochiensis* and *Globodera pailida* (potato cyst nematodes). Lesion nematodes include but are not limited to *Pratylenchus* spp. Fungal pests include those that cause leaf, yellow, stripe and stem rusts.

An "herbicide resistance protein" or a protein resulting from expression of an "herbicide resistance-encoding nucleic acid molecule" includes proteins that confer
30 upon a cell the ability to tolerate a higher concentration of an herbicide than cells that do not express the protein, or to tolerate a certain concentration of an herbicide for a longer period of time than cells that do not express the protein. In one embodiment, the polynucleotide that confers tolerance to herbicide of interest comprises an ALS inhibitor tolerant polypeptide which confers tolerance of a dose of sulfonylurea,

imidazolinone, triazolopyrimidines, pyrimidinyoxy(thio)benzoates, and/or
sulfonamino-carbonyl-triazonine herbicide. Sulfonamide and imidazolinone
herbicides inhibit growth of higher plants by blocking acetolactate synthase (ALS), also
known as, acetohydroxy acid synthase (AHAS). For example, plants containing
5 particular mutations in ALS (*e.g.*, the S4 and/or HRA mutations) are tolerant to
sulfonamide herbicides. The production of sulfonamide-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
10 incorporated herein by reference in their entireties for all purposes. In specific
embodiments, the ALS inhibitor tolerant polypeptide comprises a sulfonamide-tolerant
acetolactate synthase, a sulfonamide-tolerant acetohydroxy acid synthase, an
imidazolinone-tolerant acetolactate synthase, or an imidazolinone-tolerant
acetohydroxy acid synthase.

15 Polynucleotides coding for resistance to herbicides that act to inhibit action of
glutamine synthase, such as phosphinothricin or basta (*e.g.*, the *bar* gene), glyphosate
(*e.g.*, the EPSPS gene and the *gat* gene; see, for example, U.S. Publication No.
20040082770 and WO 03/092360) or other such genes known in the art can also be
used. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes
20 resistance to the antibiotics kanamycin and geneticin, and the ALS gene mutants
encode resistance to the herbicide chlorsulfuron.

Glyphosate resistance is imparted by mutant 5-enolpyruvyl-3-phosphokimate
synthase (EPSP) and *aroA* genes. See, for example, U.S. Patent No. 4,940,835 to Shah
et al., which discloses the nucleotide sequence of a form of EPSPS which can confer
25 glyphosate resistance. U.S. Patent No. 5,627,061 to Barry et al. also describes genes
encoding EPSPS enzymes. See also U.S. Patent Nos. 6,248,876 B1; 6,040,497;
5,804,425; 5,633,435; 5,145,783; 4,971,908; 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
30 97/04103; WO 97/04114; WO 00/66746; WO 01/66704; WO 00/66747 and WO
00/66748, which are incorporated herein by reference for this purpose. Glyphosate
resistance is also imparted to plants that express a gene that encodes a glyphosate
oxido-reductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and
5,463,175, which are incorporated herein by reference for this purpose. In addition

glyphosate resistance can be imparted to plants by the over expression of genes encoding glyphosate N-acetyltransferase. See, for example, U.S. Patent Nos 7,462,481, 7,405,074, and 7,803,992 each of which are herein incorporated by reference.

Polypeptides conferring tolerance to herbicides which inhibit the enzyme
5 glutamine synthase, such as phosphinothricin or glufosinate (e.g., the bar gene) can also be used. Glutamine synthetase (GS) appears to be an essential enzyme necessary for the development and life of most plant cells, and inhibitors of GS are toxic to plant cells. Glufosinate herbicides have been developed based on the toxic effect due to the inhibition of GS in plants. These herbicides are non-selective; that is, they inhibit
10 growth of all the different species of plants present. The development of plants containing an exogenous phosphinothricin acetyltransferase is 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, which are incorporated herein by reference in their entireties for all purposes. Mutated phosphinothricin acetyltransferase having this
15 activity are also disclosed.

In still other embodiments, polypeptides conferring tolerance to herbicides which inhibit protox (protoporphyrinogen oxidase) can be used. Protox is necessary for the production of chlorophyll, which is necessary for all plant survival. The protox enzyme serves as the target for a variety of herbicidal compounds. These herbicides
20 also inhibit growth of all the different species of plants present. The development of plants containing altered protox activity which are resistant to these herbicides are described in U.S. Patent Nos. 6,288,306; 6,282,837; and 5,767,373; and international publication WO 01/12825, which are incorporated herein by reference in their entireties for all purposes.

In still other embodiments, polypeptides involving other modes of herbicide
25 resistance are employed. For example, hydroxyphenylpyruvatedioxygenases are enzymes that catalyze the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. Molecules which inhibit this enzyme and which bind to the enzyme in order to inhibit transformation of the HPP into homogentisate are
30 useful as herbicides. Plants more resistant to certain herbicides are described in U.S. Patent Nos. 6,245,968; 6,268,549; and 6,069,115; and international publication WO 99/23886, which are incorporated herein by reference in their entireties for all purposes. Mutated hydroxyphenylpyruvatedioxygenase insensitive to HPPD inhibitors are also disclosed. For example, those polynucleotides disclosed in US Utility Application

_____, entitled "Compositions and Methods Comprising Sequences Having Hydroxyphenylpyruvate Dioxygenase (HPPD) Activity" filed concurrently herewith and herein incorporated by reference may be operably linked to the HPPD promoters and/or chimeric promoters disclosed herein. Polynucleotides such as *aad1*, *aad12*, and
5 *dmo* can be employed for their herbicide resistance properties. See for example, US Patent No. 7,838,733 and 7,884,262.

Additional herbicides, include but are not limited to, an acetyl Co-A carboxylase inhibitor such as quizalofop-P-ethyl, a synthetic auxin such as quinclorac, a protoporphyrinogen oxidase (PPO) inhibitor herbicide (such as sulfentrazone), a
10 pigment synthesis inhibitor herbicide such as a hydroxyphenylpyruvate dioxygenase inhibitor (*e.g.*, mesotrione or sulcotrione), a phosphinothricin acetyltransferase or a phytoene desaturase inhibitor like diflufenican, pigment synthesis inhibitor, auxin herbicides such as 2,4-D and dicamba, see for example US Patent No. 5,877,115 and 5,175,353.

15 Polynucleotides that improve crop yield include dwarfing genes, such as *Rht1* and *Rht2* (Peng *et al.* (1999) *Nature* 400:256-261), and those that increase plant growth, such as ammonium-inducible glutamate dehydrogenase. Polynucleotides that improve desirability of crops include, for example, those that allow plants to have a reduced saturated fat content, those that boost the nutritional value of plants, and those
20 that increase grain protein. Polynucleotides that improve salt tolerance are those that increase or allow plant growth in an environment of higher salinity than the native environment of the plant into which the salt-tolerant gene(s) has been introduced.

Polynucleotides that influence amino acid biosynthesis include, for example, anthranilate synthase (AS; EC 4.1.3.27) which catalyzes the first reaction branching
25 from the aromatic amino acid pathway to the biosynthesis of tryptophan in plants, fungi, and bacteria. In plants, the chemical processes for the biosynthesis of tryptophan are compartmentalized in the chloroplast. See, for example, US Pub. 20080050506, herein incorporated by reference. Additional sequences of interest include Chorismate Pyruvate Lyase (CPL) which refers to a gene encoding an enzyme which catalyzes the
30 conversion of chorismate to pyruvate and pHBA. The most well characterized CPL gene has been isolated from *E. coli* and bears the GenBank accession number M96268. See, US Patent No. 7,361,811, herein incorporated by reference.

As noted, the polynucleotide of interest operably linked to an HPPD promoter and/or a chimeric promoter as described herein may encode a suppression element,

such as an RNAi element. "RNAi" refers to a series of related techniques to reduce the expression of genes (See for example U.S. Patent No. 6,506,559). Older techniques referred to by other names are now thought to rely on the same mechanism, but are given different names in the literature. These include "antisense inhibition," the
5 production of antisense RNA transcripts capable of suppressing the expression of the target protein, and "co-suppression" or "sense-suppression," which refer to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference). Such techniques rely on the use of constructs
10 resulting in the accumulation of double stranded RNA with one strand complementary to the target gene to be silenced.

i. HPPD polynucleotides of interest

The HPPD polypeptide converts hydroxyphenylpyruvate, derived from the
15 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
20 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 are of significant value, especially for managing weed resistance to glyphosate.

25 As used herein, "Hydroxyphenylpyruvate dioxygenase" and "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. In organisms that degrade tyrosine, the
30 reaction catalyzed by HPPD is the second step in the tyrosine degradation pathway. In plants, formation of homogentisate is necessary for the synthesis of plastoquinone, an essential redox cofactor, and tocopherol.

Various variants of HPPD sequences are also known. See, for example, US Provisional Application 61/401,456, filed August 13, 2010, *Compositions and Methods*

Comprising Sequences having Hydroxyphenylpyruvate Dioxygenase (HPPD) Activity, herein incorporated by reference in its entirety. See, also, US 2003/0066102, WO97/49816, US 2010/0197503, US Patent No. 7,312,379, US Patent No. 6,768,044, US Patent No. 6,245,698, US Patent No. 6,268,549, and US Patent No. 6,118,050, the contents of each is herein incorporated by reference in its entirety. A review of the various structures of HPPD polypeptides from microbes, mammals and plants can be found, for example, in Moran *et al.* (2005) *Archives of Biochemistry and Biophysics* 433:117-128, herein incorporated by reference in its entirety.

HPPD polynucleotides may further comprise a chloroplast transit peptide (CTP) sequence to direct the transport of the expressed polypeptide to the chloroplast. CTP sequences are known. In one embodiment, the CTP sequence employed comprises a CTP as disclosed in U.S. Provisional application No. 61/393,507, entitled "*Methods and Compositions for Targeting Sequences of Interest to the Chloroplast*", filed October 15, 2010.

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 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 maintains the viability of the cell. As used herein, an "HPPD polynucleotide" refers to a polynucleotide encoding an active HPPD polypeptide.

HPPD activity can be assayed by utilizing the differences in light absorbance properties between 4-hydroxyphenylpyruvate (HPP) and maleylacetoacetate. HPPD catalyzes the conversion of HPP to homogentisate and homogentisate dioxygenase (HGD) catalyzes the conversion of homogentisate into maleylacetoacetate. While no difference in absorbance exists between HPP and homogentisate, a difference in absorbance can be observed at 320nm between HPP and maleylacetoacetate. Thus, by combining HPP with both HPPD and HGD under the appropriate reaction conditions HPPD activity can be assayed.

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, WO2005/053407 herein incorporated by reference.

HPPD promoters and/or chimeric promoters disclosed herein can express HPPD polypeptides in different levels to provide different levels of tolerance to different HPPD-inhibitor herbicides. While a given promoter may provide a useful level of expression to offer tolerance to some HPPD-inhibitor herbicides it may be quite inadequate to provide expression levels 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, various promoters and HPPD polypeptides 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. Thus, in specific embodiments, the promoters and chimeric promoters disclosed herein are operably linked to an HPPD polynucleotide in order to allow sufficient expression of an active HPPD polypeptide, insensitive to HPPD inhibitors, to impart HPPD tolerance to the plant.

The insensitivity to an HPPD inhibitor can be determined by assaying the insensitivity of a cell, a plant, a plant cell expressing the HPPD polypeptide or active fragment or variant thereof. In such instances, the cell, plant, or plant cell expressing an HPPD sequence displays an insensitivity to an 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 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 "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.

5 G. Plants

Plants, plant cells, plant parts and seeds, and grain having the HPPD promoter and/or the chimeric promoter disclosed herein are provided. In specific embodiments, the plants and/or plant parts have stably incorporated at least one heterologous HPPD promoter or chimeric promoter disclosed herein or an active variant or fragment
10 thereof. Thus, plants, plant cells, plant parts and seed are provided which comprise at least one heterologous HPPD promoter as set forth in any one of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76 or any one of other active fragments or variants disclosed herein. In another embodiment, the plants and/or plant parts have stably incorporated at least one heterologous chimeric promoter as
15 disclosed herein or active variants or fragments thereof. Thus plants, plant cells, plant parts and seeds are provided which comprise at least one heterologous chimeric promoter as set forth in any on of SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 77, or 78 or active variants or fragments thereof. In specific embodiments, the HPPD promoter sequences are characterized as having
20 transcriptional regulatory activity such that when the promoter sequence is operably linked to an HPPD polynucleotide having HPPD activity and insensitivity to an HPPD inhibitor, the HPPD polynucleotide is expressed in sufficient levels to impart tolerance of the plant to an HPPD inhibitor.

As used herein, the term plant includes plant cells, plant protoplasts, plant cell
25 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. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species.
30 Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides.

The promoter sequences and active variant 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.,
5 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*),
10 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*),
15 almond (*Prunus amygdalus*), sugar 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 (*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 the present invention include, for
25 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 (*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
30 (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*), and Poplar and Eucalyptus. In specific embodiments, plants of the present invention are 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 soybean 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, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants
5 include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

In some embodiments, the polynucleotides comprising the HPPD promoter or the chimeric promoters operably linked to the polynucleotide encoding the polypeptide of interest are engineered into a molecular stack. Thus, the various plants, plant cells
10 and seeds 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.

15 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
20 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 promoters. In certain cases, it may be desirable to introduce a transformation
25 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, WO99/25821,
30 WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

Thus, in specific embodiments, the promoters and chimeric promoters disclosed herein are operably linked to an HPPD polynucleotide in order to allow sufficient expression of an active HPPD polypeptide, insensitive to HPPD inhibitors, to impart

HPPD tolerance to the plant. Such constructs can then be stacked with any other sequence of interest, including any other herbicide tolerance conferring sequences. Non-limiting examples of such sequences are disclosed elsewhere herein.

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 and seeds 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.

When one wants to express HPPD polypeptides, one can express such polypeptides as a stack, with promoters such as HPPD promoter polynucleotides, chimeric promoters, or any combination thereof. Thus, in one embodiment, the plants, plant cells or plant part having the promoters and chimeric promoters described herein operably linked to an HPPD polynucleotide or active variants or fragments thereof is stacked with at least one other HPPD sequence. Such 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.

In still other embodiments, plants, plant cells, explants and expression cassettes comprising the promoters and chimeric promoters described herein operably linked to 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 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, the plant or plant cells having the promoters and chimeric promoters described herein operably linked to HPPD polynucleotides or active variants or fragment thereof may be stacked with other herbicide-tolerance traits to create a transgenic plant of the invention with further improved properties. Other 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-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; 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 include those conferring tolerance to sulfonylurea and/or imidazolinone, for example, as 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.

In other embodiments, the plants or plant cell or plant part having the promoters and chimeric promoters described herein operably linked to an HPPD sequence or an active variant or fragment thereof is stacked with, for example, a sequence which confers tolerance to an 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 example, sulfonylurea, imidazolinone, triazolopyrimidines, pyrimidinyoxy(thio)benzoates, and/or sulfonylaminocarbonyltriazolinone 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 tolerance to all four
5 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
10 nucleotide mutation resulting in one 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,
15 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
20 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.

25 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,
30 which are incorporated herein by reference in their entirety 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, the plants or plant cell or plant part having the promoters and chimeric promoters described herein operably linked to an HPPD sequence or an active variant or fragment thereof is stacked with, or example, a sequence which confers tolerance to an ALS inhibitor and glyphosate tolerance. In one
5 embodiment, the promoters described hererin operably linked to a 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, the plant or plant cell or plant part having the
10 promoters and chimeric promoters described herein operably linked to a 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
15 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 promoters and chimeric promoters described herein operably linked to a HPPD sequence or an active variant or fragment thereof
20 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
25 synthase. Other examples of herbicide-tolerance traits that could be combined with the plants or plant cell or plant part having the promoters and chimeric promoters described herein operably linked to a 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;
30 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 promoters and chimeric promoters described

herein operably linked to a 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 invention as well as methods of use thereof.

In still further embodiments, the HPPD sequences operably linked to the promoters and chimeric promoters described herein 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.

The plant or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof operably linked to the promoters and chimeric promoters described herein 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 OLC (high oleic acid content; see, *e.g.*, Fernandez-Moya *et al.* (2005) *J. Agric. Food Chem.* 53: 5326-5330).

The plant or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof operably linked to the promoters and chimeric promoters described herein 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, the plant or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof operably linked to the promoters and chimeric promoters described herein 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.

In another embodiment, the plant or plant cell or plant part having the HPPD sequence or an active variant or fragment thereof operably linked to the promoters and chimeric promoters described herein can also be combined with the Rcg1 sequence or biologically active variant or fragment thereof. The Rcg1 sequence is an anthracnose

stalk rot resistance gene in corn. See, for example, U.S. Patent Application No. 11/397,153, 11/397,275, and 11/397,247, each of which is herein incorporated by reference.

5 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.

10 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 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

15 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, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein

20 incorporated by reference.

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 cell so altered and which comprises the alteration. A "control" or "control plant" or "control plant cell" provides a reference point for

25 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

30 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.

II. Methods of Introducing

5 The methods of the invention comprise regulating the expression of a polynucleotide of interest by stably incorporating a polynucleotide comprising a HPPD promoter and/or a chimeric promoter disclosed herein into the genome of a plant or plant cell. The methods provided herein do not depend on a particular method for introducing a sequence into the host cell, only that the polynucleotide gains access to
10 the interior of at least one cell of the host. Methods for introducing polynucleotides into host cells (i.e., plants) are known in the art and include, but are not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

"Stable transformation" is intended to mean that the nucleotide construct
15 introduced into a host (i.e., 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 host (i.e., a plant) and expressed temporally.

Transformation protocols as well as protocols for introducing polynucleotide
20 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 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 transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, 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, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via
25 Microprojectile Bombardment," 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*
 5 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes,
 U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646;
 Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile
 Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed.
 Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol.*
 10 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-
 Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bowen *et al.*, 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*
 15 *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
 20 are herein incorporated by reference. Methods of homologous recombination can also
 be employed. See, for example, US Publication No. 2010-0192253 and
 WO20051049842

In specific embodiments, the HPPD promoter and/or chimeric promoter
 disclosed herein can be provided to a plant using a variety of transient transformation
 25 methods. Such transient transformation methods include, but are not limited to, the
 introduction of the promoter polynucleotides or variants and fragments thereof directly
 into the plant. 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.*
 30 *91*: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science* 107:775-784, all of
 which are herein incorporated by reference. Alternatively, the HPPD promoter
 polynucleotides can be transiently transformed into the plant using techniques known in
 the art. Such techniques include viral vector system and the precipitation of the
 polynucleotide in a manner that precludes subsequent release of the DNA. Thus, the

transcription from the particle-bound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use particles coated with polyethylimine (PEI; Sigma #P3143).

In other embodiments, HPPD promoter and/or chimeric promoter disclosed
5 herein may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. 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.
10 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
15 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, the polynucleotide of the invention can be contained in transfer cassette flanked by two non-identical recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its genome a target site which is flanked by
20 two non-identical 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.

The cells that have been transformed may be grown into plants in accordance
25 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
30 stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having an HPPD promoter and/or chimeric promoter disclosed herein, stably incorporated into their genome.

III. Methods of Use

A method for modulating the expression of a polynucleotide of interest is provided. Such methods comprise stably incorporating in the genome of a plant or
5 plant cell a polynucleotide sequence of interest operably linked to an HPPD promoter and/or chimeric promoter as described herein.

Depending on the polynucleotide of interest operably linked to the HPPD promoter and/or chimeric promoter as described herein, the transgenic plants, plant cells or seeds may have a change in phenotype, including, but not limited to, an altered
10 pathogen or insect defense mechanism, an increased resistance to one or more herbicides, an increased ability to withstand stressful environmental conditions, a modified ability to produce starch, a modified level of starch production, a modified oil content and/or composition, a modified ability to utilize, partition and/or store nitrogen, and the like.

15 In specific embodiments, the HPPD promoters and/or chimeric promoters of the invention modulate expression of an HPPD polynucleotide. Methods disclosed herein employ the HPPD promoters and/or chimeric promoters of the invention to express polynucleotides of interest encoding polypeptides conferring tolerance to herbicides, as described in detail elsewhere herein. In specific embodiments, the HPPD promoters
20 and/or chimeric promoters of the invention modulate expression of a polynucleotide of interest in any plant. In specific embodiments, the plant comprises a dicot, and in further embodiments the dicot is soybean.

In some embodiments, the promoters and chimeric promoters disclosed herein modulate expression of an HPPD polynucleotide encoding an HPPD polypeptide
25 having HPPD activity and having insensitivity to HPPD inhibitors such that the plant is tolerant to the HPPD inhibitors. Thus, a method of controlling weeds is provided comprising planting a plant or seed comprising the chimeric promoter and/or HPPD promoter disclosed herein operably linked to a polynucleotide encoding an HPPD polypeptide that is insensitive to an HPPD inhibitor and retains HPPD activity. An
30 effective amount of an HPPD inhibitor is applied to the seed, plant, and/or area of cultivation, such that the growth of the weeds in the field is controlled and/or prevented and/or reduced. In such an embodiment, the transcriptional regulatory activity of the HPPD promoter or the chimeric promoter is sufficient to direct transcription of the

HPPD polypeptide at sufficient levels and/or at a specific temporal and/or tissue specific matter to confer to the plant tolerance to the HPPD inhibitor.

Soybean HPPD proteins and methods of use

5 Compositions are further provided comprising the soybean HPPD protein comprising the native soybean HPPD CTP, the polynucleotide encoding the same, and active variants and fragments thereof. Such sequences include the polynucleotide set forth in SEQ ID NO: 57 and the polypeptide set forth in SEQ ID NO: 58, and active variants and fragments thereof. Such polypeptides are capable of being transported into
10 the chloroplast of a plant cell. In some embodiments, the polynucleotide set forth in SEQ ID NO: 57 or an active variant or fragment thereof is operably linked to a heterologous promoter. See, for example, US Utility Application _____, entitled “Compositions and Methods Comprising Sequences Having Hydroxyphenylpyruvate Dioxygenase (HPPD) Activity” filed concurrently herewith and herein incorporated by
15 reference.

In specific embodiments, active fragments and variants of the HPPD sequence as set forth in SEQ ID NO: 57 are provided. Such fragments 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 1,450 contiguous nucleotides, or up to the number of
20 nucleotides present in SEQ ID NO: 57. Generally, variants of SEQ ID NO: 57 will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 57 as determined by sequence alignment programs and parameters described elsewhere herein. Active fragments and variants of SEQ ID NO: 57 will continue to
25 encode a polypeptide having HPPD activity and which can be transported into the chloroplast of a plant cell.

The HPPD promoter as described in SEQ ID NO:1 leads to the production of at least two major transcripts from at least two transcription start sites (TSS1 and TSS2, see Figure 5). The longer transcript initiates SEQ ID NO: 57 (encoding SEQ ID NO:
30 58). Parts of the genomic sequence transcribed to produce the longer transcript also act to promote transcriptional regulatory activity for the shorter transcript that initiates SEQ ID NO: 79 (encoding SEQ ID NO: 61). Various polynucleotide sequences are known in the art comprising multiple transcriptional start sites that encode products targeted to multiple cellular compartments. See for example, Small, *Plant Mol. Biol.*,

1998, 38:265-277 and Thatcher, *J of Biol. Chem.*, 2007, 282:28915-28928. SEQ ID NO: 58 polypeptide is localized to the chloroplast, while SEQ ID NO: 61 polypeptide is localized to the cytosol.

Further provided are variant HPPD proteins as set forth in SEQ ID NO: 58.

5 “Variant” protein is intended to mean a protein derived from the native protein by 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 by the present invention are biologically active, that is they continue to possess the desired biological activity of the native
10 protein, that is, HPPD activity and wherein the protein is transported into the chloroplast of a plant cell. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a HPPD proteins disclosed herein will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or
15 more sequence identity to the amino acid sequence set forth in SEQ ID NO: 58 as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the invention may differ from SEQ ID NO: 58 by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

20 Fragments of amino acid sequences include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of a HPPD protein, or a partial-length protein and exhibiting HPPD activity but which include fewer amino acids than the full-length HPPD-related proteins disclosed herein. A biologically active portion of a HPPD protein can be a polypeptide that is, for example,
25 10, 25, 50, 100, 150, 200 contiguous amino acids in length, or up to the total number of amino acids present in a full-length HPPD protein of the current invention (i.e., of SEQ ID NO: 58). Such biologically active portions can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native HPPD protein, including but not limited to transport into the chloroplast of a plant cell. As
30 used herein, a fragment comprises at least 5 contiguous amino acids of SEQ ID NO: 58. The invention encompasses other fragments, however, such as any fragment in the protein greater than 6, 7, 8, or 9 amino acids.

The polynucleotide encoding SEQ ID NO: 58 or active fragments and variants thereof can be provided in an expression cassette for expression in a plant or organism

of interest. The expression cassette can include 5' and 3' regulatory sequences operably linked to the polynucleotide of the invention. 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. In some embodiments, the polynucleotide set forth in SEQ ID NO: 57 can be operably linked to a heterologous promoter. 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 polynucleotide to be cotransformed into the organism. Alternatively, the additional polypeptide(s) can be provided on multiple expression cassettes. Expression cassettes can be provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

Further provided are plants, plant cells, and seeds having a heterologous polynucleotide construct comprising an expression cassette having a promoter operably linked to a polynucleotide encoding the polypeptide set forth in SEQ ID NO: 58 or an active variant or fragment thereof, wherein the promoter is heterologous to said polynucleotide.

20

Chloroplast Transit Peptides

The compositions provided herein further include recombinant polynucleotides comprising a nucleotide sequence encoding a novel chloroplast transit peptide (CTP) operably linked to a nucleotide sequence encoding a polypeptide of interest. In one embodiment, the CTP comprises the polypeptide sequence set forth in SEQ ID NO: 60, or active variants or fragments thereof, or comprises the polynucleotide sequence as set forth in SEQ ID NO: 59, or active variants and fragments thereof. Such CTP-encoding sequences, when assembled within a DNA construct such that the CTP-encoding sequence is operably linked to a nucleotide sequence encoding the polypeptide of interest, facilitate co-translational or post-translational transport of the peptide of interest to the chloroplast of a plant cell. See, for example, US Utility Application _____, entitled "Methods and Compositions for Targeting Sequences of Interest to a Chloroplast" filed concurrently herewith and herein incorporated by reference.

30

Fragments and variants of the CTP-sequences (i.e. SEQ ID NO: 59 and 60) are also encompassed herein. 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 CTP activity when
5 reconstituted in a CTP and are thus capable of facilitating the translocation of a polypeptide of interest into the chloroplast of a plant. Thus, fragments of a nucleotide sequence may range from at least about 10, 20, 30, 40, 50, 60, 70, 80 nucleotides or up to the full length CTP.

A fragment of a polynucleotide that encodes a biologically active portion of a
10 CTP-polypeptide will encode at least 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 contiguous amino acids, or up to the total number of amino acids present in SEQ ID NO: 60.

"Variant" CTP is intended to mean a protein derived from the CTP (i.e. SEQ
15 ID NO: 60) 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 CTP and/or substitution of one or more amino acids at one or more sites in the CTP, and/or substitution of one or more of the N-terminal, central, or C-terminal domains of the CTP and/or substitution of a portion of one or more of the N-terminal, central, or C-
20 terminal domains of the CTP. Variant proteins encompassed are biologically active, that is they continue to possess the desired biological activity of the CTP, that is, have CTP activity when reconstituted in a CTP. Such variants may result from, for example, genetic polymorphism or from human manipulation.

Biologically active variants of a CTP provided herein (and the polynucleotide
25 encoding the same) will have at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the polypeptide of SEQ ID NO: 60 or to any N-terminal domain or portion thereof, any central domain or portion thereof or any C-terminal domain or portion thereof from any one of SEQ ID NOS: 60 or any
30 of the other CTPs disclosed herein. Variants of CTP polynucleotides provided herein will have at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the polynucleotide of SEQ ID NO: 59, and encode an active CTP.

The CTP-sequences 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 CTPs can be prepared by

5 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.

10 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.

15 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

20 derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different CTP-sequences can be manipulated to create a new CTP 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

25 homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the CTP sequences disclosed herein and other known CTPs to obtain a new polynucleotide coding for a polypeptide with an improved property of interest, such as an improved efficiency of transport to the chloroplast. Strategies for such DNA shuffling are known

30 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.

The nucleotide sequences encoding the CTPs disclosed herein (i.e., SEQ ID NO: 60, 59 or active variant or fragments thereof) can be operably linked to any polynucleotide of interest. Such constructs, when operably linked to a promoter active in a plant, will allow for the translocation of the encoded polypeptide to the chloroplast.

5 Thus, further provided are plants, plant cells and seed having stably incorporated into their genome a DNA construct comprising a promoter active in a plant operably linked to a nucleotide sequences encoding the CTP sequences disclosed herein (i.e., SEQ ID NO: 60, 59 or active variant or fragments thereof) operably linked to any polynucleotide of interest.

10 The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

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

15 individually indicated to be incorporated by reference.

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

20 claims.

Table 1.

SEQ ID NO:	Description
1	Nucleotide sequence of native Glycine max HPPD region 5' to annotated ATG gene coding region start site, comprising the HPPD promoter.
2	SHP101C, 613 nucleotide deletion in the 3'end of seq. 1 (with KpnI site added at the end)
3	SHP102C, 485 nucleotide deletion in the 3'end of seq. 1 (with KpnI site added at the end)
4	SHP103C, 122 nucleotide deletion in the 3'end of seq. 1 (with KpnI site added at the end)
5	SHP104C, A to C point mutation of seq. 1 at position -20 relative to putative transcription start site (with KpnI site added at the end).
6	SHP105C, T to C point mutation of seq. 5 at position -563 relative to the putative transcription site (with KpnI site added at the end).
7	SHP106C, T to G point mutation of seq. 6 at position -190 relative to the

	putative transcription site (with KpnI site added at the end).
8	SHP107C, TAT to GCA mutation of seq. 1 at position -21 relative to the putative transcription site (with KpnI site added at the end).
9	SHP108C, ATA to CCG mutation of seq. 8 at position -564 relative to the putative transcription site (with KpnI site added at the end).
10	SHP109C, TAT to CAG mutation of seq. 9 at position -192 relative to the putative transcription site (with KpnI site added at the end).
11	SHP110C, 227 nucleotide deletion in the 3' end of seq. 1 (with KpnI site added at the end).
12	SHP111C, TAT to CAG mutation of seq. 1 at position -192 relative to the putative transcription site (with KpnI site added at the end).
13	SHP112C, TAT to CAG mutation of seq. 1 at position -396 relative to the putative transcription site (with KpnI site added at the end).
14	SHP113C, TAT to CAG mutation of seq. 1 at position -263 relative to the putative transcription site (with KpnI site added at the end).
15	SHP114C, TAT to CAG mutation of seq. 8 at position -263 relative to the putative transcription site (with KpnI site added at the end).
16	SHP115C, TAT to CAG mutation of seq. 12 at position -263 relative to the putative transcription site (with KpnI site added at the end).
17	SHP116C, TAT to CAG mutation of seq. 10 at position -263 relative to the putative transcription site (with KpnI site added at the end).
18	SHP117C, TAT to CAG mutation of seq. 8 at position -396 relative to the putative transcription site (with KpnI site added at the end).
19	SHP118C, TAT to CAG mutation of seq. 17 at position -396 relative to the putative transcription site (with KpnI site added at the end).
20	SHP0C, 314 nucleotide deletion in the 3' end of seq. 1 (with KpnI site added at the end).
21	Element I including SynII core and soy 5'UTR (with KpnI site added at the end).
22	Element II including Rsyn7, SynII core, and soy 5'UTR (with KpnI site added at the end).
23	SHP101, seq. 21 joined at 3' end of seq. 2.
24	SHP102, seq. 21 joined at 3' end of seq. 3.
25	SHP103, seq. 21 joined at 3' end of seq. 4.
26	SHp104, seq. 21 joined at 3' end of seq. 5.
27	shp105, seq. 21 joined at 3' end of seq. 6.
28	Shp106, seq. 21 joined at 3' end of seq. 7.
29	shp107, seq. 21 joined at 3' end of seq. 8.
30	shp108, seq. 21 joined at 3' end of seq. 9.
31	shp109, seq. 21 joined at 3' end of seq. 10.
32	shp201, seq. 22 joined at 3' end of seq. 2.
33	shp202, seq. 22 joined at 3' end of seq. 3.
34	shp203, seq. 22 joined at 3' end of seq. 4.
35	shp204, seq. 22 joined at 3' end of seq. 5.
36	shp205, seq. 22 joined at 3' end of seq. 6.
37	shp206, seq. 22 joined at 3' end of seq. 7.
38	shp207, seq. 22 joined at 3' end of seq. 8.
39	shp208, seq. 22 joined at 3' end of seq. 9.
40	shp209, seq. 22 joined at 3' end of seq. 10.

41	SHP110, TATA5 of SEQ ID NO: 1 is replaced by the partial SynII core (SEQ ID NO 71)
42	Forward primer: GCAAGTATTTCAATACAATAGC
43	Reverse primer: GTTATCTGATATGATGTTGC
44	TATA1: GTATAAATAA
45	TATA2: CCAATATATG
46	TATA3: CCTTATATATC
47	TATA4: TATATAATAA
48	TATA5: GAATATAAG
49	5' fragment of "long" HPPD protein: GTAATAAAAAAAGAGAGAAGCCGCATCAA
50	5' fragment of "short" HPPD protein: AAGCAGCAGCATCACACCACACCAATGCC
51	Forward primer hp0234: GTTTTCCGCGGGTGTGATCC
52	Reverse primer hp2296: TCATTGGTACCTGGTGTGGTGTGATGCTGC
53	Reverse primer hp2154: AGCATGGTACCTTGCGTCTGGGTTGAG
54	Reverse primer hp1962: AGGAGGTACCGTCAAATCCACCTAG
55	Reverse primer hp1663: TCCTTGGTACCTGATGCACTATATAACG
56	Putative 5' UTR: ACAACCACCAAGCTCAATCTCAAGCAGCAGCATCACACCACACCA
57	Nucleotide sequence of the soybean HPPD polynucleotide (including the region encoding the native CTP) predicted from the longer transcript (5' TSS)
58	Amino Acid sequence of the soybean HPPD including the native CTP
59	5' region of SEQ ID NO: 57 encoding native CTP
60	N-terminal region of SEQ ID NO: 58 comprising native soybean CTP
61	Soybean HPPD protein predicted from shorter transcript (3' TSS)
62	Reverse primer hp2048: ATCTGGTACCTGATGTTGATGCGGC
63	Reverse primer hp1791: AGCCTGGTACCTTGTGTGTAAAAAAGATAAGAC
64	Upstream HPPD genomic sequence of <i>Arabidopsis thaliana</i>
65	Upstream HPPD genomic sequence of <i>Medicago truncatula</i>
66	Upstream HPPD genomic sequence of Poplar
67	Upstream HPPD genomic sequence of <i>Brassica rapa</i>
68	Upstream genomic sequence of <i>Vitis vinifera</i>
69	Upstream HPPD genomic sequence of <i>Sorghum bicolor</i>
70	SynII core (SEQ ID NO: 1 from US 6,072,050)
71	Partial SynII core sequence (containing TATA box and transcriptional start site) used to construct Element I and II
72	Predicted HPPD 5'UTR
73	Rsyn7 (US6072050 SEQ ID 2)
74	SHP120C; A to T mutation at nucleotide 1945 of SEQ ID NO: 1; this eliminates the start codon of the upORF element
75	SHP121C; A to T mutation at nucleotide 2128 of SEQ ID NO: 1; this introduces a translation stop codon just upstream of the +7 transcription start site
76	SHP122C; deletion of everything downstream of nucleotide 2040 in SEQ ID NO: 1
77	SHP111; deletion of TATA3 through transcription start site at +7 (nucleotide 2106-2140 of SEQ ID NO: 1) and replaced with the Partial SynII Core

78	SHP210; deletion of TATA5 through transcription start site at -231 (1867-1905 of SEQ ID NO: 1) and replaced with Element III (SEQ ID NO: 83)
79	Nucleotide sequence of the soybean HPPD protein predicted from the shorter transcript (3' TSS)
80	Amino acid sequence of N-terminal end of longer HPPD transcript (<i>G. max</i>)
81	Predicted upstream ORF in longer HPPD transcript (<i>G. max</i>)
82	Amino acid sequence of N-terminal end of shorter HPPD transcript (<i>G. max</i>)
83	Element III, partial Element II (SEQ ID 22) sequence including Rsyn7 and SynII Core

Non-limiting embodiments include:

1. A chimeric promoter construct comprising
 - a first polynucleotide comprising a regulatory region of a 4-hydroxyphenylpyruvate dioxygenase (HPPD) promoter operably linked to a second polynucleotide comprising a heterologous core promoter functional in a plant, wherein said core promoter modulates the regulatory activity of said regulatory region of the HPPD promoter when compared to the regulatory activity of said regulatory region of the HPPD promoter alone;
 - wherein said chimeric promoter has transcriptional regulatory activity in a plant.
2. The chimeric promoter construct of embodiment 1, wherein said regulatory region of the HPPD promoter comprises a variant or a fragment of SEQ ID NO:1.
3. The chimeric promoter construct of embodiment 2, wherein said transcriptional regulatory activity of said regulatory region of the HPPD promoter in the absence of said core promoter comprises less than 10% of the transcriptional regulatory activity of the HPPD promoter set forth in SEQ ID NO: 1.
4. The chimeric promoter construct of embodiment 2, wherein said transcriptional regulatory activity of said regulatory region of the HPPD promoter in the absence of said core promoter comprises at least 10% of the regulatory activity of the HPPD promoter as set forth in SEQ ID NO: 1.

5. The chimeric promoter construct of any one of embodiments 1-4, wherein said regulatory region of the HPPD promoter comprises a deletion selected from the group consisting of:

- (a) a deletion of a TATA motif; or
5 (b) a deletion of at least one of the TATA1, TATA2, TATA3, TATA4 or TATA5 motifs.

6. The chimeric promoter construct of embodiment 5, wherein said regulatory region of the HPPD promoter comprises

- 10 a) the polynucleotide set forth in any one of SEQ ID NO: 2, 3, 4, 11 or 20;
b) a polynucleotide having at least 90% sequence identity to any one of SEQ ID NO: 2, 3, 4, 11, or 20; or
c) a polynucleotide comprising a fragment comprising at least 300
15 consecutive nucleotides of SEQ ID NO: 2, 3, 4, 11, or 20.

7. The chimeric promoter construct of any one of embodiments 1-4, wherein said regulatory region of the HPPD promoter comprises at least one or more alterations in at least one of the TATA1, TATA2, TATA3, TATA4 or TATA5
20 elements.

8. The chimeric promoter construct of embodiment 7, wherein said regulatory region of the HPPD promoter comprises

- a) the polynucleotide set forth in any one of SEQ ID NO: 5, 6, 7, 8, 9,
25 10, 12, 13, 14, 15, 16, 17, 18, or 19;
b) a polynucleotide having at least 90% sequence identity to any one of SEQ ID NO: 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, or 19; or
c) a polynucleotide comprising a fragment comprising at least 300
consecutive nucleotides of SEQ ID NO: 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, or
30 19.

9. The chimeric promoter construct of any one of embodiments 1-8, wherein said core promoter increases the regulatory activity of said regulatory region of

the HPPD promoter when compared to the regulatory activity of said regulatory region of the HPPD promoter alone.

10. The chimeric promoter construct of any one of embodiments 1-8,
5 wherein said core promoter decreases the regulatory activity of said regulatory region of the HPPD promoter when compared to the regulatory activity of said regulatory region of the HPPD promoter alone.

11. The chimeric promoter construct of any one of embodiments 1-8,
10 wherein said transcriptional regulatory activity of said chimeric promoter construct mimics the level of transcriptional regulatory activity of the HPPD promoter set forth SEQ ID NO:1.

12. The chimeric promoter construct of any one of embodiments 1-11,
15 wherein said chimeric promoter, when operably linked to a polynucleotide encoding a HPPD polypeptide having HPPD activity and insensitivity to an HPPD inhibitor, allows for a sufficient level of expression of said HPPD polypeptide in a plant to impart tolerance of the plant to an HPPD inhibitor.

20 13. The chimeric promoter construct of any one of embodiments 1-12, wherein said core promoter comprises

- a) the polynucleotide set forth in SEQ ID NO:71;
- b) a polynucleotide having at least 90% sequence identity to SEQ ID NO:71, where said polynucleotide continues to have core promoter activity;
- 25 c) a polynucleotide comprising a fragment comprising at least 30 consecutive nucleotides of SEQ ID NO: 71;
- d) the polynucleotide set forth in SEQ ID NO: 21; or
- e) the polynucleotide set forth in SEQ ID NO: 83.

30 14. The chimeric promoter construct of embodiment 13, wherein said core promoter further comprises

- a) a second polynucleotide as set forth in SEQ ID NO:72;

b) a second polynucleotide having at least 90% sequence identity to SEQ ID NO:72, where said second polynucleotide modulates the activity of the core promoter; or

5 c) a second polynucleotide comprising a fragment comprising at least 20 consecutive nucleotides of SEQ ID NO: 72.

15. The chimeric promoter construct of embodiment 13 or 14, wherein said core promoter further comprises

a) the polynucleotide set forth in SEQ ID NO:73;

10 b) a polynucleotide having at least 90% sequence identity to SEQ ID NO:73, where said polynucleotide continues to have core promoter activity;

c) a polynucleotide comprising a fragment comprising at least 30 consecutive nucleotides of SEQ ID NO: 73; or

d) the polynucleotide set forth in SEQ ID NO: 22.

15

16. The chimeric promoter construct of any one of embodiments 1-15, wherein said polynucleotide comprises

a) the sequence set forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78 ;

20 b) a sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40; 41, 74, 75, 76, 77, or 78 or

c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 25 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78.

d) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of a sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78.

30

17. An expression cassette comprising a promoter operably linked to a polynucleotide of interest, wherein said promoter comprises the chimeric promoter construct of any one of embodiments 1-16.

18. The expression cassette of embodiment 17, wherein said polynucleotide of interest encodes a polypeptide or a suppression element.

19. The expression cassette of embodiment 18, wherein said polynucleotide
5 of interest encodes an HPPD polypeptide having HPPD activity and having insensitivity to an HPPD inhibitor.

20. An expression vector comprising the expression cassette of any one of
10 embodiments 17-19.

21. A plant having stably incorporated into its genome at least one
expression cassette of any one of embodiments 17-19.

22. The plant of embodiment 21, wherein said plant is a dicot.

15

23. The plant of embodiment 22, wherein said dicot is soybean.

24. The plant of embodiment 22, wherein said dicot is Brassica, sunflower,
cotton, or alfalfa.

20

25. The plant of embodiment 21, wherein said plant is a monocot.

26. The plant of embodiment 25, wherein said monocot is maize, wheat,
rice, barley, sorghum, or rye.

25

27. A method of regulating the expression of a polynucleotide of interest,
said method comprising stably incorporating in the genome of a plant or plant cell the
polynucleotide sequence of interest operably linked to a promoter wherein said
promoter comprises the chimeric polynucleotide of any one of embodiments 1-16 or
30 stably incorporating in the genome of the plant or plant cell an expression cassette of
any one of embodiments 17-20.

28. The method of embodiment 27, wherein said plant is a dicot.

29. The method of embodiment 28, wherein said dicot is soybean.

30. The method of embodiment 28, wherein said dicot is Brassica, sunflower, cotton, or alfalfa.

5

31. The method of embodiment 27, wherein said plant is a monocot.

32. The method of embodiment 31, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

10

33. A polynucleotide comprising a promoter capable of regulating transcription comprising:

(a) a nucleotide sequence comprising SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76;

15 (b) a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76, wherein said polynucleotide has regulatory activity in a plant;

(c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76, wherein said polynucleotide has transcriptional regulatory activity in a plant; or

(d) a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity to a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76.

34. An expression cassette comprising a promoter operably linked to a polynucleotide of interest, wherein said promoter comprises the polynucleotide of embodiment 33.

30

35. The expression cassette of embodiment 34 wherein said polynucleotide of interest encodes a polypeptide or a suppression element.

36. The expression cassette of embodiment 36, wherein said polynucleotide of interest encodes an HPPD polypeptide having HPPD activity and having insensitivity to an HPPD inhibitor.

5 37. An expression vector comprising the expression cassette of any one of embodiments 34-36.

38. A plant having stably incorporated into its genome at least one expression cassette comprising a polynucleotide of interest operably linked to a promoter, wherein said promoter comprises the polynucleotide of embodiment 33 or
10 the expression cassette of any one of embodiments 34-36.

39. The plant of embodiment 38, wherein said plant is a dicot.

15 40. The plant of embodiment 39, wherein said dicot is soybean.

41. The plant of embodiment 39, wherein said dicot is Brassica, sunflower, cotton, or alfalfa.

20 42. The plant of embodiment 38, wherein said plant is a monocot.

43. The plant of embodiment 42, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

25 44. A method of expressing a polynucleotide of interest, said method comprising stably incorporating in the genome of a plant or plant cell the polynucleotide of interest operably linked to a promoter, wherein said promoter comprises the polynucleotide of embodiment 33 or stably incorporating into the genome of the plant or plant cell the expression cassette of any one of embodiments 34-
30 36.

45. The method of embodiment 44, wherein said plant is a dicot.

46. The method of embodiment 45, wherein said dicot is soybean.

47. The method of embodiment 45, wherein said dicot is Brassica, sunflower, cotton, or alfalfa.

5 48. The method of embodiment 44, wherein said plant is a monocot.

49. The method of embodiment 48, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

10

EXPERIMENTAL

Example 1. Isolation of a chromosomal region comprising *Glycine max* HPPD promoter

An EST (sgc5c.pk001.j9) coding for soybean HPPD was identified from DuPont/Pioneer's proprietary *Glycine max* EST database using conventional
15 bioinformatic tools including BLAST as described in US Patent No. 7,226,745. The soybean HPPD coding region sequence (US Patent No. 7,226,745 SEQ ID NO: 35 and 36) was cloned into a T7-based bacterial expression vector. Expression, purification and characterization of the *G. max* HPPD protein proved that the encoded protein is a functional HPPD, able to catalyze the reaction from 4-hydroxyphenylpyruvate to
20 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 approximately 2kb virtual genomic sequence upstream of the EST. To validate the virtual genomic sequence, polymerase chain reaction (PCR) primers (Forward primer: GCAAGTATTTCAATACAATAGC (SEQ ID NO:42) and Reverse
25 primer: GTTATCTGATATGATGTTGC (SEQ ID NO: 43)) were designed and used to amplify the HPPD locus from genomic DNA isolated from an elite soybean variety and the common Jack variety. Genomic DNA isolation protocols including those from Qiagen for plant DNA were followed. PCR reaction parameters were: Cycle1: 94C, 2min; Cycle 2 to 30: 94C, 30s; 65C, 1min; 72C, 5min; Cycle 31: 72C, 10min. A proof-
30 reading DNA polymerase, *pfu* Turbo from Stratagene was used for PCR amplification. A 4306bp 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) and fully sequenced. The sequence comprises 3' sequences, HPPD coding region, and upstream genomic sequence. The locus is highly conserved in both soy

varieties, with an overall of 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. SEQ ID NO: 1 is the 2166 bp upstream genomic sequence of elite *G. max* comprising the HPPD promoter sequence.

5

Example 2. Characterization of the genomic sequence upstream of *G. max* HPPD coding region

The 1228 bp genomic sequence at the 3' end of SEQ ID NO:1 was subjected to promoter analysis using Pioneer's proprietary promoter analysis software, Promoter REAPer and Promoter Delineator (US2010/0138952A1). Genomic sequence from other species including *Arabidopsis thaliana*, (SEQ ID NO: 64), *Medicago truncatula* (SEQ ID NO: 65), Poplar (SEQ ID NO: 66), *Brassica rapa* (SEQ ID NO: 67), *Vitis vinifera* (SEQ ID NO: 68), and the monocot *Sorghum bicolor* (SEQ ID NO: 69) were included for comparison in this analysis. With the program Promoter REAPer, regions were identified in soy HPPD promoter that are predicted to be important for its activity based on the sequence conservation of a set of DNA motifs across seven plant species. A total of eleven regions, each 7 to 13bp long and located no further than 880 bp from the translation start codon were predicted to have a high level of importance (see Figure 1). About ten other 7bp regions show a medium level of importance. Five putative TATA boxes, GTATAAATAA (TATA1; SEQ ID NO: 44), CCAATATATG (TATA2; SEQ ID NO:45), CCTTATATATC (TATA3; SEQ ID NO:46), TATATAATAA (TATA4; SEQ ID NO: 47), and GAATATAAG (TATA5; SEQ ID NO:48) were identified. TATA3 (closest to the coding region) was predicted to be the primary TATA box for HPPD promoter activity. The encoded protein, starting from the first ATG after TATA3 (SEQ ID NO: 46), would have four more amino acid sequence (MPIP) compared with the annotated *G. max* HPPD protein sequence in Genbank EF608178. Using Promoter Delineator, the transcription start site (TSS) "A" was predicted and designated as +1.

RNA ligation mediated (RLM) 5' rapid cloning of cDNA ends (5' RACE) revealed alternate transcription start sites for the native Gm HPPD promoter. Using total RNA extracted from young soy leaves and the First Choice RLM-RACE kit (Ambion) per manufacturer's protocol, two major amplification products were observed, indicating two TSS. Cloning and sequencing of these PCR products revealed one transcript beginning with the sequence

GTAATAAAAAAAGAGAGAAGCCGCATCAA (SEQ ID NO: 49) at position -231 relative to the predicted TSS. A second transcript began with the sequence AAGCAGCAGCATCACACCACACCAATGCC (SEQ ID NO: 50) at position +7 relative to the predicted TSS. Sequence of multiple clones for each 5' RACE product
5 indicated an approximately 4 nucleotide variation between individual transcripts for both sites.

Open reading frame (ORF) analysis of the TSS-231 mRNA indicates a short ORF (upORF) encoding 7 AAs starting at position -189. Downstream of this, another ORF begins at position -93 and continues in frame through the catalytic portion of the
10 HPPD protein; the protein from this ORF begins with MPMY. The TSS+7 mRNA contains a single ORF, in the same reading frame as the protein encoded by the TSS-231 mRNA, but beginning 41 amino acids (123 nucleotides) downstream; the protein encoded by this ORF begins with MPIP.

Linked in vitro transcription and translation indicates both mRNAs are
15 translated (see Fig. 2). The +7 transcript is translated to produce a single protein, designated the "short" protein. The -231 transcript yields two proteins, with the lower molecular weight band at the same MW as the single protein produced from the +7 transcript. The higher MW protein produced from the -231 transcript is designated the "long" protein.

20

Example 3. Synthetic promoter analysis by *Agrobacterium*-mediated transient expression in plants

This example describes qualitative and quantitative assigned ranking of HPPD promoter variants using *Agrobacterium* infection and subsequent transient expression
25 of the red fluorescence marker DsRed2 (Clontech, Mountain View, CA). Agro-infiltration is a well described method (Kapila *et. al.* (1997) *Plant Science* 122: 101-108) 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.

30

Leaf tissues of bush bean (common bean, *Phaseolus vulgaris*), were agro-infiltrated with normalized bacterial cell cultures of test and control strains. 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 DsRed Filter set; no. 10447412), and acquiring images (Leica Microsystems - Wetzlar, Germany; DFC300 FX R2) of representative examples at fixed exposure time of 7 seconds. Analysis of promoter strength in samples was determined 5 days post infection by protein extraction and quantitative measure of (red) fluorescence using a Typhoon Trio+ Variable Mode Imager configured appropriately for DsRed detection; 532nm Excitation laser and 580nm BP30 Emission filter, PMT = 375-400V, 100um pixel size resolution, and Image Quant TL image analysis software (GE Healthcare – Life Sciences, Piscataway, NJ). Prior to scanning, homogenized plant extracts were prepared in 500ul extraction buffer (100mM potassium phosphate pH7.8, 1mM EDTA, 7mM beta-mercaptoethanol, 1% Triton X100, 10% glycerol), in 2ml micro-centrifuge tubes using a Geno-Grinder 2000 (Spex CertiPrep, Metuchen, NJ). 250ul of supernatant was collected and filtered through a Milipore MultiScreen-HV (Millipore – Billerica, MA; cat no. MAHVN4550) multi-well filter-plate and then normalized to 50ug total extracted protein, as determined by Bradford protein assay (Bio-Rad - Hercules, CA; Quick Start™ Bradford Protein Assay). 100ul of normalized extract of samples was scanned in 96-well plates. Purified recombinant DsRed2 standard protein (Clonotech Cat. No. 632436) was scanned simultaneously to treated sample extracts and the data was represented as calculated ng concentration DsRed2, per 50ug sample.

Example 4. Mutagenesis analysis of the HPPD promoter

A 2061 bp fragment corresponding to nucleotides 103-2163 of SEQ ID NO:1 was created by PCR with primers (Forward primer hp0234: GTTTTCCGCGGGTGTTGATCC (SEQ ID NO: 51) and Reverse primer hp2296: TCATTGGTACCTGGTGTGGTGTGATGCTGC (SEQ ID NO: 52)) to introduce SacII and KpnI sites. This fragment was isolated via gel-purification, digested with restriction enzymes SacII and KpnI, and ligated with a DsRed2 marker gene (ClonTech) together with the transcription terminator sequence PIN II from potato, to form an expression unit. To evaluate the predicted TATA boxes in promoter activity, nested deletion mutants of GmHPPD PRO (SEQ ID NO: 1) were created using PCR with primers (Forward primer hp0234: GTTTTCCGCGGGTGTTGATCC (SEQ ID NO: 51) and Reverse primers hp2154: AGCATGGTACCTTGCGTCTGGGTTGAG

(SEQ ID NO: 53), hp2048: ATCTGGTACCTGATGTTGATGCGGC (SEQ ID NO: 62) hp1962: AGGAGGTACCGTCAAATCCACCTAG (SEQ ID NO: 54), hp1791: AGCCTGGTACCTTGTGTGTGTAAGATAAGAC (SEQ ID NO: 63), and hp1663: TCCTTGGTACCTGATGCACTATATAACG (SEQ ID NO: 55)) as depicted
5 in Fig 3. Resultant deletion mutant promoter fragments SHP0C (SEQ ID NO: 20), SHP101C (SEQ ID NO 2), SHP102C (SEQ ID NO 3), SHP103C (SEQ ID 4), and SHP110C (SEQ ID 11), were fused with DsRed2 to create various expression cassettes for expression activity analysis in infiltrated leaf tissues.

These expression cassettes were introduced into a plant transformation binary
10 vector via Gateway cloning (Invitrogen) and then introduced into *Agrobacterium* strain AGL1 via electroporation according to Shen and Forde (1989, *Nucleic Acids Res.* 17: 8385). As described in Example 3, the resultant *Agrobacterium* strains were infiltrated into bush bean leaf tissues. Infiltrated leaf discs were visually examined under a fluorescent microscope and scanned with Typhoon Trio+ Variable Mode Imager for
15 red fluorescence produced from DsRED2 protein accumulation. Fig.4 shows an example of the image obtained from Typhoon scanning. Infiltration analysis confirmed that the GmHPPD PRO DNA fragment was able to drive the expression of DsRed2 in infiltrated leaf tissues compared with fluorescent background from leaf tissues infiltrated with a dMMV (Dey and Matai, (1999) *Transgenics* 3:61-70) GUS construct
20 (Fig 4. A and D).

As shown in Fig. 4, deletion of 613 bp DNA fragment, including all 5 putative TATA boxes, from the predicted transcription start site in SEQ ID: 1 resulted in a DNA fragment SHP101C that was not able to drive DsRed2 expression to a level above the background red fluorescence produced in leaf tissues infiltrated with a dMMV-GUS
25 construct (A and E, Fig. 4). GUS protein is well known to not fluoresce under these detection conditions set for DsRed2. Nested deletion fragments SHP110C, SHP0C, and SHP102C (Fig.3) containing deletion of TATA2 and 3, TATA2, 3, and 5, or TATA2, 3, 4, and 5, respectively, did not show promoter activity (Table 3). The next nested deletion mutant fragment SHP103C (Fig. 3) brings TATA2 back so that only TATA3 is
30 deleted. This fragment was able to drive Ds-Red expression to a level similar to that of the native HPPD promoter (Fig.4, D and G), suggesting that TATA2 functions as the primary TATA element for the native HPPD promoter. Because the long transcript starts before TATA2, TATA5 and/or TATA4 are likely important elements in the promoter, operating together with TATA2 to drive the transcription of -231 mRNA. As

shown in Example 2, this transcript could produce two HPPD variants, one starts translation at +31 and the other at -93. The resultant short HPPD variant starts with MPIP and the long variant starts with MPMY with 41 amino acids added to the N-term of the previously annotated soybean HPPD protein (SEQ ID 61). TATA3, positioned
5 160bp downstream of TATA2, would encode part of the protein (see Example 2 for more details).

In addition to visual observations, relative promoter strength was determined by quantitative measurement of the red fluorescence generated from expressed DsRed2 protein in infiltrated leaf tissues. Briefly, 50ug of total extracted protein from infiltrated
10 leaf discs was scanned using a Typhoon Trio+ Variable Mode Imager as described in Example 3. Red fluorescence in the protein sample was quantitatively measured. Infiltration experiments for each construct were repeated at least three times. For each repeat experiment, the background red fluorescence detected in control leaf tissues infiltrated with a non-DsRED expressing construct, dMMV-GUS or promoterless
15 DsRED, 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 GmHPPD PRO construct (the entire 2061 promoter fragment), which was set to 100%. Table 2 shows the calculation of relative promoter strength for SHP101C, SHP102C, and SHP103C. Average relative promoter strength for all mutant promoter
20 constructs was obtained with data from 3 or more repeat infiltration experiments and listed in Table 3. With 0% relative promoter strength, deletions in SHP101C and SHP102C eliminated the activity of the HPPD promoter, indicating that the upstream sequence through TATA1 has no promoter activity in the transient expression in bushbean experiments. With 2-3% relative promoter strength, deletion mutants SHP0C
25 and SHP110C led to very low but detectable expression of DsRED2, suggesting that the upstream sequence through TATA4 and TATA5 could serve as a weak promoter. Deletion mutant SHP103C in which only TATA3 is deleted retained 90% of the native HPPD promoter activity, suggesting that the upstream sequence through TATA2 is a fully functional promoter sequence.

30

Table 2.

Promoter Variant	Description	Test 1			Test 2			Test 3			Average relative pro strength (%)	Standard deviation
		DsRED reading (PMT=375)	Background subtraction	Relative pro strength (%)	DsRED reading (PMT=400)	Background subtraction	Relative pro strength (%)	DsRED reading (PMT=400)	Background subtraction	Relative pro strength (%)		
dMMV	dMMV-GUS (negative control)	16,829	0	0	24,943	0	0	47000	0	0	0	0.0
H2B	H2B-DsRED2 (positive control)	447,374	430,545	65	2,540,112	2,515,169	104	2700000	2,653,000	99	89	21.1
GmHPPD PRO	Native Glycine max HPPD promoter region	676,198	659,369	100	2,439,513	2,414,570	100	2730000	2,683,000	100	100	0.0
SHP101C	Deletion of all 5 TATA	18,294	1,465	0	22,788	-2,155	0	61000	14,000	1	0	0.3
SHP102C	Deletion of TATA 2, 3, 4, and 5	13,977	-2,852	0	23,684	-1,259	0	69000	22,000	1	0	0.6
SHP103C	Deletion of TATA3	613,261	596,432	90	1,996,294	1,971,351	82	2334000	2,287,000	85	86	4.4

Example 5. Site-directed mutagenesis study of the HPPD promoter

5 Using site-directed mutagenesis (SDM), mutations in the putative TATA boxes were created to generate another set of promoters (SHP104C to 109C and 111C to 118C) with varied strength. Site-directed mutagenesis protocols can be found in Stratagene's Quick Change manual. In SEQ ID NO: 5, a single A to C transversion in TATA3 of SEQ ID NO: 1 at position -20 relative to the putative transcription start site

10 was created. In SEQ ID NO: 6, a T to C transition in TATA1 at position -563 relative to putative transcription start site was generated in addition to the A to C transversion at -20. In SEQ ID NO: 7, a third point mutation was added to SEQ ID NO: 6 at position -190 relative to the putative transcription start site (T to G transversion). These new promoter sequences, as SacII-KpnI fragments, were fused to DsRed2 to form

15 expression units and analyzed for promoter activity as described in examples 3 and 4. As summarized in Table 3, with a point mutation in TATA3, the promoter activity of the resultant DNA fragment was reduced to 23% of the native HPPD promoter. In all cases if TATA3 is mutated, no matter what other mutations are present in the other TATA boxes, promoter activity is low but clearly detectable. This result provides

20 evidence that TATA3 is important in maintaining full activity of the soy HPPD promoter but that other elements are also functional. Additional site-directed mutagenesis with two to three nucleotide changes in similar positions in the three putative TATA boxes 1, 2, and 3 was performed to generate another set of promoters. In SHP107C (SEQ ID NO: 8), a three nucleotide change was made in TATA3 (TAT to

25 GCA) at position -21 relative to the putative transcription start site. In SHP108C (SEQ ID NO: 9), another set of three nucleotide changes (ATA to CCG) in TATA1 at position -564 was made in addition to the TAT to GCA change in TATA3. In

SHP109C (SEQ ID NO: 10), in addition to the nucleotide changes in TATA1 and TATA3 as in SHP108C, three nucleotide changes (TAT to CAG) in TATA2 at position -192 were made. These promoter variants directed DsRed2 expression at a level slightly lower than the respective single point mutation variants (SHP104C, 105C, and 106C). Additional three nucleotide changes in TATA4 or 5 of fragments SHP107C and SHP109C were made. The resultant mutant fragments SHP114C, SHP116C, SHP117C, and SHP118C all resulted in DsRed2 expression at a further reduced level (Table 3). For example, SHP109C in which three nucleotide mutations were made in TATA1, 2, and 3 has 19% of native HPPD promoter activity. Adding three-nucleotide changes in TATA4 and 5 resulted in a variant SHP118C in which all five putative TATA boxes were mutated. SHP118C has only 9% of native HPPD promoter activity. This result shows that cryptic or unknown sites in the promoter region of SEQ ID NO: 1 may be involved in promoter function.

The next set of promoter variants was created with three nucleotide changes in other TATA boxes except for TATA3. Three nucleotide changes in TATA2 (TAT to CAG) at position -192 (SHP111C, SEQ ID NO: 12) allowed retention of 93% of the native HPPD promoter activity (Table 3). In fact, as long as TATA3 remain unchanged, SDM mutants in TATA2 or 4 or 5 or combinations, such as in the example of SHP115C, are all similar, having approximately 90% of native HPPD promoter activity (SEQ ID NO: 12, 13, 14, and 16, Table 3).

Taken together, these results suggest that the native HPPD promoter may have duplicate TATA elements in TATA2 and TATA3. TATA3 is essential for the full promoter activity to drive expression of HPPD protein. When TATA3 remains unchanged, mutations in other TATA boxes did not significantly alter the strength of native HPPD promoter. When TATA3 is mutated, either by point mutation or three nucleotide changes, plus or minus additional mutations in other putative TATA boxes (1,2, 4, and 5), promoter activity of the resultant promoter variants is reduced to 9-30% of that of the native HPPD promoter. TATA4 and 5 may help with promoter activity that utilizes TATA3. TATA2 could be an important element for the upstream promoter (comparing SHP110C and SHP103C). When TATA3 is deleted, the promoter fragment still retained 93% of promoter activity, strongly suggest that the putative long transcript with 2 ATGs, one at -93 and the other -87, in-frame with HPPD protein, could be produced. Example 2 provides experimental proof that two transcripts of different lengths are indeed produced from the native HPPD promoter. TATA4,

TATA5, or some other element must be functioning for the polymerase recognition for the generation of the long transcript.

Table 3. Comparison of promoter activity among variants of the native *G. max* HPPD promoter

SEQ ID	Promoter Variant	Description	Average Relative Promoter Strength (%) from extract
1	GmHPPD PRO	Native Glycine max HPPD promoter region	100
20	SHP0C	Deletion of TATA2, TATA3, and TATA5	2
2	SHP101C	Deletion of all 5 TATA	0
3	SHP102C	Deletion of TATA 2, 3, 4, and 5	0
4	SHP103C	Deletion of TATA3	90
5	SHP104C	A to C mutation in TATA3	23
6	SHP105C	T to C in TATA1 and A to C in TATA3	30
7	SHP106C	T to C in TATA1, T to G in TATA2, and A to C in TATA3	21
8	SHP107C	TAT to GCA mutation in TATA3	15
9	SHP108C	ATA to CCG in TATA1 and TAT to GCA in TATA3	19
10	SHP109C	ATA to CCG in TATA1, TAT to CAG in TATA2, and TAT to GCA in TATA3	19
11	SHP110C	Deletion of TATA2 and TATA3	3
12	SHP111C	TAT to CAG mutation in TATA2	93
13	SHP112C	TAT to CAG mutation in TATA4	97
14	SHP113C	TAT to CAG mutation in TATA5	89
15	SHP114C	TAT to CAG in TATA5, TAT to GCA in TATA3	11
16	SHP115C	TAT to CAG in TATA5, TAT to CAG in TATA2	90
17	SHP116C	ATA to CCG in TATA1, TAT to CAG in TATA2, TAT to GCA in TATA3, TAT to CAG in TATA5	12
18	SHP117C	TAT to CAG mutation in TATA4, TAT to GCA mutation in TATA3	9
19	SHP118C	ATA to CCG in TATA1, TAT to CAG in TATA2, TAT to GCA in TATA3, TAT to CAG in TATA4, TAT to CAG in TATA5	9
74	120C	Point mutation to eliminate upORF start codon	105
75	121C	Point mutation to insert stop codon just upstream of downstream TSS	92

Example 6. Construction of synthetic promoters and their activity

SHP101C and SHP102C lack promoter activity due to deletion of TATA binding sites. To create synthetic promoters using these non-promoter DNA fragments, synthetic element I (SED ID NO: 21) and II (SEQ ID NO: 22), each flanked by restriction sites XhoI and KpnI, were synthesized and ligated with the 3' ends of the HPPD promoter-derived DNA fragments. Synthetic element I comprises the SynII core (derived from US 6,072,050 SEQ ID NO: 1) sequences followed by the 45bp putative 5'UTR sequence including the predicted transcription start site from the soybean native HPPD gene (ACAACCACCAAGCTCAATCTCAAGCAGCAGCATCACACCACACCA, (SEQ ID NO: 56) nucleotides between TATA3 and ATG, see Figure 1). Synthetic element II contains the Rsyn7 region derived from US 6,072,050 SEQ ID NO: 2 immediately upstream of synthetic element I (SEQ ID NO:21). These synthetic promoters were then fused with DsRed2 to evaluate their ability to drive DsRed2 expression in infiltrated leaf tissues. Table 4 contains results from various synthetic promoters and their relative promoter strength determined by DsRed2 expression as outlined in Example 3. As shown in Table 4, the addition of synthetic element II (SEQ ID NO: 22) to the non-promoter DNA fragments SHP101C and 102C restored promoter activity to 13% and 23% of that of the native HPPD promoter. Replacing synthetic element II (SEQ ID NO: 22) in these two SHP promoters with synthetic element I (SEQ ID NO: 21) resulted in low activity promoters.

With synthetic element I, synthetic promoters SHP103, SHP104, SHP105, SHP106, SHP107, SHP108, and SHP109 were created. SHP203, 204, 205, 206, 207, 208, and 209 were created with synthetic element II. These synthetic promoters were all more active than SHP101/102 and SHP201/202, with approximately one to four fold higher promoter activity when compared to that of the native HPPD promoter (Table 4).

Example 7. Use of SHP promoters to achieve appropriate transgene expression

The rare representation of HPPD ESTs in leaf tissue in the soybean database suggests that soy HPPD is transcribed at an extremely low level and as inferred from tissue distribution may be expressed in a tissue-specific and/or developmental-stage specific manner. Cis-control elements embedded in the promoter region can be used in

a chimeric promoter to drive expression of a transgene mimicking the expression pattern of the native HPPD. Examples of such transgenes include marker genes such as DsRed2 and GUS, genes in tyrosine metabolic pathway such as HPPD, PDH, and ADH, and genes involved in other metabolic pathways. In one example, to visualize native HPPD expression patterns, DsRed2 or GUS was fused with synthetic promoters such as SHP103 (SEQ ID NO: 25) or the native promoter (SEQ ID NO: 1) and delivered into Arabidopsis via floral-dip, a well-known *Agrobacterium*-mediated transformation procedure (Clough and Bent, Plant J. 1998. 16(6):735-43). Transgenic seeds, selected by kanamycin resistance, were germinated and plants were examined for the expression pattern of DsRed2. Similar constructs are also delivered into tobacco via *Agrobacterium*-mediated transformation and expression of the marker gene is analyzed by Northern, Western, and fluorescence scanning. In this way, sequences important for the tissue and temporal specific properties of the soy HPPD promoter can be explored.

The above mentioned synthetic promoters can also be used to drive HPPD transgene expression in *G. max* to enable successful engineering of an HPPD-inhibitor tolerance trait in plants. To mimic the level and pattern of native HPPD expression in soy, the maize wild-type HPPD and insensitive variants such as those disclosed in U.S. Provisional Patent Application 61/401,456, filed August 13, 2010 were constructed with various SHP promoters and delivered into *G. max* via particle bombardment.

In one example, the insensitive maize HPPD variants were constructed with SHP promoters such that the 5' end of the long transcript is fused with the coding sequence, with or without N terminal truncation, of the insensitive variants to create a translational fusion between the soy HPPD coding sequence and the maize insensitive variants. Under the direction of SHP promoters, two transcripts are produced in the transgenic soybean plants in similar time and spatial pattern to the native HPPD transcripts but with higher expression level in one or both mRNA species. The resultant fusion proteins, one with an additional 41 amino acids at the N-terminus, are distributed in subcellular locations in a similar way to that of the native HPPD protein. Such transgenic plants are produced via particle bombardment-mediated transformation or *Agrobacterium*-mediated transformation. T0 plants are sprayed with 2x HPPD-inhibiting herbicides to test for gene efficacy and the next generations of plants are evaluated either in the greenhouse or in the field for agronomic evaluations. Transcription efficiency and pattern are examined using RT-PCR, northern, and primer

extension analysis. Protein expression level and pattern are determined by western, Mass Spectrometry, and immuno-localization.

Example 8. Modifications in synthetic promoters to alter promoter activity

5 The level or pattern of transgene expression conferred by designed HPPD promoters described above can be changed with further modifications in the sequence. In general, addition of enhancer elements increases promoter activity. For example, when a 2X 35S enhancer element was added to SHP101 and SHP103 (SEQ ID NOS: 23 and 25, respectively), greater than 10 fold higher promoter activity was observed.

10 Alternatively, changes made to the 5'UTR sequence in SHP promoters can alter promoter activity. Furthermore, insertion of SynII core or SynII core plus Rsyn7 or other TATA elements into upstream sequence of mutant promoter fragments described in Example 4 and 5 can also alter promoter activity. In SHP110 (SEQ ID NO: 41), partial SynII core sequence is inserted in place of TATA5. The transcription of the long

15 mRNA is enhanced in SHP110. In SHP210 (SEQ ID NO:78) TATA5 through transcription start site at -231 is deleted (deletion = nt 1867-1905 of SEQ ID 1) and replaced with Rsyn7 + the partial SynII core sequence; transcription from the -231 TSS is expected to be further elevated compared to SHP110. In SHP111 (SEQ ID NO:77) TATA3 through transcription start site at +7 is deleted (deletion = nt 2106-2140 of

20 SEQ ID 1) and replaced with the partial SynII Core; transcription from the +7 TSS is expected to be elevated relative to the native promoter. In SHP120C (SEQ ID NO:74) an A to T mutation at nt 1945 of SEQ ID 1 eliminates the start codon of the upORF element and may be expected to upregulate translation of mRNA from the -231 transcription start site. Other modifications include the addition of an intron in the

25 5'UTR. Further trimming at the 5' end of the SHP sequences can be made to further modulate promoter activity.

Table 4. Relative strength of SHP promoters

SEQ ID	Promoter name	Base DNA Fragment	Added Sequence (SEQ ID)	Relative Strength (% Native HPPD Promoter)
1	GmHPPD PRO	GmHPPD PRO	NA	100
23	SHP101	SHP101C	21	3
24	SHP102	SHP102C	21	4
25	SHP103	SHP103C	21	310
26	SHP104	SHP104C	21	181
27	SHP105	SHP105C	21	210
28	SHP106	SHP106C	21	377
29	SHP107	SHP107C	21	129
30	SHP108	SHP108C	21	150
31	SHP109	SHP109C	21	266
32	SHP201	SHP101C	22	23
33	SHP202	SHP102C	22	13
34	SHP203	SHP103C	22	272
35	SHP204	SHP104C	22	277
36	SHP205	SHP105C	22	152
37	SHP206	SHP106C	22	195
38	SHP207	SHP107C	22	231
39	SHP208	SHP108C	22	213
40	SHP209	SHP109C	22	230
41	SHP110	NA	70	78

Example 9. Transient expression of Gm HPPD-AcGFP fusion proteins

5 Numerous genes have been found to have two or more in-frame ATGs at the 5' end (For review, see Small et al., *Plant Molecular Biology*, 1998. 38: 265-277). Many of such genes are known to have multiple transcription starts to enable the production of two proteins from the same gene. Often, the "long" protein contains plastid targeting signal at the N-terminal while the "short" protein does not. Appropriate distribution of

10 the "long" and "short" protein variants between two subcellular compartments is desired for the respective protein function to be carried out normally. The soy HPPD gene described here falls into this class of genes. No other HPPD gene is known to share the same description.

Transient expression experiments indicate that the long HPPD protein (SEQ ID NO: 58) is imported to chloroplasts, while the short protein (SEQ ID NO: 61) remains in the cytosol. Plant expression cassettes were constructed fusing portions of the N-terminus of Gm HPPD to an *Aequorea coerulea* green fluorescent protein 1 (AcGFP1). One fusion contained amino acid residues 1-86 of the long Gm HPPD protein. Another contained residues 1-44 of the short HPPD protein (this corresponds to residues 42-86 of the long protein). These cassettes were incorporated into binary vectors which also contained an untargeted DsRed2 expression cassette and introduced into *A. tumefaciens* strain AGL1 and then used to infect leaf discs of *G. max* as described in Example 3. As shown in Fig. 10 below, green fluorescence is clearly visible in the chloroplasts of infected cells when AcGFP is fused to amino acid residues 1-86 of Gm HPPD. When the fusion is made with residues 42-86, corresponding to the 44 N-terminus residues of the short protein, green fluorescence is visible only in the cytoplasm.

15

Example 10. HPPD promoter expression profiling in Arabidopsis

This example describes qualitative assessment of spatial and temporal expression pattern for engineered red fluorescence reporter (*DsRed2*; Clontech, Mountain View, CA USA) driven by variants of the *G. max* HPPD promoter in stably transformed Arabidopsis plants.

Agrobacterium mediated transformation and selection of ecotype Columbia (*Col-0*) *Arabidopsis thaliana* was performed according to published protocols by Clough S.J., Bent A. 1998, *Plant J.* 16: 735–743, employing “floral spray” technique of bacterial suspension application to flowering plants, described by Chung M.H., Chen M.K., Pan S.M. 2000, *Transgenic Res.* 9: 471–476.

Harvested seed were sown on sterile agar plates with antibiotic selection and grown at 22°C /125umol m⁻² m⁻¹/18 hour photoperiod in a growth chamber. Resistant seedlings were identified and transferred to soil (Sunshine Redi-earth Plug & Seedling; Sun Grow Horticulture Inc, Bellvue, WA USA) in 3” square pots at 8 days. Some seedlings were transferred to fresh selection medium in 110mm Petri-dishes for further characterizing early vegetative expression pattern. Potted plants were grown under 22°C/225umol m⁻² m⁻¹/18 hour photoperiod in growth rooms while Petri dishes were retained under growth chamber conditions.

30

Inspection and documentation of expression pattern was performed at 15, 24 and 33 days after sowing representing early and late vegetative and early reproductive plant development stages, respectively. Event populations were inspected and imaged under stereo epi-fluorescent microscope (M165 FC; Leica Microsystems - Wetzlar, Germany; with DsRed Filter set; no. 10447412 and DFC300 FX R2 digital camera system), acquiring fluorescence images of representative examples at fixed illumination, magnification and image capture time. Further examination of DsRed expression profiles were determined by Typhoon Trio+ Variable Mode Imager (p/n 63-0055-89; GE Healthcare – Life Sciences, Piscataway, NJ USA) configured appropriately for DsRed2 detection; 532nm excitation laser and 580nm BP30 emission filter, PMT = 400V, 50um pixel size resolution, and analyzed with provided Image Quant TL image analysis software. Prior to scanning, whole plant samples were removed from and rinsed of soil, arrayed on 96-well black plates (part no. 655090; Greiner Bio-One N.A. -Monroe, NC USA), and covered with a universal assay plate lid (Costar #3099; Corning Incorporated, Corning NY USA) to immobilize and position for uniform presentation on scanner platen. Scanned image size and contrast were normalized between scans to accurately represent relative pattern of DsRed2 expression between events and test constructs.

Test vectors and controls (Table 5) represent: Native GM-HPPD promoter, deletions and mutations (SHP promoters), an H2B promoter - DsRed2 reference and untransformed wild type Col-0 control. Expression pattern and level were found to vary depending on the changes made in the HPPD promoter (Table 5 and Figure 11).

25

THAT WHICH IS CLAIMED:

1. A chimeric promoter construct comprising
a first polynucleotide comprising a regulatory region of a 4-
5 hydroxyphenylpyruvate dioxygenase (HPPD) promoter operably linked to a second
polynucleotide comprising a heterologous core promoter functional in a plant,
wherein said core promoter modulates the regulatory activity of said
regulatory region of the HPPD promoter when compared to the regulatory activity of
said regulatory region of the HPPD promoter alone;
10 wherein said chimeric promoter has transcriptional regulatory activity in
a plant.
2. The chimeric promoter construct of claim 1, wherein said regulatory
region of the HPPD promoter comprises a variant or a fragment of SEQ ID NO:1.
15
3. The chimeric promoter construct of claim 2, wherein said transcriptional
regulatory activity of said regulatory region of the HPPD promoter in the absence of
said core promoter comprises less than 10% of the transcriptional regulatory activity of
the HPPD promoter set forth in SEQ ID NO: 1.
20
4. The chimeric promoter construct of claim 2, wherein said transcriptional
regulatory activity of said regulatory region of the HPPD promoter in the absence of
said core promoter comprises at least 10% of the regulatory activity of the HPPD
promoter as set forth in SEQ ID NO: 1.
25
5. The chimeric promoter construct of any one of claims 1-4, wherein said
regulatory region of the HPPD promoter comprises a deletion selected from the group
consisting of:
 - (a) a deletion of a TATA motif; or
 - 30 (b) a deletion of at least one of the TATA1, TATA2, TATA3, TATA4 or
TATA5 motifs.
6. The chimeric promoter construct of claim 5, wherein said regulatory
region of the HPPD promoter comprises

a) the polynucleotide set forth in any one of SEQ ID NO: 2, 3, 4, 11 or 20;

b) a polynucleotide having at least 90% sequence identity to any one of SEQ ID NO: 2, 3, 4, 11, or 20; or

5 c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 2, 3, 4, 11, or 20.

7. The chimeric promoter construct of any one of claims 1-4, wherein said regulatory region of the HPPD promoter comprises at least one or more alterations in at
10 least one of the TATA1, TATA2, TATA3, TATA4 or TATA5 elements.

8. The chimeric promoter construct of claim 7, wherein said regulatory region of the HPPD promoter comprises

a) the polynucleotide set forth in any one of SEQ ID NO: 5, 6, 7, 8, 9,
15 10, 12, 13, 14, 15, 16, 17, 18, or 19;

b) a polynucleotide having at least 90% sequence identity to any one of SEQ ID NO: 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, or 19; or

c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, or
20 19.

9. The chimeric promoter construct of any one of claims 1-8, wherein said core promoter increases the regulatory activity of said regulatory region of the HPPD promoter when compared to the regulatory activity of said regulatory region of the
25 HPPD promoter alone.

10. The chimeric promoter construct of any one of claims 1-8, wherein said core promoter decreases the regulatory activity of said regulatory region of the HPPD promoter when compared to the regulatory activity of said regulatory region of the
30 HPPD promoter alone.

11. The chimeric promoter construct of any one of claims 1-8, wherein said transcriptional regulatory activity of said chimeric promoter construct mimics the level

of transcriptional regulatory activity of the HPPD promoter set forth SEQ ID NO:1.

5 12. The chimeric promoter construct of any one of claims 1-11, wherein said chimeric promoter, when operably linked to a polynucleotide encoding a HPPD polypeptide having HPPD activity and insensitivity to an HPPD inhibitor, allows for a sufficient level of expression of said HPPD polypeptide in a plant to impart tolerance of the plant to an HPPD inhibitor.

10 13. The chimeric promoter construct of any one of claims 1-12, wherein said core promoter comprises

- a) the polynucleotide set forth in SEQ ID NO:71;
- b) a polynucleotide having at least 90% sequence identity to SEQ ID NO:71, where said polynucleotide continues to have core promoter activity;
- 15 c) a polynucleotide comprising a fragment comprising at least 30 consecutive nucleotides of SEQ ID NO: 71;
- d) the polynucleotide set forth in SEQ ID NO: 21; or
- e) the polynucleotide set forth in SEQ ID NO: 83.

20 14. The chimeric promoter construct of claim 13, wherein said core promoter further comprises

- a) a second polynucleotide as set forth in SEQ ID NO:72;
- b) a second polynucleotide having at least 90% sequence identity to SEQ ID NO:72, where said second polynucleotide modulates the activity of the core promoter; or
- 25 c) a second polynucleotide comprising a fragment comprising at least 20 consecutive nucleotides of SEQ ID NO: 72.

30 15. The chimeric promoter construct of claim 13 or 14, wherein said core promoter further comprises

- a) the polynucleotide set forth in SEQ ID NO:73;
- b) a polynucleotide having at least 90% sequence identity to SEQ ID NO:73, where said polynucleotide continues to have core promoter activity;

- c) a polynucleotide comprising a fragment comprising at least 30 consecutive nucleotides of SEQ ID NO: 73; or
- d) the polynucleotide set forth in SEQ ID NO: 22.

5 16. The chimeric promoter construct of any one of claims 1-15, wherein said polynucleotide comprises

a) the sequence set forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78 ;

b) a sequence having at least 85% sequence identity to the sequence set
10 forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40; 41, 74, 75, 76, 77, or 78 or

c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78.

d) a polynucleotide comprising a fragment comprising at least 300
15 consecutive nucleotides of a sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 74, 75, 76, 77, or 78.

20 17. An expression cassette comprising a promoter operably linked to a polynucleotide of interest, wherein said promoter comprises the chimeric promoter construct of any one of claims 1-16.

25 18. The expression cassette of claim 17, wherein said polynucleotide of interest encodes a polypeptide or a suppression element.

19. The expression cassette of claim 18, wherein said polynucleotide of interest encodes an HPPD polypeptide having HPPD activity and having insensitivity to an HPPD inhibitor.

30

20. An expression vector comprising the expression cassette of any one of claims 17-19.

21. A plant having stably incorporated into its genome at least one expression cassette of any one of claims 17-19.

5

22. The plant of claim 21, wherein said plant is a dicot.

23. The plant of claim 22, wherein said dicot is soybean.

24. The plant of claim 22, wherein said dicot is Brassica, sunflower, cotton, or alfalfa.

10

25. The plant of claim 21, wherein said plant is a monocot.

26. The plant of claim 25, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

15

27. A method of regulating the expression of a polynucleotide of interest, said method comprising stably incorporating in the genome of a plant or plant cell the polynucleotide sequence of interest operably linked to a promoter wherein said promoter comprises the chimeric polynucleotide of any one of claims 1-16 or stably incorporating in the genome of the plant or plant cell an expression cassette of any one of claims 17-20.

20

28. The method of claim 27, wherein said plant is a dicot.

25

29. The method of claim 28, wherein said dicot is soybean.

30. The method of claim 28, wherein said dicot is Brassica, sunflower, cotton, or alfalfa.

30

31. The method of claim 27, wherein said plant is a monocot.

32. The method of claim 31, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

33. A polynucleotide comprising a promoter capable of regulating transcription comprising:

(a) a nucleotide sequence comprising SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76;

5 (b) a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76, wherein said polynucleotide has regulatory activity in a plant;

10 (c) a polynucleotide comprising a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76, wherein said polynucleotide has transcriptional regulatory activity in a plant; or

15 (d) a polynucleotide comprising a nucleotide sequence having at least 85% sequence identity to a fragment comprising at least 300 consecutive nucleotides of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 74, 75, or 76.

34. An expression cassette comprising a promoter operably linked to a polynucleotide of interest, wherein said promoter comprises the polynucleotide of claim 33.

20

35. The expression cassette of claim 34 wherein said polynucleotide of interest encodes a polypeptide or a suppression element.

25 36. The expression cassette of claim 36, wherein said polynucleotide of interest encodes an HPPD polypeptide having HPPD activity and having insensitivity to an HPPD inhibitor.

37. An expression vector comprising the expression cassette of any one of claims 34-36.

30

38. A plant having stably incorporated into its genome at least one expression cassette comprising a polynucleotide of interest operably linked to a promoter, wherein said promoter comprises the polynucleotide of claim 33 or the expression cassette of any one of claims 34-36.

39. The plant of claim 38, wherein said plant is a dicot.
40. The plant of claim 39, wherein said dicot is soybean.
- 5
41. The plant of claim 39, wherein said dicot is Brassica, sunflower, cotton,
or alfalfa.
42. The plant of claim 38, wherein said plant is a monocot.
- 10
43. The plant of claim 42, wherein said monocot is maize, wheat, rice,
barley, sorghum, or rye.
44. A method of expressing a polynucleotide of interest, said method
15 comprising stably incorporating in the genome of a plant or plant cell the
polynucleotide of interest operably linked to a promoter, wherein said promoter
comprises the polynucleotide of claim 33 or stably incorporating into the genome of the
plant or plant cell the expression cassette of any one of claims 34-36.
- 20
45. The method of claim 44, wherein said plant is a dicot.
46. The method of claim 45, wherein said dicot is soybean.
47. The method of claim 45, wherein said dicot is Brassica, sunflower,
25 cotton, or alfalfa.
48. The method of claim 44, wherein said plant is a monocot.
49. The method of claim 48, wherein said monocot is maize, wheat, rice,
30 barley, sorghum, or rye.

Application number / Numéro de demande: 2,805,937

Figures: _____

Pages: 1, 2, 10, 11, 12

Unscannable items received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
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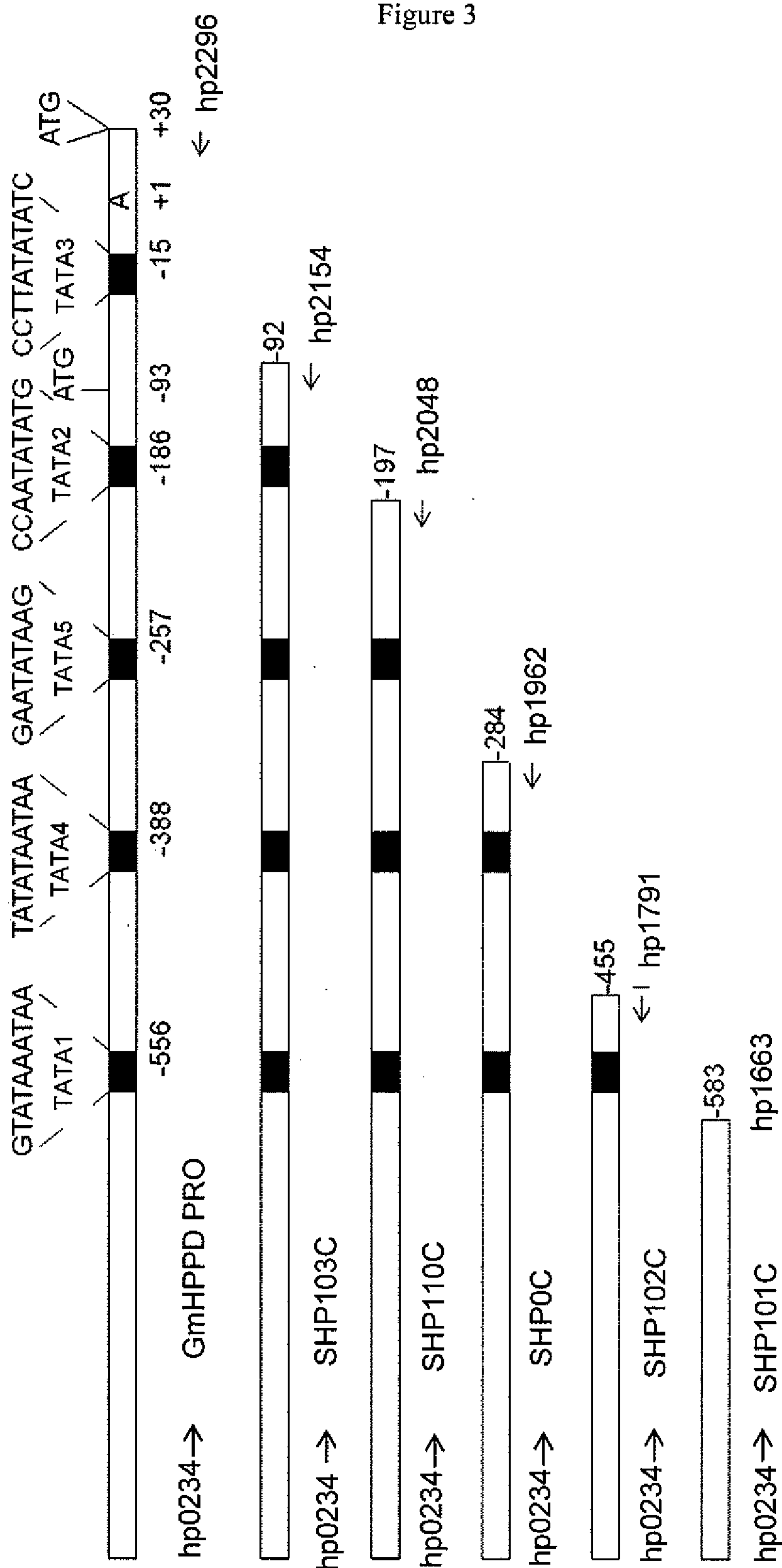


Figure 4

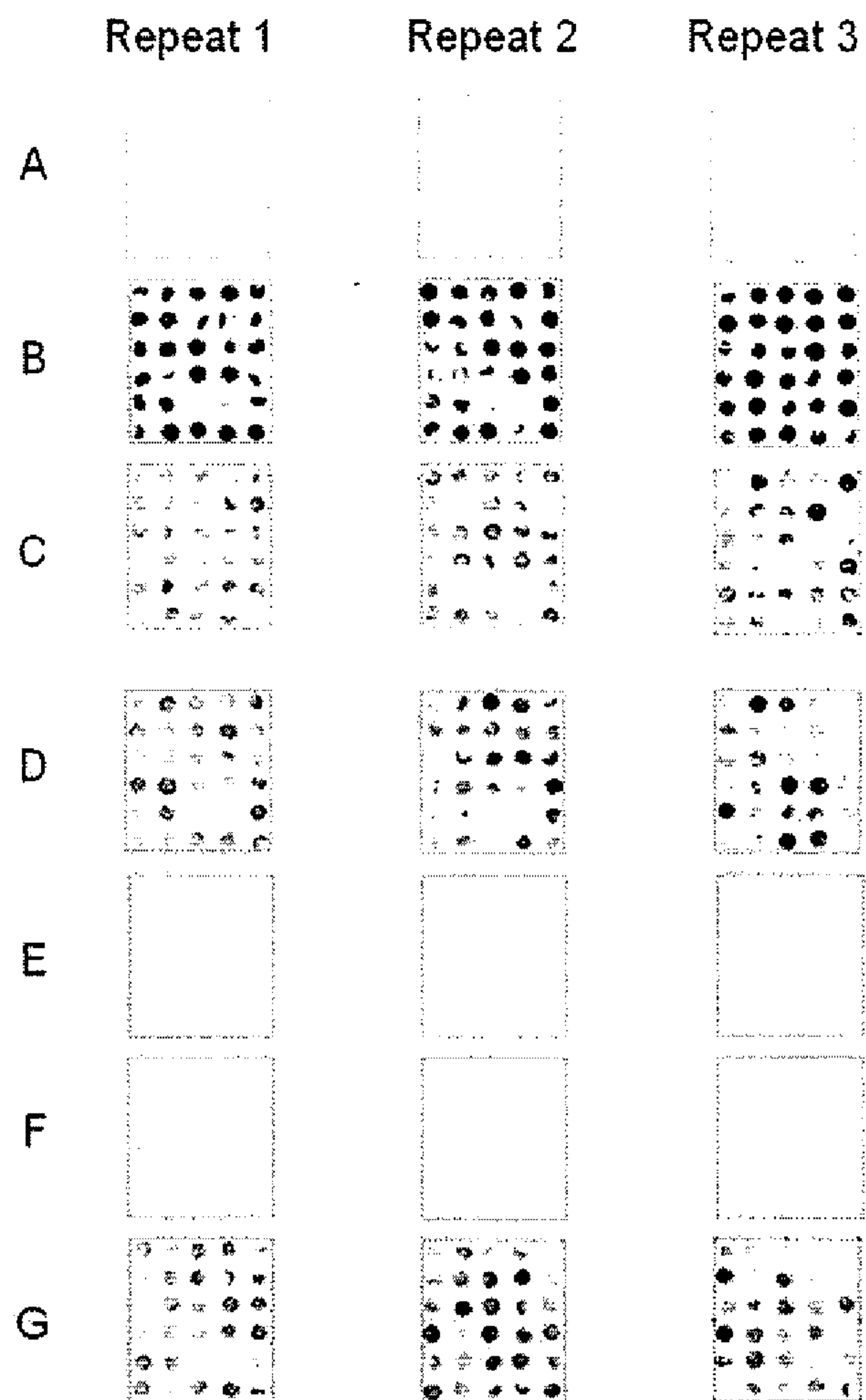
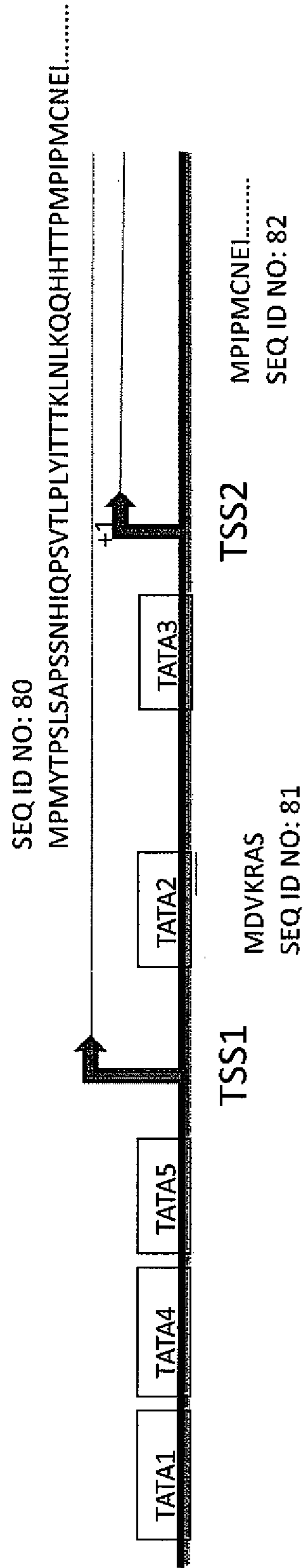


Figure 5

Long transcript translation leads to chloroplast localization










uORF in long transcript may regulate translation

Short transcript translation leads to cytosolic localization

Figure 6

HPPD Promoters

Native Soy HPPD Promoter		<u>Experimental Name</u>	
T1 T4 T5 T2 T3 	SEQ ID NO: 1	GmHPPD PRO	
T1 T4 T5 T2 	SEQ ID NO: 4	SHP103C	
T1 T4 T5 	SEQ ID NO: 11	SHP110C	
T1 T4 	SEQ ID NO: 20	SHP0C	
T1 	SEQ ID NO: 3	SHP102C	
	SEQ ID NO: 2	SHP101C	
T1 T4 T5 T2 T3 	SEQ ID NO: 76	SHP122C	

- | | |
|--------------|-------------------|
| ■ TATA1 (T1) | ■ TATA4 (T4) |
| ■ TATA2 (T2) | ■ TATA5 (T5) |
| ■ TATA3 (T3) | * Point mutations |

Figure 7












HPPD Promoters containing mutations

		<u>Experimental Name</u>
T1 T4 T5 T2 T3	SEQ ID NO: 1	GmHPPD PRO
T1 T4 T5 T2 T3	SEQ ID NO: 5	SHP104C
T1 T4 T5 T2 T3	SEQ ID NO: 6	SHP105C
T1 T4 T5 T2 T3	SEQ ID NO: 7	SHP106C
T1 T4 T5 T2 T3	SEQ ID NO: 8	SHP107C
T1 T4 T5 T2 T3	SEQ ID NO: 9	SHP108C
T1 T4 T5 T2 T3	SEQ ID NO: 10	SHP109C
T1 T4 T5 T2 T3	SEQ ID NO: 12	SHP111C
T1 T4 T5 T2 T3	SEQ ID NO: 13	SHP112C
T1 T4 T5 T2 T3	SEQ ID NO: 14	SHP113C
T1 T4 T5 T2 T3	SEQ ID NO: 15	SHP114C
T1 T4 T5 T2 T3	SEQ ID NO: 16	SHP115C
T1 T4 T5 T2 T3	SEQ ID NO: 17	SHP116C
T1 T4 T5 T2 T3	SEQ ID NO: 18	SHP117C
T1 T4 T5 T2 T3	SEQ ID NO: 19	SHP118C
T1 T4 T5 T2 T3	SEQ ID NO: 74	SHP120C
T1 T4 T5 T2 T3	SEQ ID NO: 75	SHP121C

■ TATA1 (T1)	■ TATA4 (T4)
■ TATA2 (T2)	■ TATA5 (T5)
■ TATA3 (T3)	* Point mutations

Figure 8

**Chimeric Promoters with Synthetic Element I (SEQ ID NO:21)
including SynII core**

	<u>Experimental Name</u>	
T1 T4 T5 T2 T3 	SEQ ID NO: 1	
	SEQ ID NO: 23	SHP101
T1 	SEQ ID NO: 24	SHP102
T1 T4 T5 T2 	SEQ ID NO: 25	SHP103
T1 T4 T5 T2 T3 	SEQ ID NO: 26	SHP104
T1 T4 T5 T2 T3 	SEQ ID NO: 27	SHP105
T1 T4 T5 T2 T3 	SEQ ID NO: 28	SHP106
T1 T4 T5 T2 T3 	SEQ ID NO: 29	SHP107
T1 T4 T5 T2 T3 	SEQ ID NO: 30	SHP108
T1 T4 T5 T2 T3 	SEQ ID NO: 31	SHP109
T1 T4 T5 T2 	SEQ ID NO: 77	SHP111

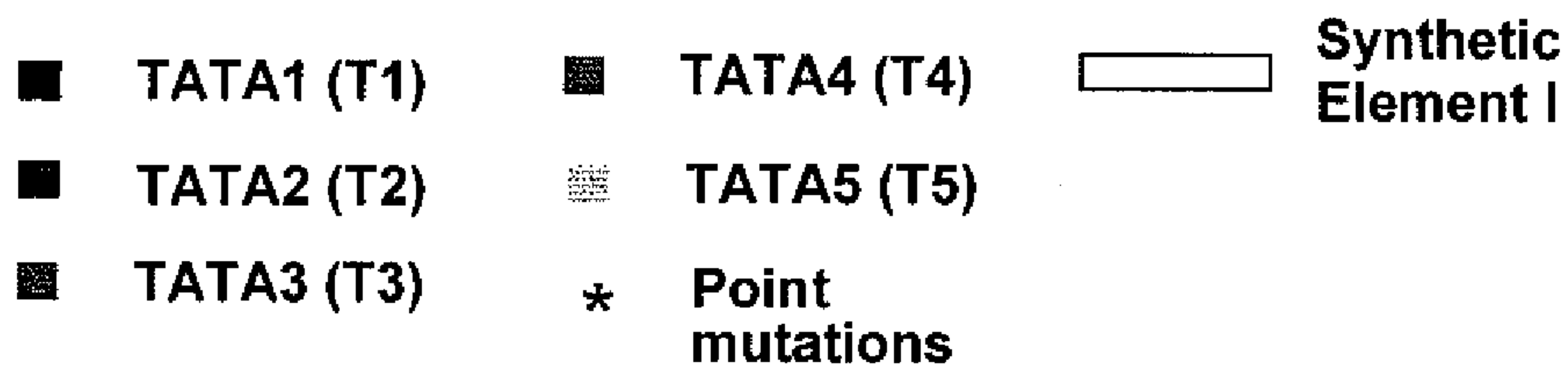


Figure 9

**Chimeric Promoters with Synthetic Element II (SEQ ID NO:22)
including Rsyn7, SynII core, and 5' UTR**

Experimental Name

