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(71) Demandeur/Applicant:
E.I. DUPONT DE NEMOURS & COMPANY, US

(72) Inventeurs/Inventors:
CAHOON, EDGAR B., US;
COUGHLAN, SEAN J., US;
CAHOON, REBECCA E., US;
BUTLER, KARLENE H., US

(74) Agent: TORYS LLP

(54) Titre : COMPOSITIONS ET PROCEDES POUR LA TRANSFORMATION DE TENEUR EN TOCOTRIENOL
(54) Title: COMPOSITIONS AND METHODS FOR ALTERING TOCOTRIENOL CONTENT

(57) **Abrégé/Abstract:**

The invention provides isolated nucleic acids and their encoded polypeptides that alter tocol content in seeds. The invention further provides expression cassettes, host cells and transformed plants containing the nucleic acids. The present invention further provides methods for altering tocol content in seeds.



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- (71) Applicant: **E.I. DUPONT DE NEMOURS & COMPANY** [US/US]; 1007 Market Street, Suite 900, DuPont Building, Wilmington, DE 19898 (US).
- (72) Inventors: **CAHOON, Edgar, B.**; 745 Tuxedo Boulevard, Webster Groves, MO 63119 (US). **COUGHLAN, Sean, J.**; 108 Stratton Drive, Canterbury Hill, Hockessin, DE 19707 (US). **CAHOON, Rebecca, E.**; 745 Tuxedo Boulevard, Webster Groves, MO 63119 (US). **BUTLER, Karlene, H.**; 844 Broadfield Drive, Newark, DE 19713 (US).
- (74) Agents: **LAPPEGARD, Kathryn, K.** et al.; 7100 N.W. 62nd Avenue, Darwin Building, Johnston, IA 50131-1000 (US).
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(54) Title: COMPOSITIONS AND METHODS FOR ALTERING TOCOTRIENOL CONTENT

(57) Abstract: The invention provides isolated nucleic acids and their encoded polypeptides that alter tocol content in seeds. The invention further provides expression cassettes, host cells and transformed plants containing the nucleic acids. The present invention further provides methods for altering tocol content in seeds.

COMPOSITIONS AND METHODS FOR ALTERING TOCOTRIENOL CONTENT

FIELD OF THE INVENTION

The present invention relates to the field of plant molecular biology,
5 particularly to the isolation of genes. The invention further relates to the use of the
genes to host cells. More specifically, this invention pertains to nucleic acid
fragments encoding homogentisate geranylgeranyl transferase in plants.

BACKGROUND OF THE INVENTION

10 Tocotrienols are vitamin E-related compounds whose occurrence in plants
is limited primarily to the seeds and fruits of most monocot species (e.g., palm,
wheat, rice and barley). Tocotrienols are structurally similar to tocopherols,
including α -tocopherol or vitamin E, which occur ubiquitously in the plant kingdom
as well as in photosynthetic microbes such as *Synechocystis*. Tocotrienols and
15 tocopherols both contain a chromanol head group that is linked to a hydrocarbon
side chain. The only structural difference between these molecules is the
presence of three double bonds in the hydrocarbon side chain of tocotrienols.
This difference is related to the biosynthetic origins of the side chains. Tocopherol
side chains are derived from phytyl-pyrophosphate (PP), and the tocotrienol side
20 chains are believed to be derived from geranylgeranyl-PP (Soll, J. et al. (1980)
Arch. Biochem. Biophys. 204:544-550).

Four forms or molecular species of tocopherols and tocotrienols occur in
nature: α , β , γ and δ . These molecular species contain different numbers of
methyl groups that are bound to the aromatic portion of the chromanol head. Like
25 tocopherols, tocotrienols are potent lipid-soluble antioxidants and therefore have
considerable nutritive value in human and animal diets (Packer, L. et al. (2001) *J.*
Nutr. 131:369S-373S). In addition, tocotrienols are believed to have therapeutic
properties including a demonstrated ability to down regulate cholesterol
biosynthesis (Theriault, A. et al. (1999) *Clin. Biochem.* 32:309-319; Qureshii, A.A.
30 et al. (1986) *J. Biol. Chem.* 261:10544-10550).

It has been speculated that the first committed step in the biosynthesis of
tocotrienols involves the condensation of geranylgeranyl-PP and homogentisate to
form 2-methyl-6-geranylgeranylbenzoquinol (Soll, J. et al. (1980) *Arch. Biochem.*
Biophys. 204:544-550). The enzyme that catalyzes this reaction can thus be
35 functionally described as a homogentisate geranylgeranyl transferase (HGGT).

Functional identification of genes or cDNAs encoding HGGT polypeptides has yet to be reported. The lack of these nucleic acids limits efforts to manipulate the biosynthesis of the nutritionally important tocotrienols in plants and microbial hosts. The problem to be solved, therefore, is in identifying the nucleic acids that
5 encode polypeptides required for tocotrienol biosynthesis in plants.

SUMMARY OF THE INVENTION

Compositions and methods for the alteration of the tocol content and composition of plants are provided. The compositions comprise novel nucleotide
10 molecules comprising nucleotide sequences for HGGT. The compositions can be used to transform plants to manipulate the synthetic pathway for tocol compounds.

Transformed plants, plant cells, plant tissues, seed and grain are provided. Transformed plants of the invention find use in methods for improving grain or seed characteristics including, but not limited to, antioxidant level or activity.

15 Expression cassettes comprising sequences of the invention are provided. Isolated polypeptides encoded by the nucleotide sequences of the invention are also provided.

DETAILED DESCRIPTION

The problem to be solved is identifying the nucleic acids that encode
20 polypeptides required for tocotrienol biosynthesis in plants. These polynucleotides may be used in plant cells and photosynthetic microbes to alter the tocols, such as tocotrienols, produced in the cells. More specifically, the polynucleotides of the instant invention may be used to significantly increase the content of vitamin E-related antioxidants such as tocotrienol in edible tissues of vegetable, fruit, and
25 agronomic crop plants, including grains such as corn and soybean seed. The availability of nucleic acid sequences encoding all or a portion of the enzyme homogentisate geranylgeranyl transferase (HGGT) would facilitate studies to better understand tocotrienol biosynthesis in plants and provide genetic tools to alter tocotrienol metabolism. The present invention has solved this problem by
30 providing nucleotide and deduced amino acid sequences corresponding to novel HGGT polynucleotides and corresponding polypeptides from barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*).

The HGGT-catalyzed reaction is analogous to the first step in tocopherol biosynthesis, which involves the condensation of homogentisate and phytyl-PP to

form 2-methyl-6-phytylbenzoquinol (Soll, J. et al., *supra*). The latter reaction is catalyzed by the enzyme homogentisate phytyltransferase (HPT).

cDNAs encoding HPT from a number of plant species have previously been disclosed in World Patent Application WO 00/68393. Given the similarity in their
5 substrates and activity, one can hypothesize that HGGT is a divergent, but related form of HPT. It is likely that HGGT is the only specialized enzyme in the tocotrienol biosynthetic pathway. Methylation and cyclization reactions that convert the HGGT product 2-methyl-6-geranylgeranylbenzoquinol into tocotrienols are likely catalyzed by enzymes that are shared between the tocopherol and
10 tocotrienol biosynthetic pathways (Schultz, G. et al. (1985) *Physiol. Plant.* 64:123-129). As such, expression of HGGT will be sufficient to confer tocotrienol biosynthesis to a plant, plant tissue, a cell from plant tissue or a photosynthetic microbe that does not normally produce tocotrienols.

Recently, genes or cDNAs for HPT have been identified and characterized
15 from the cyanobacterium *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana* based on their sequence similarity to chlorophyll synthases (Schledz, M. et al. (2001) *FEBS* 499:15-20; Collakova, E. and DellaPenna, D. (2001) *Plant Physiol.* 127:1113-1124). cDNAs for homologs of the *Arabidopsis* HPT have also been identified from wheat, rice, corn and soybean (Collakova, E. and DellaPenna, D.
20 (2001) *Plant Physiol.* 127:1113-1124).

The invention is drawn to compositions and methods for altering tocols. The compositions and methods find use in improving the antioxidant quality of grain for use as food for humans and feed for livestock. Furthermore, the tocols can be extracted, purified or further altered via processing. As used herein,
25 "grain" means the mature seed produced by commercial growers for purposes other than reproducing the species and/or immature seed as an integral part of whole plant corn harvested for silage. As used herein, grain includes plant parts commonly categorized as a fruit, nut or vegetable.

As used herein, "wild-type" refers to untransformed organisms and
30 descendants of untransformed organisms.

The term "tocol" refers generally to any of the tocopherol and tocotrienol molecular species (e.g., α -, β -, γ -, and δ -) that are known to occur in biological systems. The term "tocol content" refers to the total amount of tocopherol and tocotrienol in a whole plant, tissue, or cell or in a microbial host. The term "tocol

composition" refers both to the ratio of the various tocols produced in any given biological system and to altered characteristics, such as antioxidant activity, of any one tocol compound. When the alteration of tocols is taught or claimed herein, such alteration can be to tocol content and/or tocol composition. When an
5 increase of tocols is taught or claimed herein, such increase refers to an increase of tocol content and/or an increase of tocol activity.

The term "tocotrienol" refers generally to any of the tocotrienol molecular species (e.g., α -, β -, γ -, and δ -) that are known to occur in biological systems. The term "tocotrienol content" refers to the total amount of tocotrienol in a whole plant,
10 tissue, or cell or in a microbial host. The term "tocotrienol composition" refers both to the ratio of the various tocotrienols produced in any given biological system and to altered characteristics, such as antioxidant activity, of any one tocotrienol compound. When the alteration of a tocotrienol is taught or claimed herein, such alteration can be to tocotrienol content and/or tocotrienol composition. When an
15 increase of tocotrienols is taught or claimed herein, such increase refers to an increase of tocotrienol content and/or an increase of tocotrienol activity.

The term "homogentisate phytyltransferase" or "HPT" refers to the enzyme that catalyzes the condensation of homogentisate (or homogentisic acid) and phytyl pyrophosphate (or phytyl diphosphate). This reaction is believed to be the
20 committed step in tocopherol biosynthesis. Other names that have been used to refer to this enzyme include "homogentisate phytyl pyrophosphate prenyltransferase" and "homogentisate phytyl diphosphate prenyltransferase". The shortened version phytyl/prenyl transferase is also used.

The term "homogentisate geranylgeranyl transferase" or "HGGT" refers to
25 the enzyme that catalyzes the condensation of homogentisate (or homogentisic acid) and geranylgeranyl pyrophosphate (or geranylgeranyl diphosphate). This reaction is an important step in tocotrienol biosynthesis and can result in the alteration of the tocol content and/or composition.

The invention provides isolated nucleotide molecules comprising nucleotide
30 sequences encoding HGGT. Also provided are isolated polypeptides encoded by such nucleotide sequences. The nucleotide sequences find use in methods for altering tocols and tocotrienols in a biological system such as a plant. The methods include improving the antioxidant activity of grain, altering tocotrienols in a plant or part thereof, and improving tocols in a host. The methods comprise

transforming a plant or host with at least one nucleotide construct comprising at least a portion of at least one nucleotide sequence of the invention. If desired, the nucleotide construct may additionally comprise an operably linked promoter that drives expression in the plant of interest. Such a nucleotide construct can be used to increase the expression of HGGT.

Among the many applications of improved tocols, tocotrienols and antioxidant activity are improved storage of grain, improved stability of oil extracted from grain, health benefits to humans and animals consuming the grain, and the production of novel tocols or tocotrienols for cosmetic, industrial and/or nutraceutical use. It is also known that the presence of tocols in plant vegetative green tissue such as leaf tissue is necessary to protect the plant from the photo-oxidative damage induced directly and indirectly by the production of free oxygen radicals in the chloroplast during oxygenic photosynthesis. It is therefore likely that ectopic expression of tocotrienols in green plant tissue, such as leaf tissue, in addition to the normal tocopherol content of the leaf will lead to an increase ability to withstand such photo-oxidative damage, and thus lead to an increase in the photosynthetic capacity of the plant. This would translate to an increase in harvestable yield for the plant over the entire growing season.

The nucleotide constructs of the invention comprise at least a portion of a nucleotide sequence of the invention. The nucleotide construct of the invention may additionally comprise at least one promoter that drives expression in a host or plant. Preferred promoters include, for corn, an embryo specific promoter such as promoters for the 16kDa and 18kDa oleosin genes, an endosperm specific promoter, such as the promoter for the 10kDa zein gene, and a vegetative promoter such as promoters for ubiquitin genes.

A nucleotide construct of the invention comprises at least a portion of one nucleotide sequence of the invention. Preferably, such a nucleotide construct additionally comprises an operably linked promoter that drives expression in a plant. If desired, two or more of such nucleotide sequences may be linked or joined together to form one polynucleotide molecule, and such a polynucleotide may be used to transform a plant. For example, a nucleotide construct comprising a nucleotide sequence encoding a HGGT can be linked with another nucleotide sequence encoding the same or another HGGT. Nucleotide sequences encoding both HGGT and HPT may also be linked in a nucleotide construct. Similarly, the

two nucleotide sequences can be provided on different nucleotide constructs, and each of the separate nucleotide sequences can be operably linked to a promoter that drives expression in a plant. For example, a construct may be used that increases total HGGT activity and decreases total HPT activity, thereby resulting in shunting the pathway towards the production of tocotrienols and decreased production of tocopherols. The alternative strategy may also be used. If separate nucleotide constructs are employed for the HGGT nucleotide sequence and an HPT nucleotide sequence, two individual plants may be transformed with the nucleotide constructs, and the plants may then be crossed to produce progeny having the desired genotype of both the HGGT and HPT nucleotide sequences.

Similarly, a construct to down-regulate the geranylgeranyl reductase responsible for producing phytol pyrophosphate, one of the precursors for tocopherol biosynthesis, may be linked in cis with a construct to express HGGT. The result of this manipulation would be an increased pool size of geranylgeranyl-pyrophosphate and a corresponding increase of flux into the tocotrienol biosynthetic pathway.

The methods of the present invention can be employed to alter tocols or tocotrienols in any plant or part thereof, and antioxidant activity may thereby be altered. Plants that may be used in the invention include field crops (e.g., alfalfa, barley, bean, maize, cotton, flax, pea, rape, rice, rye, safflower, sorghum, oats, millet, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); and fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon) and Arabidopsis. Some methods of the invention involve altering the antioxidant levels in grain and other parts of a plant that may be subjected to post-harvest processing or can be used as food source for humans, livestock and other animals, such as pets. With post-harvest processing, the tocols or tocotrienols so produced can be a valuable source of recovery for millers and other processors.

Methods for assessing tocopherol content and tocopherol composition (including tocopherol activity) are known in the art. Tocopherol content and composition may be measured by HPLC in combination with fluorescence detection. Such methods are described in Example 2 and in numerous literature references (e.g., Kamal-Eldi A., Gorgen S., Pettersson J., Lampi A.M. (2000) J Chromatogr A 881:217-227; Bonvehi J.S., Coll F.V., Rius I.A. (2000) J. AOAC Intl. 83:627-634; Goffman F.D. and Böhme T. (2001) J Agric. Food Chem. 49:4990-4994). Such methods typically involve the resolution of tocopherol molecular species contained in complex mixtures by use of a normal or reverse phase HPLC matrix. Eluted tocopherol molecular species are then detected by fluorescence of the chromanol head group with an excitation wavelength typically in the range of 290 to 295 nm and an emission wavelength typically in the range of 325 to 335 nm. Using this methodology, the composition of a tocopherol mixture can be determined by comparing the retention times of separated molecular species with those of known standards. The content of each tocopherol molecular species can be measured by the relative intensity of its fluorescence emission at the selected wavelength. The absolute amount of each tocopherol species can be determined by measuring the intensity of fluorescence emission relative to that of an internal standard, which is added in a known amount to the tocopherol mixture prior to HPLC analysis. A suitable internal standard can include a tocopherol analog that is not normally found in nature (e.g., 5,7-dimethyltol) or a naturally occurring tocopherol molecular species that is not present in a given tocopherol mixture. The total tocopherol content of a complex mixture of compounds can be derived by summing the absolute amount of each of the component tocopherol molecular species as determined by HPLC analysis.

Methods for assessing tocotrienol content and tocotrienol composition (including tocotrienol activity) are known in the art. Tocotrienol content and composition may be measured by HPLC using methods described above for the analysis of tocopherol content and composition. Using HPLC techniques described in Example 2 and elsewhere (e.g., Podda M., Weber C., Traber M.G., Packer L. (1996) J. Lipid Res. 37:893-901), tocotrienol molecular species can be readily resolved from tocopherol molecular species in a complex mixture. The occurrence and structural identification of tocotrienols in a complex mixture can be

determined by gas chromatography-mass spectrometry as described by Frega N., Mozzon M., and Bocci F. (1998) J. Amer. Oil Chem. Soc. 75:1723-1728.

In addition, lipophilic antioxidant activity may be measured by assays including the inhibition of the coupled auto-oxidation of linoleic acid and β -carotene and oxygen radical absorbance capacity (ORAC) as described elsewhere (Serbinova E.A. and Packer L. (1994) Meth. Enzymol. 234:354-366; Emmons C. L., Peterson D.M., Paul G.L. (1999) J. Agric. Food Chem. 47:4894-4898); Huang D et al (2002) J.Agric. Food Chem.) in the press. Such methods typically involve measuring the ability of antioxidant compounds (i.e., tocols) in test materials to inhibit the decline of fluorescence of a model substrate (fluorescein, phycoerythrin) induced by a peroxy radical generator (2',2'-azobis[20amidinopropane]dihydrochloride).

The nucleotide constructs of the invention can also be used to decrease or suppress the expression of endogenous HGGT in a plant. Decreasing the expression of HGGT involved in the production of tocotrienol can serve to shift the tocol pathway toward the production of tocopherol. A plant can be transformed with the HGGT nucleotide sequences in the sense orientation for co-suppression or sense suppression of gene expression. Alternatively, the plant can be transformed with the HGGT nucleotide sequences in the antisense orientation for antisense suppression. Expression of HGGT polypeptides can also be suppressed by modifying genomic sequences in a plant by chimeraplasty. Generally, such modifications will alter the amino acid sequence of the polypeptides encoded by the genomic sequence as to reduce or eliminate the activity of a HGGT in a plant, particularly in a seed.

Compositions of the invention include nucleotide sequences encoding HGGT polypeptides that are involved in regulating tocols or tocotrienols. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID Nos: 2, 4, 6, 8 and 10. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID Nos: 1, 3, 5, 7, and 9, and fragments and variants thereof.

The invention encompasses isolated or substantially purified nucleic acid or polypeptide compositions. An "isolated" or "purified" nucleic acid molecule or

polypeptide, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, 0.3kb or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating polypeptide. When the polypeptide of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, 5%, 3% or 1% (by dry weight) of chemical precursors or non-polypeptide-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and polypeptides encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence. Fragments of a nucleotide sequence may encode polypeptide fragments that retain the biological activity of the native protein and hence HGGT activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode polypeptides retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 30 nucleotides, about 50 nucleotides, about 70 nucleotides, about 100 nucleotides, about 150 nucleotides and up to the full-length nucleotide sequence encoding the polypeptides of the invention.

A fragment of a HGGT nucleotide sequence that encodes a biologically active portion of an HGGT polypeptide of the invention will encode at least 15, 25, 30, 50, 75, 100, or 125 contiguous amino acids, or up to the total number of amino acids present in a full-length HGGT polypeptide of the invention (for example, 407, 408, 404, 380 and 361 amino acids for SEQ ID NO: 2, 4, 6, 8 and 10 respectively). Fragments of a HGGT nucleotide sequence that are useful as hybridization probes

or PCR primers generally need not encode a biologically active portion of an HGGT polypeptide.

Thus, a fragment of an HGGT nucleotide sequence may encode a biologically active portion of an HGGT polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an HGGT polypeptide can be prepared by isolating a portion of one of the HGGT nucleotide sequences of the invention, expressing the encoded portion of the HGGT polypeptide (e.g., by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the HGGT polypeptide. Conserved motifs for HGGT sequences are identified in SEQ. ID Nos: 56-66.

Nucleic acid molecules that are fragments of an HGGT nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, or 700 nucleotides, or up to the number of nucleotides present in a full-length HGGT nucleotide sequence disclosed herein (for example, 1457, 1365, 1242, 1730, and 1769 nucleotides for SEQ ID NO: 1, 3, 5, 7 and 9, respectively). The coding sequences for the conserved motifs identified in SEQ. ID Nos: 56-66 can also be easily identified from the HGGT sequences provided herein. The same is also true of degenerate sequences coding for the conserved motifs identified in SEQ. ID Nos: 56-66.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the HGGT polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode an HGGT polypeptide of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 80% generally at least about 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere

herein using default parameters. Optionally, variants will also encode for at least any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the conserved motifs identified in SEQ. ID Nos: 56-66.

By "variant" polypeptide is intended a polypeptide derived from the native polypeptide by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native polypeptide; deletion or addition of one or more amino acids at one or more sites in the native polypeptide; or substitution of one or more amino acids at one or more sites in the native polypeptide. Variant polypeptides encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native polypeptide, that is, HGGT activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native HGGT polypeptide of the invention will have at least about 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to the amino acid sequence for the native polypeptide as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a polypeptide of the invention may differ from that polypeptide 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. Optionally, variants will also comprise at least any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the conserved motifs identified in SEQ. ID Nos: 56-66.

The polypeptides of the invention 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 of the HGGT polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence 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; US Patent No. 4,873,192; Walker and Gastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide 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 preferred.

Thus, the genes and nucleotide sequences of the invention include both the
5 naturally occurring sequences as well as mutant forms. Likewise, the polypeptides of the invention encompass both naturally occurring polypeptides as well as variations and modified forms thereof. Such variants will continue to possess the desired HGGT activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame
10 and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the polypeptide sequences encompassed herein are not expected to produce radical changes in the characteristics of the polypeptide. However, when it is difficult to predict the exact
15 effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by assays for HGGT activity.

Variant nucleotide sequences and polypeptides also encompass sequences and polypeptides derived from a mutagenic and recombinogenic
20 procedure such as DNA shuffling. With such a procedure, one or more different HGGT coding sequences can be manipulated to create a new HGGT polypeptide 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
25 identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the HGGT polynucleotides of the invention and/or other HGGT genes to obtain a new gene coding for a polypeptide with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such
30 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.* (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.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence
5 homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire HGGT nucleotide sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended polynucleotides derived from a common ancestral gene and which are
10 found in different species as a result of speciation. Polynucleotides found in different species are considered orthologs when their nucleotide sequences and/or their encoded polypeptide sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

15 In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor
20 Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using
25 paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

For clarification, "PCR" or "polymerase chain reaction" is a technique for the synthesis of large quantities of specific DNA segments, consists of a series of
30 repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P , or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the HGGT sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire HGGT sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding HGGT sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among HGGT sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Polynucleotide sequences useful as probes include the polynucleotide sequences encoding the conserved motifs set forth in SEQ. ID Nos: 56-66. Such probes may be used to amplify corresponding HGGT sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or

washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than
5 about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes
10 (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C.
15 Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. The duration of hybridization is generally less than about 24 hours, usually about 4
20 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41$
25 $(\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a
30 perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m)

for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Isolated sequences that encode for a HGGT polypeptide and which hybridize under stringent conditions to the HGGT sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

Nucleotides (usually found in their T-monophosphate form) are often referred to herein by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "W" for A or T, "H" for A or C or T, "D" for A or G or T, "M" for A or C, "S" for C or G, "V" for A or C or G, "B" for C or G or T, "I" for inosine, and "N" for A, C, G, or T.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

5 (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) for optimal alignment of the two sequences.

10 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.

15 Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent 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 homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman *and Wunsch* (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-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.

25 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

30 Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic*

Acids Res. 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN
5 program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a polypeptide of the invention.
10 BLAST polypeptide searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a polypeptide 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)
15 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 polypeptides) can be used. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by
20 inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent
25 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 the preferred program.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:
30 443-453, 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 Wisconsin Genetics Software Package for polypeptide 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 selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored 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 Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

For purposes of the present invention, comparison of nucleotide or polypeptide sequences for determination of percent sequence identity to the HGGT sequences disclosed herein is preferably made using CLUSTAL with the following changes from the default parameters. For amino acid sequence comparisons a Gap Penalty of 10 and Gap Length Penalty of 10 was used for multiple alignments and a KTUPLE of 2, Gap Penalty of 3, Window of 5 and Diagonals Saved of 5 was used for pairwise alignments. For nucleotide sequence comparisons, a Gap Penalty of 10 and Gap Length Penalty of 10 was used for multiple alignments and a KTUPLE of 2, Gap Penalty of 5, Window of 4 and Diagonals Saved of 4 was used for pairwise alignments. Any equivalent program can also be used to determine percent sequence identity. 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 the preferred program.

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

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

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of polypeptides encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C lower than the T_m , depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "nucleotide constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the nucleotide constructs of the present invention encompass all nucleotide constructs that can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

Furthermore, it is recognized that the methods of the invention may employ a nucleotide construct that is capable of directing, in a transformed plant, the expression of at least one polypeptide, or at least one RNA, such as, for example, an antisense RNA that is complementary to at least a portion of an mRNA. Typically such a nucleotide construct is comprised of a coding sequence for a polypeptide or an RNA operably linked to 5' and 3' transcriptional regulatory regions. Alternatively, it is also recognized that the methods of the invention may employ a nucleotide construct that is not capable of directing, in a transformed plant, the expression of a polypeptide or an RNA.

In addition, it is recognized that methods of the present invention do not depend on the incorporation of the entire nucleotide construct into the genome, only that the plant or cell thereof is altered as a result of the introduction of the nucleotide construct into a cell. In one embodiment of the invention, the genome may be altered following the introduction of the nucleotide construct into a cell. For example, the nucleotide construct, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome of the present invention include, but are not limited to, additions, deletions, and substitutions of nucleotides

in the genome. While the methods of the present invention do not depend on additions, deletions, or substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprise at least one nucleotide.

5 The nucleotide constructs of the invention also encompass nucleotide constructs that may be employed in methods for altering or mutating a genomic nucleotide sequence in an organism, including, but not limited to, chimeric vectors, chimeric mutational vectors, chimeric repair vectors, mixed-duplex
10 oligonucleotides, self-complementary chimeric oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use, such as, for example, chimeraplasty, are known in the art. Chimeraplasty involves the use of such nucleotide constructs to introduce site-specific changes into the sequence of genomic DNA within an organism. See, U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of
15 which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; herein incorporated by reference.

 The HGGT sequences of the invention are provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3'
20 regulatory sequences operably linked to a HGGT nucleotide sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences
25 being linked are contiguous and, where necessary to join two polypeptide coding regions, contiguous and 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.

30 Such an expression cassette is provided with a plurality of restriction sites for insertion of the HGGT nucleotide sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a HGGT polynucleotide sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of HGGT in the plant, plant cell or other host. Thus, the phenotype of the plant, plant cell or other host is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (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 Acid Res.* 15:9627-9639.

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

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

expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

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

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

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can
30 be combined with constitutive, chemically regulated, tissue-preferred, or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985)

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

Chemically regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator.

Depending upon the objective, the promoter may be a chemical inducible promoter, where application of the chemical induces gene expression, or a chemical repressible promoter, where application of the chemical represses gene expression. Chemical inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemically regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced HGGT expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993)

Plant J. 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

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

Root-specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2): 207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β -glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2):343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-

772); and rolB promoter (Capano *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

“Seed-preferred” promoters include both “seed-specific” promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as “seed-germinating” promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); and milps (myo-inositol-1-phosphate synthase); (see WO 00/11177, herein incorporated by reference). The 27 kDa gamma-zein promoter is a preferred endosperm-specific promoter. The maize globulin-1 and oleosin promoters are preferred embryo-specific promoters. For dicots, seed-specific promoters include, but are not limited to, bean β -phaseolin, napin, β -conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, promoters of the 15 kDa beta-zein, 22 kDa alpha-zein, 27 kDa gamma-zein, waxy, shrunken 1, shrunken 2, globulin 1 and oleosin genes. See also WO 00/12733, where seed-preferred promoters from *end1* and *end2* genes are disclosed; herein incorporated by reference.

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

The HGGT polypeptides of the invention can be targeted to specific compartments within the plant cell. Methods for targeting polypeptides to a specific compartment are known in the art. Generally, such methods involve modifying the nucleotide sequence encoding the polypeptide in such a manner as to add or remove specific amino acids from the polypeptide encoded thereby. Such amino acids comprise targeting signals for targeting the polypeptide to a

specific compartment such as, for example, a the plastid, the nucleus, the endoplasmic reticulum, the vacuole, the mitochondrion, the peroxisome, the Golgi apparatus, and for secretion from the cell. Targeting sequences for targeting a polypeptide to a specific cellular compartment, or for secretion, are known to those of ordinary skill in the art. Chloroplast-targeting or plastid-targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol.* 30:769-780; Schnell *et al.* (1991) *J. Biol. Chem.* 266(5):3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb.* 22(6):789-810); tryptophan synthase (Zhao *et al.* (1995) *J. Biol. Chem.* 270(11):6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem.* 272(33):20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem.* 268(36):27447-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem.* 263:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; and Shah *et al.* (1986) *Science* 233:478-481.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase, as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). 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.

The invention involves transforming host cells with the nucleotide constructs of the invention. Generally, the nucleotide construct will comprise a HGGT nucleotide sequence of the invention, either a full length sequence or functional fragment thereof, operably linked to a promoter that drives expression in the host cell of interest. Host cells include, but are not limited to: plant cells; animal cells; fungal cells, particularly yeast cells; and bacterial cells.

The methods of the invention involve introducing a nucleotide construct into a plant. By "introducing" is intended presenting to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a nucleotide construct to a plant, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

By "stable transformation" is intended that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleotide construct introduced into a plant does not integrate into the genome of the plant.

Transformation protocols as well as protocols for introducing nucleotide 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

nucleotide sequences into plant cells and subsequent insertion into the plant genome 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 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* 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.* 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 al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996)

Nature Biotechnology 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The nucleotide constructs of the invention may also 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. It is recognized that a HGGT of the invention may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant polypeptide. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing nucleotide constructs into plants and expressing a polypeptide encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

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

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

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic

identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. Transformed plants include those plants directly transformed as provided herein, as well as plants that have the directly transformed plants in their pedigree and retain the change in genotype, such as the inclusion of the expression cassette, created by the original transformation.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar 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 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 (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, barley, rice, sorghum, rye, millet, tobacco, etc.), more preferably cereal plants, yet more preferably corn, wheat, barley, rice, sorghum, rye and millet plants.

It is recognized that with these nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for a HGGT sequence can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a nucleotide construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The following examples are presented by way of illustration, not by way of limitation.

EXPERIMENTAL

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred
 5 embodiments of the invention, are given by way of illustration, not by way of limitation. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various
 10 modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Example 1: Composition of cDNA Libraries;
 Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) tissues were prepared. The cDNA libraries representing mRNAs from barley
 20 (*Hordeum vulgare*) and rice (*Oryza sativa*) developing seeds are described in Examples 2 and 4, respectively. The characteristics of the corn (*Zea mays*) and wheat (*Triticum aestivum*) libraries are described in Table 1 below.

TABLE 1

cDNA Libraries from Corn and Wheat

Library	Tissue	Clone
cco1n	Corn (<i>Zea mays</i>) cob of 67 day old plants grown in green house*	cco1n.pk087.l17:fis
wdk2c	Wheat (<i>Triticum aestivum</i>) developing kernel, 7 days after anthesis	wdk2c.pk012.f2:fis
p0058	Sweet Corn (<i>Zea mays</i>) hybrid (Honey N Pearl) shoot culture. It was initiated on 2/28/96 from seed derived meristems. The culture was maintained on 273N medium.	p0058.chpbj67r:fis

*These libraries were normalized essentially as described in U.S. Patent No. 5,482,845 (the contents of which are hereby incorporated by reference).

cDNA libraries may be prepared by any one of many methods available.

5 For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). The Uni-ZAP™ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid
10 vector pBluescript. In addition, the cDNAs may be introduced directly into pre-cut Bluescript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial
15 colonies containing recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al.
20 (1991) *Science* 252:1651-1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

Full-insert sequence (FIS) data is generated utilizing a modified transposition protocol. Clones identified for FIS are recovered from archived glycerol stocks as single colonies, and plasmid DNAs are isolated via alkaline
25 lysis. Isolated DNA templates are reacted with vector primed M13 forward and reverse oligonucleotides in a PCR-based sequencing reaction and loaded onto automated sequencers. Confirmation of clone identification is performed by sequence alignment to the original EST sequence from which the FIS request is made.

30 Confirmed templates are transposed via the Primer Island transposition kit (PE Applied Biosystems, Foster City, CA) which is based upon the *Saccharomyces cerevisiae* Ty1 transposable element (Devine and Boeke (1994) *Nucleic Acids Res.* 22:3765-3772). The *in vitro* transposition system places unique binding sites randomly throughout a population of large DNA molecules.

The transposed DNA is then used to transform DH10B electro-competent cells (Gibco BRL/Life Technologies, Rockville, MD) via electroporation. The transposable element contains an additional selectable marker (named DHFR; Fling and Richards (1983) *Nucleic Acids Res.* 11:5147-5158), allowing for dual selection on agar plates of only those subclones containing the integrated transposon. Multiple subclones are randomly selected from each transposition reaction, plasmid DNAs are prepared via alkaline lysis, and templates are sequenced (ABI Prism dye-terminator ReadyReaction mix) outward from the transposition event site, utilizing unique primers specific to the binding sites within the transposon.

Sequence data is collected (ABI Prism Collections) and assembled using Phred/Phrap (P. Green, University of Washington, Seattle). Phrep/Phrap is a public domain software program which re-reads the ABI sequence data, re-calls the bases, assigns quality values, and writes the base calls and quality values into editable output files. The Phrap sequence assembly program uses these quality values to increase the accuracy of the assembled sequence contigs. Assemblies are viewed by the Consed sequence editor (D. Gordon, University of Washington, Seattle).

In some of the clones the cDNA fragment corresponds to a portion of the 3'-terminus of the gene and does not cover the entire open reading frame. In order to obtain the upstream information one of two different protocols are used. The first of these methods results in the production of a fragment of DNA containing a portion of the desired gene sequence while the second method results in the production of a fragment containing the entire open reading frame. Both of these methods use two rounds of PCR amplification to obtain fragments from one or more libraries. The libraries some times are chosen based on previous knowledge that the specific gene should be found in a certain tissue and some times are randomly-chosen. Reactions to obtain the same gene may be performed on several libraries in parallel or on a pool of libraries. Library pools are normally prepared using from 3 to 5 different libraries and normalized to a uniform dilution. In the first round of amplification both methods use a vector-specific (forward) primer corresponding to a portion of the vector located at the 5'-terminus of the clone coupled with a gene-specific (reverse) primer. The first method uses a sequence that is complementary to a portion of the already known gene sequence

while the second method uses a gene-specific primer complementary to a portion of the 3'-untranslated region (also referred to as UTR). In the second round of amplification a nested set of primers is used for both methods. The resulting DNA fragment is ligated into a pBluescript vector using a commercial kit and following the manufacturer's protocol. This kit is selected from many available from several vendors including Invitrogen (Carlsbad, CA), Promega Biotech (Madison, WI), and Gibco-BRL (Gaithersburg, MD). The plasmid DNA is isolated by alkaline lysis method and submitted for sequencing and assembly using Phred/Phrap, as above.

Example 2: Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Barley Seed

In an attempt to identify cDNAs for homogentisate geranylgeranyl transferase (HGGT), degenerate PCR oligonucleotides were designed based on partially conserved domains in amino acid sequences deduced from homogentisate phytyl transferase (HPT) cDNAs disclosed in WO 00/68393 (the contents of which are hereby incorporated by reference). Degenerate sense and antisense oligonucleotides were designed that consist of the following sequences: 5'-TAYRTNGTNGGNHTNAAAYCA-3' (SEQ ID NO:20) and 5'-GCRTARAANARNTTCCADATRAA-3' (SEQ ID NO:22). These oligonucleotides were designated HPT5' (SEQ ID NO:20) and HPT3' (SEQ ID NO:22), respectively, and correspond to the amino acid sequences: Y(I/V)VG(I/L/F/M)NQ (SEQ ID NO:21) and FIW(K/N)(I/L/M)FYA (SEQ ID NO:23).

It is known that tocotrienols are enriched in the seed endosperm of monocotyledonous plants, including barley (*Hordeum vulgare*) (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129-131; Qureshi, A.A. et al., (1986) *J. Biol. Chem.* 261:10544-10550). Such tissues therefore represent likely sources of expressed genes for the tocotrienol biosynthetic enzyme HGGT. As a first step towards the identification of an HGGT cDNA, total RNA was isolated from developing seeds of barley (cultivar Barsoy) using Trizol reagent (Life Technologies) according to the manufacturer's protocol. First strand cDNA was then prepared from 2 µg of the isolated RNA by using oligo-dT priming and Superscript II reverse transcriptase (Life Technologies) in a 25 µL reaction as described in the manufacturer's protocol. PCR amplification

was then conducted using Advantage cDNA polymerase mix (Clontech) and 2 μ L of the first strand cDNA synthesis reaction as template in a total volume of 50 μ L. Oligonucleotides HPT5' (SEQ ID NO: 20) and HPT3' (SEQ ID NO: 22) were included in the amplification reaction as sense and antisense primers. Forty
5 cycles of amplification were conducted with annealing and extension temperatures of 50 °C and 72 °C, respectively. The resulting PCR products displayed an approximate size of 700 nucleotides, as expected for the coding sequence of HPT-related enzymes. PCR products were subsequently subcloned into the vector pPCR-Script AMP (Stratagene) according to the manufacturer's protocol
10 and transformed into *E. coli* DH10B cells (Gibco-BRL). Nucleotide sequence was obtained from the cDNA inserts of plasmids from twelve of the resulting colonies. The sequence of one cDNA insert (SEQ ID NO: 11) was found to encode 234 amino acids of an HPT-related polypeptide (SEQ ID NO: 12) that shared 58 to 61% identity with the analogous portion of HPT polypeptides from *Arabidopsis*
15 (SEQ ID NO: 13), soybean (SEQ ID NO: 14), rice (SEQ ID NO: 15) and maize (SEQ ID NO: 16) that were disclosed in WO 00/68393. Over the same portion of their amino acid sequences, the *Arabidopsis*, soybean, rice and maize HPTs share >75% identity. Thus, the partial cDNA sequence identified from barley seeds encoded a divergent form of HPT that was subsequently determined to
20 correspond to the polynucleotide sequence for HGGT.

In order to establish the function of the polypeptide encoded by the partial cDNA from barley seed, the complete 5' and 3' ends of the cDNA were amplified from a barley developing seed cDNA library using nested PCR. As used herein, the term "nested PCR" refers to a polymerase chain reaction (PCR) technique in
25 which the product or products of a PCR reaction are reamplified by using an oligonucleotide primer combination in which one or both primers correspond to a portion of the target DNA that lies within the sequence amplified in the initial reaction. For the library construction, polyA⁺-RNA was enriched from the developing seed total RNA described above using the QuickPrep mRNA
30 purification kit (Pharmacia Biotech) according to the manufacturer's protocol. cDNA inserts were prepared from the poly-A⁺ RNA using a Uni-ZAP XR cDNA synthesis kit (Stratagene) and cloned into the *EcoRI/XhoI* sites of the pBluescript SK(+) vector as previously described (Cahoon et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:12935-12940). Approximately 200 ng of the resulting plasmid library was

used as template in nested PCR reactions described below. The reactions were conducted in 50- μ L volumes, and amplification was achieved using Advantage cDNA polymerase mix (Clontech) with an annealing temperature of 55°C.

Reactions contained primer pairs that were designed based on sequence from the pBluescript SK(+) vector and from the partial cDNA amplified from barley seed

(SEQ ID NO: 11). Thirty-five cycles were conducted in each of the PCR reactions

described below. For amplification of the complete 5' end of the putative barley HGGT cDNA, reactions were performed with the following sense and antisense

primers: 5'-AAATTAACCCTCACTAAAGGG-3' (modified T3 primer) (SEQ ID NO:

24) and 5'-ATACATGATGCAGCGAGGAGC-3' (SEQ ID NO:25). The unpurified

products of this reaction were diluted six-fold, and 1 μ L of this dilution was used as template in a second reaction that contained the following nested sense and

antisense primers: 5'-CTCTAGAACTAGTGGATCCC-3' (modified SK primer)

(SEQ ID NO: 26) and 5'-GTATTCCTATGCTAAAGCTC-3' (SEQ ID NO: 27). For

amplification of the complete 3' end of the putative barley HGGT cDNA, reactions were conducted with the following sense and antisense primers: 5'-

GAATTTTCAGTAGCAACTGG-3' (SEQ ID NO: 28) and 5'-

GTAAAACGACGGCCAGT-3' (M13 - 20 primer) (SEQ ID NO: 29). The unpurified

products of this reaction were diluted six-fold, and 1 μ L of this dilution was used as

template in a second reaction that contained the following nested sense and

antisense primers: 5'-CTCCTCGCTGCATCATGTATC-3' (SEQ ID NO: 30) and 5'-

GTAATACGACTCACTATAGGGC-3' (T7 primer) (SEQ ID NO: 31). The products

of the nested reactions above were subcloned into the vector pGEM-T Easy

(Promega), and transformed into *E. coli* DH10B cells. DNA sequences

corresponding to the amplified 5' and 3' ends of the putative barley HGGT cDNA

were then obtained from cDNA inserts of plasmids from several independent

transformants. These sequences together with that from SEQ ID NO: 11 were

assembled to generate the sequence of the full-length cDNA for the putative

barley HGGT (SEQ ID NO: 1). The resulting full-length cDNA was designated

"bdl2c.pk006.o2". The bdl2c.pk006.o2 cDNA (SEQ ID NO: 1) was found to

encode a 407 amino acid polypeptide (SEQ ID NO: 2) that shares 45 to 47%

identity with HPTs from *Arabidopsis* (SEQ ID NO: 13), soybean (SEQ ID NO: 14),

rice (SEQ ID NO: 15) and maize (SEQ ID NO: 16) that were disclosed in WO

00/68393. By comparison, the *Arabidopsis*, soybean, rice, and maize HPTs share 61 to 69% identity with each other.

To examine the tissue-specific expression of the gene for the putative barley HGGT polypeptide, Northern blot analysis was conducted using polyA⁺-enriched RNA isolated from leaf, roots and developing seeds of barley. For this experiment, total RNA was isolated from these tissues using the Trizol reagent (Life Technologies) according to the manufacturer's protocol, and polyA⁺ RNA was then enriched from the total RNA extract using the PolyATract mRNA isolation system (Promega). Approximately 2 µg of polyA⁺ from each tissue was electrophoresed in a 1% (w/v) agarose gel and then transferred from the gel to Bright Star-Plus nylon membrane (Ambion) using NorthernMax transfer buffer (Ambion). The RNA was fixed to the membrane by baking at 80°C for 2 h. The membrane was rinsed with 2X SSC and then hybridized with ³²P-labeled probes for 18 h at 42°C in NorthernMax hybridization buffer (Ambion). Probes were prepared from the full-length barley HPT-like cDNA (bdl2c.pk006.o2; SEQ ID NO: 1) and were labeled using random hexamer priming. Following incubation with probes, blots were washed for 15 min with 2X SSC/ 0.1% SDS at room temperature, then washed for a 15 min at room temperature with 0.2X SSC/0.1% SDS, and finally washed for 15 min at 42°C with 0.2X SSC/0.1% SDS. Radioactivity on filters was detected by phosphorimaging. As a control for RNA loading, the Northern blot was re-hybridized with a labeled probe prepared from a barley α-tubulin cDNA, which corresponds to a constitutively expressed gene.

A Northern blot analysis to assess the expression of a nucleotide sequence encoding a barley HGGT was performed. Enriched polyA⁺ RNA isolated from leaf (L), root (R), and developing seed (S) of barley were analyzed. A barley α-tubulin gene, which is known to be a constitutively expressed gene in plants was analyzed using the same Northern blot as for HGGT gene expression and showed that the loading of polyA⁺ RNA from each tissue was approximately equal.

Expression of the gene for the putative barley HGGT was detected in developing seeds but was not detected in leaves and roots. The seed specific expression of the gene for the putative barley HGGT is consistent with the occurrence of tocotrienols in barley seeds and the corresponding absence of these compounds from leaves and roots (Cahoon, unpublished observation).

To determine the function of the putative barley HGGT, the full-length bdl2c.pk006.o2 cDNA (SEQ ID NO: 1) encoding this enzyme was operably linked to the cauliflower mosaic virus 35S promoter and expressed in tobacco callus. The barley cDNA was initially amplified by PCR to generate flanking *NcoI* and *EcoRI* sites for cloning into the plant expression vector. The sequence of the sense oligonucleotide used in the amplification reaction was 5'-
5 ttccatggCGAGGATGCAAGCCGTCACGG -3' (SEQ ID NO: 32), and the sequence of the antisense oligonucleotide was 5'-
ttgaattcACACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 33). (Note: The bases
10 in lower case contain the added restriction sites, which are underlined, and flanking sequence to facilitate restriction enzyme digestion.) Thirty cycles of PCR amplification were conducted in a 100 μ L volume using *Pfu* polymerase (Stratagene) and approximately 300 ng of the barley developing seed cDNA library described above as template. The product of this reaction was purified by
15 agarose electrophoresis, and then digested with the restriction enzymes *NcoI* and *EcoRI*. The amplified barley cDNA was then cloned into the corresponding sites of the plant expression vector pML63. A detailed description of vector pML63 has been previously disclosed in WO 00/11176 (the contents of which are hereby incorporated by reference). The resulting plasmid pBHGGT-35S contained the
20 putative barley HGGT cDNA operably linked at its 5' end to the cauliflower mosaic virus 35S promoter and at its 3' end to termination sequence from the nopaline synthase (*nos*) gene. This expression cassette was then removed from pBHGGT-35S following digestion with the restriction enzyme *SaI* and cloned into the corresponding restriction site of the binary vector pZS199 to generate plasmid
25 pSH24. Vector pZS199 was described in detail in WO 00/11176. The vector contains right and left T-DNA borders for integration of the expression cassette into the host plant genome and a neomycin phosphotransferase II gene linked to a cauliflower mosaic virus 35S promoter, which confers kanamycin selection for transgenic plant cells.

30 Plasmid pSH24, which contains a fusion of the barley putative HGGT cDNA with the cauliflower mosaic virus 35S promoter and the *nos* termination sequences in vector pZS199, was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Cultures derived from these cells were used for transformation of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks according to the protocol

described by Rogers, S.G., Horsch, R.B., and Fraley, R.T. (1986) *Methods Enzymol.* 118:627-648. Tobacco leaf disks were also transformed with *A. tumefaciens* harboring only the pZS199 vector. Transformed tobacco callus was selected by the ability of cells to grow on media containing kanamycin at a concentration of 300 mg/L. In addition, expression of the transgene in these cells was confirmed by Northern blot analysis using a radiolabeled probe derived from the full-length barley putative HGGT cDNA (bdl2c.pk006.o2 cDNA; SEQ ID NO: 1).

Transformed callus arising from leaf disks was analyzed for tocotrienol production following ten to twelve days of growth on fresh kanamycin-containing media. Analyses were conducted using 10 to 50 mg (dry weight) of lyophilized tobacco callus. The transgenic tissue was initially homogenized in 3 mL of methanol:chloroform (2:1 v/v) in a 13 x 100 mm glass test tube. Following 2 h of incubation, 1 mL of chloroform and 1.8 mL of water were added. The organic and aqueous layers were thoroughly mixed and then partitioned by centrifugation. The organic layer was recovered, dried under nitrogen, and resuspended in 175 μ L of heptane. The tocopherol and tocotrienol content of the organic extract was then determined using HPLC. Resolution of these compounds was achieved using a Hewlett Packard LiChroSpher Si 60 column (25 cm length; 5 μ particle size) and a solvent system consisting of heptane:isopropanol (99:1 v/v) with a flow rate of 1 mL/min. Analytes were detected and quantified by fluorescence with excitation at 292 nm and emission at 335 nm. Tocopherol and tocotrienol molecular species were identified by mobility relative to standard compounds and quantified relative to δ -tocopherol, which was added as an internal standard. Using this methodology, tobacco callus expressing the barley putative HGGT cDNA was found to contain the following tocotrienol species: α -, β -, γ - and δ -tocotrienol. In contrast, no tocotrienols were detected in tobacco callus transformed with only the pZS199 expression vector. In these studies, expression of the barley HGGT cDNA was mediated by the cauliflower mosaic virus 35S promoter. The analyses were conducted using an equivalent amount of tobacco callus.

This result thus demonstrates that the barley bdl2c.pk006.o2 cDNA disclosed in SEQ ID NO: 1 encodes a functional homogentisate geranylgeranyl transferase (HGGT) whose expression in transgenic plant cells results in tocotrienol production. Amounts of tocotrienol in tobacco callus expressing the

barley HGGT cDNA were as high as 164.7-mg/kg dry weight, and the total tocopherol and tocotrienol content of these cells was as much as ten-fold higher than that in cells transformed with only the pZS199 vector (Table 2).

TABLE 2

Total tocopherol and tocotrienol content of tobacco callus transformed with the binary vector pZS199 (Vector Control) or with the barley HGGT cDNA operably linked to the cauliflower mosaic virus 35S promoter in pZS199 (+Barley HGGT cDNA). (The values shown were obtained from independent transgenic events.)

Transgenic Events	Total Tocopherol Content (mg/kg dry weight)	Total Tocotrienol Content (mg/kg dry weight)
Vector Control		
Event 1	17.5	n.d.*
Event 2	16.4	n.d.
Event 3	12.8	n.d.
Event 4	14.6	n.d.
+Barley HGGT cDNA		
Event 1	11.8	161.1
Event 2	14.0	155.4
Event 3	12.3	118.5
Event 4	14.3	164.7
Event 5	9.7	121.1

*n.d., not detected.

To confirm the identity of tocotrienols in transgenic tobacco callus expressing the barley HGGT cDNA, the organic extract from these cells was analyzed by gas chromatography-mass spectrometry. These analyses were performed using a Hewlett Packard 6890 gas chromatograph interfaced with a Hewlett Packard 5973 mass selective detector (MSD). Samples were separated with a 15-m x 0.25-mm (inner diameter) DB-1HT column (J&W Scientific). The

oven temperature was programmed from 125°C (4-min hold) to 240°C (12-min hold) at a rate of 10°C/min. The ionization potential of the MSD was 70 eV. Using these conditions, compounds were detected with mass spectra identical to those of tocotrienol standards. The mass spectra of α - and γ -tocotrienol that were
5 detected in extracts of tobacco callus expressing the barley HGGT cDNA contained molecular ions for α -tocotrienol (m/z 424) and γ -tocotrienol (m/z 410) as well as M^+ -219 ions arising from loss of the side chain and M^+ -259 ions arising from cleavage of the chroman ring and accompanying rearrangement as described (Nair and Zenaida (1968) *Arch. Biochem. Biophys.* 127:413-418).

10 These results conclusively demonstrate the ability to produce tocotrienols and to increase tocol content in transgenic plant cells by overexpression of the barley HGGT cDNA.

15 Example 3: Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Wheat Seed

Wheat seeds and derivatives such as wheat bran and kernel are enriched in tocotrienols (Shin, T.S. (1994) *J. Chromatogr. A* 678:49-58) and thus represent potential sources of an HGGT cDNA. Homology searches were conducted using
20 the nucleotide sequence of the barley HGGT cDNA (SEQ ID NO: 1) and expressed sequence tags (ESTs) generated from developing wheat kernel. These searches resulted in the identification of wheat EST wdk2c.pk012.f2: fis (SEQ ID NO: 34) that shared 94% identity with the barley HGGT cDNA (SEQ ID NO:1) over a span of 321 nucleotides. This high degree of sequence identity suggested that
25 EST wdk2c.pk012.f2: fis (SEQ ID NO: 34) encodes an HGGT polypeptide. Based on sequence comparisons with the cDNA for barley HGGT (SEQ ID NO: 1), the cDNA corresponding to EST wdk2c.pk012.f2: fis (SEQ ID NO: 34) lacked coding sequence for at least 200 N-terminal amino acids. Functional characterization of the wheat HGGT-like polypeptide corresponding to EST wdk2c.pk012.f2: fis (SEQ
30 ID NO: 34) thus required isolation of additional 5' coding sequence.

Nested PCR was conducted to isolate a full-length cDNA for the wheat HGGT-like polypeptide. The template for the initial amplification reaction consisted of 25 ng of a mixture of cDNA libraries prepared from developing kernels, roots and seedlings of wheat. The libraries consisted of cDNA inserts
35 cloned into the plasmid pBluescript SK(+). The sense oligonucleotide primer for

this reaction corresponded to sequence in pBluescript SK(+) that flanks the 5' end of cDNA inserts, and the antisense primer corresponded to sequence in the EST wdk2c.pk012.f2: fis (SEQ ID NO: 34). The sequences of the corresponding primers were as follows: 5'-GCCAAGCTCGGAATTAACCCTCA-3' (sense) (SEQ ID NO: 35) and 5'-CACAGTACAAGGAAAATCCAAGCA-3' (antisense) (SEQ ID NO: 36). The reaction was conducted in a volume of 20 μ L, and amplification was achieved using Advantage cDNA polymerase mix. The initial PCR cycling conditions were as follows: denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 4 min. The annealing temperature was then lowered by 0.5°C for each of the subsequent 9 cycles. This was then followed by 25 cycles with an annealing temperature of 63°C. The unpurified product of this reaction was then diluted 200-fold and used as template in a reaction that was conducted using the same amplification conditions as in the first reaction. The respective sense and antisense primers for this reaction corresponded to sequences in pBluescript SK(+) and EST wd2kc.pk012.f2: fis (SEQ ID NO: 34) that were flanked by the primers from the first reaction. The sequences of these “nested” oligonucleotide primers used in the second reaction were as follows: 5'-GCCGCTCTAGAACTAGTGGATCCCC-3' (sense) (SEQ ID NO: 37) and 5'-TCCAAGCATTGGATAGGGTATCA-3' (antisense) (SEQ ID NO: 38). The product of this second PCR reaction was subcloned into the vector pGEM-T Easy (Promega) according to the manufacturer's protocol, and complete DNA sequence was subsequently obtained from the subcloned PCR products. Using this methodology, full-length coding sequence was obtained for a 408 amino acid polypeptide that shared 86.7% identity with the barley HGGT. The sequences of the full-length cDNA (designated “wdk2c.pk012.f2:cgs”) from wheat and the corresponding polypeptide are disclosed in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

In order to establish the function of the wheat HGGT-like polypeptide in SEQ ID NO: 4, the corresponding wdk2c.pk012.f2:cgs cDNA (SEQ ID NO: 3) was operably linked to a cauliflower mosaic virus 35S promoter and expressed in tobacco callus. The complete open-reading frame of the wheat cDNA was initially amplified by PCR with oligonucleotides that contained added restriction enzyme sites to facilitate cloning into the plant expression vector. The oligonucleotide primers used for the PCR reaction were as follows: 5'-

ttggaattcGTGGCCGCCCGGCGAGGATGC-3' (sense) (SEQ ID NO: 39) and 5'-
ttggtaccTCACATCTGCTGGCCCTTGTAC-3' (antisense) (SEQ ID NO: 40). (Note:
The bases in lower case contain the added restriction sites, which are underlined,
and flanking sequence to facilitate restriction enzyme digestion.) The template for
5 the reaction was the wheat HGGT-like cDNA (wdk2c.pk012.f2:cgs; SEQ ID NO: 3)
cloned in pGEM-T Easy, and amplification was achieved using *Pfu* polymerase
(Stratgene). The product of this reaction was purified by agarose gel
electrophoresis, digested with *EcoRI* and *KpnI*, and cloned into the corresponding
sites of the plant expression vector pML135 to generate the plasmid pWhtHGGT-
10 35S. The vector pML135 was generated from vector pML63, which has been
described previously in WO 00/11176 (the contents of which are hereby
incorporated by reference), by replacement of the beta-glucuronidase (GUS) gene
with a polylinker that contained the restriction enzyme recognition sites for *PmlI*,
EcoRI, *SmaI*, and *KpnI*. pWhtHGGT-35S contains the open-reading frame of the
15 wheat HGGT-like cDNA (wdk2c.pk012.f2:cgs; SEQ ID NO: 3) flanked on its 5'-end
by the cauliflower mosaic virus 35S promoter and on its 3'-end by transcriptional
termination sequence for the nopaline synthase gene. This expression cassette
was subsequently moved as a *SaII* fragment from pWhtHGGT-35S and cloned
into the corresponding site of the binary vector pZS199 (described in Example 2)
20 to generate the plasmid pEC17. *Agrobacterium tumefaciens* LBA4404 cells were
subsequently transformed as described in Example 2. The transformed *A.*
tumefaciens cells were then used for infection of tobacco (*Nicotiana tabacum* cv.
Xanthi) leaf disks using protocols detailed in Example 2.

The resulting tobacco callus transformed with the wheat HGGT-like cDNA
25 (wdk2c.pk012.f2:cgs; SEQ ID NO: 3) operably linked to the cauliflower mosaic
virus 35S promoter was maintained and analyzed for tocopherol and tocotrienol
content as described in Example 2. Tobacco callus expressing the wheat HGGT
cDNA accumulated molecular species of tocotrienols including α -, β -, γ - and δ -
tocotrienol. Of five independent events analyzed, amounts of tocotrienols
30 detected were as high as 140 mg/kg (dry weight). In contrast, no tocotrienol
accumulation was detected in tobacco callus transformed with the pZS199 binary
vector lacking cDNA insert. The analyses were conducted using an equivalent
amount of tobacco callus.

These results thus demonstrate that the wheat wdk2c.pk012.f2:cgs cDNA disclosed SEQ ID NO: 3 encodes a functional HGGT polypeptide whose expression in transgenic plant cells results in the production of tocotrienols.

5 Example 4: Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Rice Seed

Rice seeds and byproducts such as rice bran are enriched in tocotrienols (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and
10 Hall, London, 1994, pp. 129-131) and are thus potential sources of homogentisate geranylgeranyl transferase (HGGT) cDNAs. Using methodology similar to that described in Example 2, isolation of a HGGT cDNA from rice seed was achieved through a PCR strategy that employed degenerate oligonucleotide primers designed from partially conserved domains in homogentisate phytyltransferases
15 (HPTs) disclosed in WO 00/68393. The term "degenerate oligonucleotide" refers to a synthesized mixture of a nucleotide sequence in which a given position within the sequence can be represented by more than one nucleotide in the mixture. The template for PCR amplification reactions was a cDNA library prepared from developing rice seeds harvested at two to five days after pollination. The
20 protocols for RNA isolation from rice seeds and synthesis of cDNA inserts were the same as those used in the preparation of a barley developing seed cDNA library described in Example 2. The resulting cDNA inserts were cloned in a 5'→3' orientation into the *EcoRI* and *XhoI* sites of the Lambda Uni-ZAP XR phage vector (Stratagene). The vector containing the developing rice seed cDNA inserts was
25 then packaged and subsequently amplified according to the manufacturer's protocol (Stratagene).

The resulting rice developing seed cDNA library was used as template for PCR amplification at a total amount of 5×10^6 plaque-forming units in a 50- μ L reaction volume. The degenerate oligonucleotides HPT5' (SEQ ID NO: 20) and
30 HPT3' (SEQ ID NO: 22) (as described in Example 2) were used as the sense and antisense primers, respectively. These primers were included in the reaction at a final concentration of 1.5 μ M. Amplification was achieved using Advantage cDNA polymerase mix (Clontech). Forty amplification cycles were conducted using the following temperatures and times for each cycle: 94°C for 1 min, 50°C for 1 min,

and 72°C for 1.25 min. The unpurified product of this reaction was diluted seven-fold, and 1 μ L of this dilution was used as template in a second PCR reaction. This reaction was conducted using the same reaction conditions as above. HPT3' (SEQ ID NO: 22) was again used as the antisense primer, and HPT5' (SEQ ID NO: 20) was replaced as the sense primer with the degenerate oligonucleotide HPT5'-2 (SEQ ID NO: 41), which contained the following sequence: 5'-ATHGAYAARRTNAAYAARCC-3' (SEQ ID NO: 41). This oligonucleotide corresponds to the amino acid sequence IDK(I/V/M)NKP (SEQ ID NO: 42), which is a partially conserved domain in HPT sequences disclosed in WO 00/68393.

The coding sequence of this domain corresponds to a region upstream of the HPT5' sequence in HPT cDNAs. Using the HPT5'-2 (SEQ ID NO: 41) and HPT3' primers (SEQ ID NO: 22), products of approximately 700 bp were obtained from the second PCR reaction. The products were then purified and subcloned into vector pGEM-T Easy (Promega) according to the manufacturer's protocol. Upon partial sequencing, one of the PCR products was found to encode a polypeptide that was more related to HGGTs from barley and wheat than to HPTs disclosed in WO 00/68393.

Functional characterization of this putative rice HGGT required isolation of additional 5' and 3' sequence for the complete N'- and C'-termini of this polypeptide. This was achieved through a series of nested PCR reactions in which the rice developing seed cDNA library (described above) was used as the template. For all PCR reactions described below, forty amplification cycles were conducted using Advantage cDNA polymerase mix (Clontech) and the temperatures and times for each cycle were as follows: 94°C for 1 min, 53°C for 1 min, and 72°C for 1.25 min. For isolation of the coding sequence of the complete C' terminus, sense oligonucleotide primers were designed based on the sequence of the PCR product encoding the partial rice HGGT-like polypeptide, and antisense primers were designed based on sequences in the Lambda Uni-Zap cDNA library vector. A PCR reaction was initially conducted that contained an aliquot of the rice developing seed cDNA library (5×10^6 plaque-forming units) and the following sense and antisense primers: 5'-GGAAGTGCATACTCTGTTGATG-3' (SEQ ID NO: 43) and 5'-GTAAAACGACGGCCAGT-3' (M13 -20 primer) (SEQ ID NO: 29). The unpurified products of this reaction were diluted 10-fold, and 1 μ L of this dilution was used as template in a second reaction that contained the

following nested sense and antisense primers: 5'-

CTTGTATACTATTTGTAAGAGC-3' (SEQ ID NO: 44) and 5'-

GTAATACGACTCACTATAGGGC-3' (T7 primer) (SEQ ID NO: 31). The product of this reaction was purified by agarose gel electrophoresis and subcloned into the
5 vector pGEM-T Easy (Promega) using the method described by the manufacturer. Nucleotide sequence was then obtained for the entire subcloned PCR product.

Comparison of the resulting sequence with those of barley and wheat HGGTs indicated that the PCR product encoded the complete C'-terminus of an HGGT-like polypeptide. For isolation of the coding sequence of the complete N'-terminus

10 of the rice HGGT-like polypeptide, sense primers were designed based on sequences in the Lambda Uni-Zap cDNA library vector. The design of antisense primers was based on the coding sequence obtained for the C'-terminus of the rice HGGT-like polypeptide. A PCR reaction was initially conducted that contained an aliquot of the rice developing seed cDNA library (5×10^6 plaque-forming units)
15 and the following sense and antisense primers: 5'-AACAGCTATGACCATG-3' (M13 reverse) (SEQ ID NO: 45) and 5'-ATAATTGCTCATGTGCATGGTC-3' (SEQ ID NO: 46). The unpurified products of this reaction were diluted 10-fold, and 1 μ L of this dilution was used as template in a second reaction that contained the following nested sense and antisense primers: 5'-

20 AAATTAACCCTCACTAAAGGG-3' (modified T3 primer) (SEQ ID NO: 24) and 5'-CATGTAAATGATGTGATCCAC-3' (SEQ ID NO: 47). The product of this reaction was purified by agarose gel electrophoresis and subcloned into the vector pGEM-T Easy (Promega) according to the manufacturer's protocol. Results of sequence analysis indicated that the PCR product contained the coding region for the
25 complete N'-terminus of an HGGT-like polypeptide (relative to barley and wheat HGGTs described in Examples 2 and 3).

Using the sequence information obtained above, the complete open-reading frame for the rice HGGT-like polypeptide was then amplified by PCR from the rice developing seed cDNA library. The oligonucleotide primers used in this
30 reaction corresponded to sequences that flanked the open-reading frame of the rice HGGT-like polypeptide. These primers contained the following sequences: 5'-ttgcggccgcAGACGATGCAAGCCTCATCGG-3' (sense) (SEQ ID NO: 48) and 5'-ttgcggccgcCTTGCCCTTGTGTATATAGTGC-3' (antisense) (SEQ ID NO: 49). (Note: The lower case, underlined sequence corresponds to an added *NotI*

restriction site. The remaining lower case sequence was added to facilitate restriction enzyme digestion.) PCR amplification was conducted using Advantage cDNA polymerase mix and an aliquot of the rice developing seed cDNA library (3.5 X 10⁶ plaque-forming units) in a 50 µL reaction volume. Thirty-five
5 amplification cycles were performed using an annealing temperature of 57°C. The resulting cDNA product was purified by agarose electrophoresis and sub-cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's protocol. The sequence obtained from this cDNA (designated "rds1c.pk007.m9") is disclosed in SEQ ID NO: 5, and the deduced amino acid sequence is disclosed in
10 SEQ ID NO: 6. The polypeptide encoded by the PCR product shared 68.6% amino acid sequence identity with the barley HGGT (SEQ ID NO: 2) and 66.3% identity with the wheat HGGT (SEQ ID NO: 4).

Functional characterization of the putative rice HGGT polypeptide (SEQ ID NO: 6) was conducted by transgenic expression in tobacco callus. For these
15 studies, the coding sequence of the putative rice HGGT (SEQ ID NO: 6) was operably linked to the cauliflower mosaic virus 35S promoter. To facilitate cloning into the plant expression vector, the coding sequence of the putative rice HGGT was amplified with primers that contained a flanking *Sma*I and *Kpn*I restriction sites, which are underlined in the sequences below. The sequences of the
20 oligonucleotide primers used in this reaction were as follows: 5'-
TTCCCGGGGAGACGATGCAAGCCTCATCG-3' (sense) (SEQ ID NO: 50) and 5'-
TTGGTACCGTGTATATAGTGCTCACTGCAC-3' (antisense) (SEQ ID NO: 51). PCR amplification was conducted with *Pfu* polymerase, and the rice putative HGGT cDNA (rds1c.pk007.m9; SEQ ID NO: 5) was used as the template. The
25 product of this reaction was purified by agarose gel electrophoresis, digested with *Sma*I and *Kpn*I, and then cloned into the corresponding restriction sites of the plant expression vector pML135 (which is described in Example 3). The resulting plasmid pRiceHGGT-35S contained an expression cassette consisting of the putative rice HGGT open reading frame flanked on its 5'-end by the cauliflower
30 mosaic virus 35S promoter and on its 3'-end by transcriptional termination sequence for the nopaline synthase (*nos*) gene. This expression cassette was then inserted as a *Sa*I restriction fragment into the corresponding site of the binary vector pZS199 (which is described in WO 00/11176 (the contents of which are hereby incorporated by reference) and in Examples 2 and 3) to generate

plasmid pEC18. *Agrobacterium tumefaciens* LBA4404 cells were subsequently transformed with pEC18 as described in Example 2. The transformed *A. tumefaciens* cells were then used for infection of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks using protocols detailed in Example 2.

5 The resulting tobacco callus transformed with the putative rice HGGT cDNA (rds1c.pk007.m9; SEQ ID NO: 5) operably linked to the cauliflower mosaic virus 35S promoter was maintained and analyzed for tocopherol and tocotrienol content as described in Example 2. Tobacco callus expressing the putative rice HGGT cDNA accumulated molecular species of tocotrienols including α -, β -, γ - and δ -
10 tocotrienol. Of four independent transformation events analyzed, amounts of tocotrienols detected were as high as 95 mg/kg (dry weight). In contrast, no tocotrienol accumulation was detected in tobacco callus transformed with the pZS199 binary vector lacking cDNA insert.

 These results thus demonstrate that the rice rds1c.pk007.m9 cDNA
15 disclosed in SEQ ID NO:5 encodes a functional HGGT polypeptide whose expression in transgenic plant cells is sufficient for the production of tocotrienols.

Example 5: Production of Tocotrienols in Somatic Soybean Embryos

20 Somatic soybean embryos have been used as model for the prediction of transgenic phenotypes in soybean seeds (Kinney, A.J. (1996) *J. Food Lipids* 3:273-292). Somatic soybean embryos and seeds are enriched in tocopherols, but contain little or no tocotrienols (Coughlan, unpublished result; The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London,
25 1994, pp. 129-131). To demonstrate the ability to produce tocotrienols in somatic soybean embryos, the barley HGGT cDNA (bdl2c.pk006.o2; SEQ ID NO: 1) was expressed in this tissue under control of a strong seed specific promoter. The open-reading frame of bdl2c.pk006.o2 was initially amplified by PCR to generate flanking *NotI* sites for cloning into the soybean expression vector. The sequences
30 of the sense and antisense oligonucleotide primers used in this reaction were as follows: 5'-ttgcggccgcAGGATGCAAGCCGTCACGGCGGCAGCCG-3' (SEQ ID NO: 52) and 5'-ttgcggccgcTTCACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 53). (Note: The lower case, underlined nucleotide sequences correspond to added *NotI* restriction sites.) PCR amplification was achieved using *Pfu*

polymerase, and an aliquot of the barley developing seed cDNA library described in Example 2 was used as the template. The product of this PCR reaction was purified by agarose gel electrophoresis and subcloned into pCR-Script-AMP (Stratagene) as described in the manufacturer's protocol. The amplified open-
5 reading frame of the barley HGGT was then released as a *NotI* fragment and cloned into the corresponding site of soybean expression vector pKS121 to generate plasmid pSH13. The construction of vector pKS121 was previously described in WO 02/00904 (the contents of which are hereby incorporated by reference). This vector contains the seed specific promoter for the Kunitz trypsin
10 inhibitor-3 (Kti3) gene (Jofuku and Goldberg (1989) *Plant Cell* 1:1079-1093) linked via a *NotI* restriction site to the 3' transcriptional termination sequence of the Kti3 gene. Bacterial selection for the pKS121 plasmid is conferred by a hygromycin B phosphotransferase gene (Gritz and Davies (1983) *Gene* 25:179-188) under control of the promoter for the T7 RNA polymerase promoter. Plasmid pSH13
15 thus contains a soybean expression cassette consisting of the barley HGGT open-reading frame operably linked on its 5' end to the Kti3 promoter and on its 3' end to the Kti3 transcription termination sequence.

Somatic soybean embryos were transformed with pSH13 using the biolistic method. For these experiments, pSH13 was co-bombarded with the plasmid
20 pKS18HH at a 10:1 molar ratio of the two plasmids. Plasmid pKS18HH (U.S. Patent No. 5,846,784 (the contents of which are hereby incorporated by reference)) contains hygromycin B phosphotransferase gene under control of the cauliflower mosaic 35S promoter, which allows for selection of transformed plant cells by resistance to the antibiotic hygromycin B. The protocol used for
25 transformation of somatic soybean embryos is described below.

To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of a soybean cultivar Jack were cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos that produce secondary embryos were then excised and placed into a
30 suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular staged embryos, the suspensions were maintained as described below.

Soybean embryogenic suspension cultures were maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour

day/night schedule. Cultures were subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures were then co-transformed with pSH13 and pKS18HH by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70; U.S. Patent No. 4,945,050). A DuPont Biolisticä PDS1000/HE instrument (helium retrofit) was used for these transformations.

To 50 mL of a 60 mg/mL 1 mm gold particle suspension were added (in order): 5 mL DNA (1 mg/mL), 20 mL spermidine (0.1 M), and 50 mL CaCl₂ (2.5 M). The particle preparation was then agitated for three minutes, spun in a microfuge for ten seconds and the supernatant removed. The DNA-coated particles were then washed once in 400 mL 70% ethanol and resuspended in 40 mL of anhydrous ethanol. The DNA/particle suspension was sonicated three times for one second each. Five mL of the DNA-coated gold particles was then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture was placed in an empty 60 x 15-mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately five to ten plates of tissue were bombarded. Membrane rupture pressure was set at 1100 psi and the chamber was evacuated to a vacuum of 28 inches mercury. The tissue was placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue was divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media was exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media was refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue was removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line was treated as an independent transformation event. These suspensions were then subcultured and maintained as clusters of immature embryos. Immature embryos at this stage produce storage products, including storage lipids that are similar in composition to zygotic embryos at a similar stage of development (see WO 94/11516 (the contents of which are hereby incorporated by reference)).

The resulting somatic embryos transformed with the barley HGGT expression cassette from pSH13 were examined for tocotrienol production using extraction and analytical techniques described in Example 2. In at least three of 19 transgenic events analyzed, the accumulation of α , γ , and δ molecular species of tocotrienol was detected. In contrast, no tocotrienols were detected in untransformed somatic soybean embryos.

These results thus demonstrate the ability to produce tocotrienols in a crop plant that does not normally accumulate these antioxidant molecules through the transgenic expression of a nucleic acid fragment encoding an HGGT polypeptide.

Example 6: Production of Tocotrienols in Corn (*Zea mays*) Seed

Corn oil, which is derived primarily from the embryo of corn seeds, is typically enriched in tocopherols but contains little or no tocotrienols (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129-131). Based on results disclosed in Examples 2 and 5, the barley HGGT cDNA (SEQ ID NO: 1) can be expressed in seed embryo of corn to produce tocotrienols and to increase the tocol antioxidant content of this tissue and the extracted oil. As described below, this can be achieved by transforming corn with the barley HGGT open reading frame operably linked on its 5' end to an embryo specific promoter, such as the promoter for the corn 16 kDa oleosin gene (Lee, K. and Huang, A.H. (1994) *Plant Mol. Biol.* 26:1981-1987).

Initially, the open reading frame of the barley HGGT cDNA (SEQ ID NO: 1) was amplified by PCR to generate flanking *PacI* restriction sites, which allow for the cDNA to be cloned into a corn embryo expression cassette. The sense and antisense oligonucleotide primers used for PCR amplification were as follows: 5'-atttaattaaGCCGGCGAGGATGCAAGCCGTC-3' (SEQ ID NO: 54) and 5'-tattaattaaTTCACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 55). (The lower case, underlined nucleotides correspond to the added *PacI* sites, and the additional lower case nucleotides are added to facilitate restriction enzyme digestion.) Amplification can be achieved using *Pfu* polymerase (Stratagene) and reaction conditions similar to those described in Example 5. The resulting PCR product derived from the barley HGGT cDNA was purified, digested with *PacI*, and then cloned into the corresponding site of the corn embryo expression vector

pTG10 to generate plasmid pKR242. Vector pTG10 contains a corn expression cassette consisting of 959 base pairs of the promoter for the corn 16 kDa oleosin gene which has been previously described in WO 99/64579 (the contents of which are hereby incorporated by reference). This promoter element is linked via a *PacI* restriction site to 330 base pairs of the 3' transcription termination sequence of the corn 16 kDa oleosin gene (Lee, K. and Huang, A.H. (1994) *Plant Mol. Biol.* 26:1981-1987). Bacterial selection in pTG10 is conferred by a hygromycin B phosphotransferase gene (Gritz, L. and Davies, J. (1983) *Gene* 25:179-188) under control of the promoter for the T7 RNA polymerase gene. Plasmid pKR242 thus contains an expression cassette composed of the barley HGGT open reading frame operably linked on its 5' end to the promoter for the corn 16 kDa oleosin gene and on its 3' end to the transcription termination sequence of the corn 16 kDa oleosin gene. The entire expression cassette is flanked by *Ascl* restriction sites. Following digestion with *Ascl*, the expression cassette of pKR242 was removed and inserted into the corresponding site of the binary vector PHP15578 to generate plasmid PHP18749. The binary vector PHP15578 has been previously described in WO 02/00904 (the contents of which are hereby incorporated by reference). PHP15578 contains right and left border regions to facilitate *Agrobacterium*-mediated transformation of corn, and plant selection is conferred by a cauliflower mosaic virus 35S promoter-bialaphos selectabe marker element.

Plasmid PHP18749 can be used for the generation of transgenic corn that expresses the barley HGGT cDNA in an embryo specific manner. An *Agrobacterium*-based protocol can be used for the transformation of corn with expression elements from PHP18749 as described below.

Transformation of corn Mediated by *Agrobacterium*:

Freshly isolated immature embryos of corn, about 10 days after pollination (DAP), can be incubated with the *Agrobacterium*. The preferred genotype for transformation is the highly transformable genotype Hi-II (Armstrong (1991) *Maize Gen. Coop. Newsletter* 65:92-93). An F₁ hybrid created by crossing a Hi-II with an elite inbred may also be used. After *Agrobacterium* treatment of immature embryos, the embryos can be cultured on medium containing toxic levels of herbicide. Only those cells that receive the herbicide resistance gene, and the

linked gene(s), grow on selective medium. Transgenic events so selected can be propagated and regenerated to whole plants, produce seed, and transmit transgenes to progeny.

5 Preparation of *Agrobacterium*:

The engineered *Agrobacterium tumefaciens* LBA4404 can be constructed to contain plasmid PHP18749, as disclosed in U.S. Patent No. 5,591,616 (the contents of which are hereby incorporated by reference). To use the engineered construct in plant transformation, a master plate of a single bacterial colony
10 transformed with PHP18749 can be prepared by inoculating the bacteria on minimal AB medium and allowing incubation at 28°C for approximately three days. (The composition and preparation of minimal AB medium has been previously described in WO 02/00904 (the contents of which are hereby incorporated by reference.) A working plate can then be prepared by streaking the transformed
15 *Agrobacterium* on YP medium (0.5% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) sodium chloride, 1.5% (w/v) agar) that contains 50 µg/mL of spectinomycin.

The PHP18749-transformed *Agrobacterium* for plant transfection and co-cultivation can then be prepared one day prior to corn transformation. Into 30 mL of minimal A medium (prepared as described in Application WO 02/009040) in a
20 flask was placed 50 µg/mL spectinomycin, 100 µM acetosyringone, and about a 1/8 loopful of *Agrobacterium* from a one to two-day-old working plate. The *Agrobacterium* can then be grown at 28°C with shaking at 200 rpm for approximately fourteen hours. At mid-log phase, the *Agrobacterium* can be harvested and resuspended at a density of 3 to 5 X 10⁸ CFU/mL in 561Q medium
25 that contains 100 µM acetosyringone using standard microbial techniques. The composition and preparation of 561Q medium was described in WO 02/009040.

Immature Embryo Preparation:

Nine to ten days after controlled pollination of a corn plant, developing
30 immature embryos are opaque and 1 - 1.5 mm long. This length is the optimal size for infection with the PHP18749-transformed *Agrobacterium*. The husked ears can be sterilized in 50% commercial bleach and one drop Tween-20 for thirty minutes, and then rinsed twice with sterile water. The immature embryos can then

be aseptically removed from the caryopsis and placed into 2 mL of sterile holding solution consisting of medium 561Q that contains 100 μ M of acetosyringone.

Agrobacterium Infection and Co-cultivation of Embryos:

5 The holding solution can be decanted from the excised immature embryos and replaced with the PHP18749-transformed *Agrobacterium*. Following gentle mixing and incubation for about five minutes, the *Agrobacterium* can be decanted from the immature embryos. Immature embryos were then moved to a plate of 562P medium, the composition of which has been previously described in WO
10 02/009040. The immature embryos can be placed on this media scutellum surface pointed upwards and then incubated at 20°C for three days in darkness. This can be followed by incubation at 28°C for three days in darkness on medium 562P that contains 100 μ g/mL carbenecillin as described in U.S. Patent No. 5,981, 840.

Selection of Transgenic Events:

15 Following incubation, the immature embryos can be transferred to 5630 medium, which can be prepared as described in WO 02/009040. This medium contains Bialaphos for selection of transgenic plant cells as conferred by the BAR
20 gene that is linked to barley HGGT expression cassette. At ten to fourteen-day intervals, embryos were transferred to 5630 medium. Actively growing putative transgenic embryogenic tissue can be after six to eight weeks of incubation on the 5630 medium.

25 Regeneration of T₀ Plants:

 Transgenic embryogenic tissue is transferred to 288W medium and incubated at 28°C in darkness until somatic embryos matured, or about ten to eighteen days. Individual matured somatic embryos with well-defined scutellum and coleoptile are transferred to 272 embryo germination medium and incubated
30 at 28°C in the light. After shoots and roots emerge, individual plants are potted in soil and hardened-off using typical horticultural methods.

 288W medium contains the following ingredients: 950 mL of deionized water; 4.3 g of MS Salts (Gibco); 0.1 g of myo-inositol; 5 mL of MS Vitamins Stock Solution (Gibco); 1 mL of zeatin (5 mg/mL solution); 60 g sucrose; 8 g of agar

(Sigma A-7049, Purified), 2 mL of indole acetic acid (0.5 mg/mL solution*); 1 mL of 0.1 mM ABA*; 3 mL of Bialaphos (1 mg/mL solution*); and 2 mL of carbenicillin (50 mg/mL solution). The pH of this solution is adjusted to pH 5.6. The solution is autoclaved and ingredients marked with an asterisk (*) are added after the media
5 has cooled to 60°C.

Medium 272 contains the following ingredients: 950 mL of deionized water; 4.3 g of MS salts (Gibco); 0.1 g of myo-inositol; 5 mL of MS vitamins stock solution (Gibco); 40 g of Sucrose; and 1.5 g of Gelrite. This solution is adjusted to pH 5.6 and then autoclaved.

Confirmation of Transformation:

Fifty putative transgenic events were recovered and initially confirmed using a leaf paint test with bialaphos herbicide. The subsequent lack of a herbicide-injury lesion indicated the presence and action of the herbicide selectable marker
15 and the plants were self pollinated and taken through seed fill and maturation.

More than 150 seeds were obtained from each of 48 of the initially selected, transformed lines. The germ from 10 seeds of each of the 48 lines and one non-transgenic control was partially removed by dissection, weighed and combined in a 1.5 ml micro-centrifuge tube. One ml of heptane, which also contained 80 µg of
20 α-tocopherol acetate as internal standard, was added and the germ material was ground using a small plastic pestle. The ground germ material was further extracted during 2 hrs of agitation at room temperature. Total tocopherols and tocotrienols were analyzed on one aliquot of extract using the HPLC separation and fluorescence detection procedure described in Example 2. The total fatty acid
25 content of the heptane extract was determined by adding 2.5 mg of Tri-heptadecanoylglyceride to a 50 µl aliquot and forming the methyl esters by transesterification in 1 ml of methanol containing 0.25% sodium methoxide. The fatty acid methyl esters were extracted into 1 ml of heptane after addition of 1 ml of 1M NaCl to the transesterification reaction. Total fatty acids were determined
30 by separation of the methyl esters by GLC, detection by flame ionization and comparison of peak areas to the peak area of the methylheptadecanoate derived from the internal standard. α-, β-, γ- and δ-Tocotrienol was detected in all of the transgenic lines but not in the germ extract of the non-transgenic control. Gamma-tocopherol was the most prominent tocopherol present and γ-tocotrienol was the

most prominent tocotrienol in the transgenic events. The total tocopherol content of the transgenic lines on an oil basis was similar to that of the non-transgenic control and the total tocotrienol varied by event but reached levels up to 3 times the endogenous tocopherol content. The results for all the events analyzed are shown in Table 3 and demonstrate that the barley HGGT gene under the control of a promoter that is active in maize embryo tissue is capable of introducing the capability to synthesize and store tocotrienol. The total oil soluble anti-oxidant content can be increased by at least 3-fold by the expressed chimeric gene.

TABLE 3

Total tocopherol per mg of oil and total tocotrienol per mg of oil in the germ of 48 transgenic maize lines expressing the barley HGGT gene under control of the maize oleosin promoter and one wild type control. Individual events are ranked by total tocotrienol content.

EVENT #	TOTAL TOCOPHEROL (NG/MG OF OIL)	TOTAL TOCOTRIENOL (NG/MG OF OIL)
A1532.064.7.25.1	2997.5	8368.5
A1532.064.8.11.1	2858.9	6274.0
A1532.064.7.14.1	2990.3	5791.1
A1532.064.8.8.1	2720.3	5273.8
A1532.064.7.16.1	3018.5	5188.9
A1532.064.8.16.1	2068.4	5146.2
A1532.064.7.8.1	2701.9	5074.8
A1532.064.7.22.1	2306.6	4601.8
A1532.064.8.17.1	1993.4	4565.4
A1532.064.7.4.1	2996.7	4372.7
A1532.064.8.15.1	2610.3	4336.6
A1532.064.7.19.1	3663.3	4186.1
A1532.064.7.5.1	1946.2	4104.0
A1532.064.7.23.1	2554.5	3914.6
A1532.064.8.20.1	2359.2	3913.3
A1532.064.8.14.1	2680.9	3728.6
A1532.064.7.20.1	2026.7	3681.1
A1532.064.8.24.1	2330.7	3679.9
A1532.064.7.18.1	2017.5	3621.9
A1532.064.7.1.1	2899.1	3477.0
A1532.064.8.22.1	2827.6	3333.6
A1532.064.7.3.1	4293.1	3248.2
A1532.064.8.7.1	3797.9	3093.1
A1532.064.7.15.1	2840.9	3091.1
A1532.064.8.9.1	3425.5	2391.8
A1532.064.8.18.1	2932.6	2391.6
A1532.064.7.7.1	2500.9	2327.0
A1532.064.8.12.1	2673.3	2315.3
A1532.064.7.24.1	3072.7	2089.2

(Table 3 Continued)

EVENT #	TOTAL TOCOPHEROL (NG/MG OF OIL)	TOTAL TOCOTRIENOL (NG/MG OF OIL)
A1532.064.8.13.1	2782.0	2062.6
A1532.064.7.12.1	2140.8	1824.4
A1532.064.7.11.1	3076.0	1805.6
A1532.064.8.3.1	1515.9	1770.7
A1532.064.7.10.1	2928.5	1692.5
A1532.064.7.17.1	4019.4	818.5
A1532.064.7.13.1	4047.7	775.1
A1532.064.8.2.1	2869.2	683.7
A1532.064.8.1.1	2667.5	601.8
A1532.064.8.25.1	3068.6	481.5
A1532.064.7.21.1	3887.7	375.8
A1532.064.7.9.1	2320.7	357.8
A1532.064.8.5.1	2797.1	81.9
A1532.064.7.2.1	1186.8	56.0
A1532.064.8.6.1	3094.8	41.6
WT	2732.0	0

Since the transgene in the seed population from a self pollinated initial transformant is expected to be genetically segregating for the presence of the transgene and its copy number, 15 single seeds from events were selected by their bulked, excised-germ, tocotrienol content. Single seeds were ground in a ball- impact single seed grinder and 100 mg of the resulting powder was weighed into 1.5 ml micro-centrifuge tubes and extracted using 1 ml of heptane and 5.4 μ g of the α -tocopherol acetate internal standard as describe for the bulk germ. Unlike bulk germ, wild type maize whole seed contains the set of α , β and γ tocotrienols due to their presence in the endosperm tissue. Extracted tocols were separated and quantified using the HPLC method described above and the results expressed as parts per million tocopherol, tocotrienol and the sum of the compound classes is shown in Table 4.

Table 4

Total tocopherols, total tocotrienols and the sum of the two expressed parts per million in grain. The results are from 15 single seeds for each of 7 transgenic events chosen to represent a sampling of all events based on their bulk germ analysis. The first entries listed as "B73" followed by a letter are single seeds from a wild type control. Entries within an event are ranked by their total tocotrienol content.

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
B73L 10/23	57.3	8.5	65.8
B73N 10/23	47.4	7.5	54.9
B73H 10/23	88.6	7.3	95.9
B73M 10/23	63.4	7.1	70.5
B73I 10/23	44.4	6.7	51.1
B73O 10/23	52.2	6.3	58.5
B73B 10/17	41.1	5.9	47.0
B73J 10/23	52.5	5.6	58.1
B73E 10/23	49.6	5.5	55.1
B73F 10/23	52.5	5.3	57.8
B73A 10/17	17.2	3.5	20.7
B73C 10/17	30.6	3.3	33.9
B73D 10/17	29.6	2.5	32.1
B73K 10/23	23.3	2.1	25.4
B73G 10/23	33.7	1.2	34.9
2I A1532.064.7.2	98.6	21.5	120.1
2J A1532.064.7.2	70.6	15.3	85.9
2L A1532.064.7.2	27.8	14.1	41.9
2H A1532.064.7.2	25.4	11.4	36.8
2B A1532.064.7.2	38.9	11.2	50.1
2C A1532.064.7.2	37.5	10.5	48.0
2M A1532.064.7.2	46.0	9.9	55.9
2N A1532.064.7.2	38.4	9.6	48.0
2A A1532.064.7.2	29.5	9.5	39.0
2K A1532.064.7.2	38.8	9.0	47.8
2G A1532.064.7.2	43.2	7.1	50.3
2E A1532.064.7.2	23.4	6.8	30.2
2O A1532.064.7.2	14.7	5.8	20.5
2F A1532.064.7.2	83.9	5.4	89.3
2D A1532.064.7.2	49.1	4.8	53.9
31G A1532.064.8.6	140.3	21.3	161.6
31J A1532.064.8.6	87.5	20.0	107.5
31K A1532.064.8.6	58.4	18.0	76.4
31A A1532.064.8.6	94.8	16.3	111.1
31M A1532.064.8.6	84.3	15.0	99.3
31L A1532.064.8.6	63.3	13.0	76.3
31I A1532.064.8.6	43.3	10.4	53.7
31D A1532.064.8.6	31.1	9.3	40.4
31H A1532.064.8.6	30.9	8.6	39.5
31F A1532.064.8.6	42.4	8.0	50.4
31B A1532.064.8.6	73.5	7.5	81.0
31C A1532.064.8.6	61.3	6.3	67.6
31E A1532.064.8.6	30.7	5.9	36.6
15A A1532.064.7.15	70.8	191.9	262.7
15M A1532.064.7.15	58.5	155.9	214.4
15N A1532.064.7.15	72.2	135.1	207.3
15I A1532.064.7.15	77.1	126.8	203.9
15O A1532.064.7.15	78.4	120.1	198.5
15J A1532.064.7.15	45.7	104.6	150.3
15E A1532.064.7.15	45.6	92.8	138.4
15L A1532.064.7.15	40.9	89.7	130.6

(Table 4 Continued)

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
15F A1532.064.7.15	52.5	82.9	135.4
15D A1532.064.7.15	35.7	78.2	113.9
15B A1532.064.7.15	37.9	69.8	107.7
15K A1532.064.7.15	27.6	58.5	86.1
15C A1532.064.7.15	26.9	52.0	78.9
15G A1532.064.7.15	30.5	10.5	41.0
15H A1532.064.7.15	17.7	7.3	25.0
25B A1532.064.7.25	29.2	295.9	325.1
25C A1532.064.7.25	54.6	258.1	312.7
25H A1532.064.7.25	34.5	258.0	292.5
25G A1532.064.7.25	77.3	246.7	324.0
25M A1532.064.7.25	52.7	235.6	288.3
25K A1532.064.7.25	72.9	226.2	299.1
25F A1532.064.7.25	66.7	221.0	287.7
25D A1532.064.7.25	40.0	196.9	236.9
25L A1532.064.7.25	60.0	195.9	255.9
25E A1532.064.7.25	39.9	181.8	221.7
25A A1532.064.7.25	51.7	176.6	228.3
25N A1532.064.7.25	61.3	133.0	194.3
25I A1532.064.7.25	39.7	114.5	154.2
25O A1532.064.7.25	32.9	60.8	93.7
25J A1532.064.7.25	109.1	13.7	122.8
36F A1532.064.8.11	55.0	293.3	348.3
36B A1532.064.8.11	62.1	285.5	347.6
36J A1532.064.8.11	91.1	249.3	340.4
36K A1532.064.8.11	87.5	242.1	329.6
36D A1532.064.8.11	63.8	195.4	259.2
36M A1532.064.8.11	60.2	185.7	245.9
36C A1532.064.8.11	142.9	163.9	306.8
36G A1532.064.8.11	79.3	161.8	241.1
36L A1532.064.8.11	138.2	152.4	290.6
36I A1532.064.8.11	85.7	147.0	232.7
36E A1532.064.8.11	79.1	147.0	226.1
36H A1532.064.8.11	75.9	143.7	219.6
36N A1532.064.8.11	64.0	137.2	201.2
36O A1532.064.8.11	78.5	129.1	207.6
36A A1532.064.8.11	110.5	128.1	238.6
41C A1532.064.8.16	77.1	307.0	384.1
41F A1532.064.8.16	45.3	235.5	280.8
41I A1532.064.8.16	127.0	228.0	355.0
41D A1532.064.8.16	63.2	209.0	272.2
41B A1532.064.8.16	51.4	202.6	254.0
41E A1532.064.8.16	122.5	195.4	317.9
41O A1532.064.8.16	91.4	189.6	281.0
41L A1532.064.8.16	63.5	188.6	252.1
41N A1532.064.8.16	57.4	188.4	245.8
41H A1532.064.8.16	125.0	167.5	292.5
41M A1532.064.8.16	88.2	163.3	251.5

(Table 4 Continued)

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
41A A1532.064.8.16	64.3	147.9	212.2
41J A1532.064.8.16	59.2	135.8	195.0
41K A1532.064.8.16	67.2	107.1	174.3
41G A1532.064.8.16	120.4	102.4	222.8
42J A1532.064.8.17	112.5	460.7	573.2
42F A1532.064.8.17	99.9	373.2	473.1
42I A1532.064.8.17	24.2	213.8	238.0
42O A1532.064.8.17	57.2	201.3	258.5
42L A1532.064.8.17	65.7	196.5	262.2
42E A1532.064.8.17	27.4	193.5	220.9
42A A1532.064.8.17	51.6	181.1	232.7
42N A1532.064.8.17	67.8	157.9	225.7
42B A1532.064.8.17	26.6	157.2	183.8
42D A1532.064.8.17	51.2	149.5	200.7
42C A1532.064.8.17	64.3	122.7	187.0
42G A1532.064.8.17	26.2	109.4	135.6
42K A1532.064.8.17	98.8	105.6	204.4
42H A1532.064.8.17	38.8	104.6	143.4
42M A1532.064.8.17	54.4	19.4	73.8

The 15 wild type seeds had an average tocopherol content of 45.6 ppm, tocotrienol contents that ranged from 1.2 to 8.5 ppm and an averaged 5.2 ppm. Event number A15232.064.8.6 which was the lowest ranked transgenic had similar average values of 64.7 and 12.3 ppm for tocopherols and tocotrienols respectively. By contrast, event A1532.064.8.17 that was the top ranked event by bulk germ tocotrienol in oil had single seed tocotrienol values that ranged from 19.4 ppm to 460.7 ppm. The average tocopherol content in the event remained at 57.8 ppm. The transgene has no apparent effect on the ability of the maize seed to store tocopherols while it greatly increase the content of tocotrienol. In a population of segregating seeds, it is reasonable to expect at least one wild type seed in a sample of 15. If the lowest ranking seed in event A1532.064.8.17 is excluded from the tocotrienol average on that basis the average becomes 194.8 ppm and the total of tocopherol and tocotrienol is 252.8 ppm. Those values are conservative estimates of the maximum phenotype expected from the transgene in this event since the population is still segregating for transgene ploidy. If one assumes that the top 25% of seed by ranking is representative of the homozygous condition (assumes one segregating genetic locus) the average tocotrienol

estimate is about 315 ppm. That value combined with the average tocopherol content gives a combined estimate of about 373 ppm.

Average expected values for total tocopherol and total tocotrienol in corn seeds is in the range 40 to 90 ppm (see Weber, E.J. 1987. Lipids of the Kernel. Page 335 in: Corn: Chemistry and Technology. S.A. Watson and P.E. Ramstad, eds. American Association of Cereal Chemists, Inc. St. Paul, MN). The expression of the barley HGGT increases the tocotrienol levels from about 10 ppm to between 100 and 400 ppm and the total tocopherol + tocotrienol content to between 120 and 450 ppm.

Example 7: Characterization of cDNA Clones Encoding Proteins
Similar to *Arabidopsis thaliana* Homogentisate
Phytyltransferase (*Also Known as Tocopherol
Polyprenyltransferase*)

BLASTP searches using amino acid sequences deduced from clones listed in Table 3 revealed similarity of these polypeptides encoded by the cDNAs homogentisate phytyltransferase from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO: 13). Shown in Table 5 are the BLASTP results for amino acid sequences deduced from the entire cDNA inserts comprising the indicated clone ("FIS").

TABLE 5

BLAST Results for Sequences Encoding Polypeptides Homologous to HGGT

Clone	Status	BLAST pLog Score
		NCBI General Identifier No. 17104828 (SEQ ID NO:13)
bdl2c.pk006.o2	FIS	94.00
cco1n.pk087.l17: fis	CGS	85.52
p0058.chpbj67r: fis	FIS	91.00
rds1c.pk007.m9	FIS	90.70
wdk2c.pk012.f2: cgs	CGS	95.00

The nucleotide sequence of clone bdl2c.pk006.o2 is shown in SEQ ID NO: 1. The amino acid sequence deduced from nucleotides 63 through 1286 of SEQ ID NO: 1 is shown in SEQ ID NO: 2. The nucleotide sequence of clone cco1n.pk087.l17:cgs is shown in SEQ ID NO: 7. The amino acid sequence deduced from nucleotides 211 through 1353 of SEQ ID NO: 7 is shown in SEQ ID NO: 8. The nucleotide sequence of clone p0058.chpbj67r:fis is shown in SEQ ID NO: 9. The amino acid sequence deduced from nucleotides 357 through 1441 of SEQ ID NO: 9 is shown in SEQ ID NO: 10. The nucleotide sequence of clone rds1c.pk007.m9 is shown in SEQ ID NO: 5. The amino acid sequence deduced from nucleotides 6 through 1220 of SEQ ID NO: 5 is shown in SEQ ID NO: 6. The nucleotide sequence of clone wdk2c.pk012.f2:cgs is shown in SEQ ID NO: 3. The amino acid sequence deduced from nucleotides 53 through 1279 of SEQ ID NO: 3 is shown in SEQ ID NO: 4.

The complete gene sequence (cgs) for clone cco1n.pk087.l17 employed a PCR based amplification of the 5'-end of the transcript. Briefly, two nested primers (SEQ ID Nos: 67 and 68) were used in a RACE protocol (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) to generate fragments between a single point in the transcript and the 5' end. Primers oriented in the 5' direction (complementary to nucleotides 598-621 and 570-591 of SEQ ID NO: 7) were designed from the instant sequences, then commercially available RACE systems (BRL) were used to isolate specific 5' cDNA fragments (Ohara *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5673-5677; Loh *et al.* (1989) *Science* 243:217-220). Products generated by the RACE procedures can be combined to generate full-length cDNAs (Frohman and Martin (1989) *Techniques* 1:165). SEQ ID No: 7 as listed is a cgs sequence and contains two adenine residues at positions 571 and 572. In contrast, the fis sequence variant of SEQ ID No: 7 contains three adenine residues spanning positions 571 to 573 (positions corresponding to SEQ ID No: 7). Primer SEQ ID No: 67 was designed with three thymine residues in order to hybridize with the three adenine residues in the fis variant of SEQ ID No: 7. The additional adenine residue in the fis variant of SEQ ID No: 7 would cause a frameshift to occur in the corresponding amino acid sequence.

Alignments of the amino acid sequences set forth in SEQ ID Nos: 2, 4, 6, 8 and 10 and the sequence from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO: 13) were performed. The data in Table 6

represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID Nos:2, 4, 6, 8 and 10 and the sequence from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO:13).

5

TABLE 6

Percent Identity of Amino Acid Sequences Deduced from the
Nucleotide Sequences of cDNA Clones Encoding
Polypeptides Homologous to HGGT

Clone	SEQ ID NO:	Percent Identity to NCBI General Identifier No. 17104828 (SEQ ID NO:13)
bdl2c.pk006.o2	2	45.3
wdk2c.pk012.f2:cgs	4	45.5
rds1c.pk007.m9	6	44.8
cco1n.pk087.l17:cgs	8	42.5
p0058.chpbj67r:fis	10	46.5

10

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) CABIOS. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a HGGT.

20

Example 8: Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the beta subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238) can be used for expression of the instant

25

polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin.

Between the 5' and 3' regions are the unique restriction endonuclease sites NcoI (which includes the ATG translation initiation codon), SmaI, KpnI and XbaI. The entire cassette is flanked by HindIII sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar Jack, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression

cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

5 To 50 μ L of a 60 mg/mL 1 μ m gold particle suspension is added (in order): 5 μ L DNA (1 μ g/ μ L), 20 μ L spermidine (0.1 M), and 50 μ L CaCl_2 (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μ L 70% ethanol and resuspended in 40 μ L of anhydrous
10 ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five μ L of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue
15 with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed
20 back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be
25 observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or
30 regenerated into whole plants by maturation and germination of individual somatic embryos.

Example 9: Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant HGGT polypeptides can be used to produce tocotrienols in microbes such as algal and cyanobacterial cells that contain an operable tocopherol biosynthetic pathway. Expression of cDNAs encoding the instant HGGT polypeptides in these cells are expected to result in the condensation of geranylgeranyl pyrophosphate and homogentisate. The product of the HGGT reaction 2-methyl-6-geranylgeranylbenzoquinol can then be converted to tocotrienols by tocopherol biosynthetic enzymes native to the host microbial cell. Tocotrienols can be produced in microbes by linking the cDNAs encoding the instant HGGT polypeptides with promoter elements that are suitable to direct gene expression in the selected host cell. The resulting chimeric genes can be introduced into the host microbial cell using techniques such as homologous recombination (Williams, J.G.K. (1988) *Methods Enzymol.* 167:766-778; Legarde, D. *et al.* (2000) *App. Environ. Microbiol.* 66:64-72). Host cells transformed with cDNAs for the instant HGGT polypeptides operably linked to functional promoters can then be analyzed for tocotrienol production using techniques described in Example 2.

Example 10: Functional Conversion of a Homogentisate Phytoltransferase(HPT) into a Homogentisate Geranylgeranyl Transferase (HGGT) by Enzyme Engineering

Numerous examples exist of the conversion of the substrate specificity of one enzyme class to that of another by replacement of specific amino acid residues (e.g., Yuan, L. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:10639-10643; Cahoon, E.B. *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4872-4877; Reznik, G.O. *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13525-13530). This conversion is typically achieved through comparisons of primary or tertiary structures of enzymes that have similar amino acid sequences but different substrate specificities. From these comparisons, one skilled in the art can recognize amino acid residues that likely contribute to the substrate recognition properties of a given functional class of enzymes. Residues that dictate substrate specificity are often ones that are conserved in members of a given functional class of enzyme but are different in other structurally related but functionally divergent classes

(Yuan, L. et al., *supra*). By replacement of these residues, one can experimentally test whether a given amino acid or group of amino acids contribute to the substrate specificity of an enzyme.

Selected amino acid residues in an enzyme can be replaced through a variety of mutagenesis methods that are well known to those skilled in the art (e.g., Cahoon, E.B. et al., *supra*). By expressing the gene or cDNA for the mutant enzyme in a host cell, one can readily monitor whether replacement of amino acids has altered substrate specificity of the enzyme. Alteration of substrate specificity can be measured by supplying the mutant enzyme with alternative substrates in an *in vivo* or *in vitro* assay. Alternatively, one skilled in the art can measure the accumulation of metabolic products of the mutated enzyme upon expression in a host cell.

As described in the instant invention, homogentisate geranylgeranyl transferases (HGGTs) (SEQ ID NOs: 2, 4, 6, 8 and 10) are members of the UbiA prenyltransferase family. Of the functionally diverse members of this family, HGGTs share the highest degree of amino acid sequence identity with homogentisate phytyltransferases (HPTs) (typically 40 to 50% identity). Despite this degree of structural relatedness, HGGTs and HPTs have divergent substrate specificities. HGGTs catalyze the condensation of homogentisate and geranylgeranyl pyrophosphate, the first committed step in tocotrienol biosynthesis. In contrast, HPTs catalyze the condensation of homogentisate and phytyl pyrophosphate, the first committed step in tocopherol biosynthesis. Sequence alignments between SEQ ID NOs: 2, 4, 6, 8, 10, 13, 14, 15, 16 and the sequence from *Synechocystis* sp. PCC 6803 (NCBI General Identification (GI) No. 16330366; SEQ ID NO: 17), the sequence from *Escherichia coli* K12 (4-hydroxybenzoate-octaprenyltransferase) (NCBI General Identification (GI) No. 16131866; SEQ ID NO: 18) and the sequence from oat (*Avena sativa*) (chlorophyll synthase) (NCBI General Identification (GI) No. 7378659; SEQ ID NO: 19) showed amino acid residues that are conserved in all HGGTs but are different in other UbiA prenyltransferase family members including HPTs. Using the sequence of the barley HGGT (SEQ ID NO: 2) as the basis for amino acid numbering, these residues include the following: arginine 72, glutamine 73, cysteine 85, cysteine 118, phenylalanine 124, isoleucine 127, isoleucine 128, glycine 129, threonine 131, proline 137, aspartate 142, phenylalanine 144, threonine 145, cysteine 161,

isoleucine 213, methionine 270, glutamine 272, leucine 279, alanine 280, isoleucine 333, threonine 338, threonine 351, glutamine 355, serine 361, glycine 364, leucine 365, glutamate 380, asparagine 381 and phenylalanine 401. It is likely that these residues or some subgroup of these residues define the substrate specificity of HGGTs. As such, these amino acids represent targets for the functional conversion of an HPT-type enzyme into an HGGT. In achieving HGGT activity, one need not alter the HPT or UbiA prenyltransferase sequence to comprise all 29 of these residues at these positions. Rather, altering the target sequence to comprise at least any 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, or 29 of these residues at the positions identified can confer HGGT activity upon the target sequence.

By replacement of the equivalent residues (shown above) in an HPT into those found in HGGTs, an HPT can be converted into an enzyme that displays enhanced substrate specificity for geranylgeranyl pyrophosphate in preference to phytyl pyrophosphate. An HPT engineered in this manner is thus the functional equivalent of a naturally occurring HGGT and can be used to produce tocotrienols and increase the tocol content in transgenic host cells as described for HGGTs in Examples 2, 3, 4, 5, 6, 8 and 9. Thus, the invention described herein contemplates HGGTs obtained from plants, including monocots, and sequences from any source that have been engineered to function in plants, and more specifically, in monocots. The term plant homogentisate geranylgeranyl transferase encompasses homogentisate geranylgeranyl transferase sequences derived from a plant source in addition to HPT or other UbiA prenyltransferase sequences from any source modified to be or to encode a functional homogentisate geranylgeranyl transferase in a plant or plant cell using the methods taught herein. The term monocot homogentisate geranylgeranyl transferase encompasses homogentisate geranylgeranyl transferase sequences derived from a monocot plant source in addition to HPT or other UbiA prenyltransferase sequences from any source modified to be or to encode a functional homogentisate geranylgeranyl transferase in a monocot plant or plant cell using the methods taught herein

Example 11: Tocotrienol Production in *Arabidopsis thaliana* by Transgenic Expression of the Barley Homogentisate Geranylgeranyl Transferase

The cDNA for barley homogentisate geranylgeranyl transferase (HGGT) (SEQ ID NO: 1) was constitutively expressed in *Arabidopsis thaliana* to further demonstrate the utility of this cDNA for tocotrienol production in transgenic plants. Plasmid pSH24 was used for *Agrobacterium*-mediated transformation of *Arabidopsis*. This plasmid, which was described in Example 2, contains the open reading frame of the barley HGGT operably linked on its 5' end to the cauliflower mosaic virus 35S promoter and on its 3' end to the transcription termination sequence of the nopaline synthase gene. This expression cassette together with a kanamycin resistance gene for transgenic plant selection is contained within right and left border regions of the plasmid. pSH24 was introduced into *Agrobacterium tumefaciens* strain C58C1-pMP90 using a freeze-thaw method of bacterial transformation (Holsters, M. et al. (1978) *Mol. Gen. Genet.* 163(2):181-187). *Arabidopsis thaliana* cv Columbia plants were then transformed with *Agrobacterium* harboring pSH24 using the vacuum infiltration method described by Bechtold, N. et al. ((1993) *C.R. Acad. Sci., Paris* 316:1194-1199). Transformed plants were selected for the ability of seeds from the infiltrated plants to germinate and for the seedlings to subsequently grow on media containing 40 µg/mL of kanamycin.

Leaves from the plants transformed with the barley HGGT cDNA linked to the cauliflower mosaic virus 35S promoter were extracted and tocotrienol accumulation measured using analytical methods described in Example 2. Leaves were collected from the second whorl of 30-day old plants. Plants were maintained under a 14 h-22°C/10 h-18°C light/dark cycle with a light intensity of 100 µmol m⁻² s⁻¹. In the organic extract from leaves of the transgenic plants, several tocotrienol molecular species were detected including α-, γ-, and δ-tocotrienols. The primary tocol form found in these leaves was γ-tocotrienol. No tocotrienols, in contrast, were detected in untransformed plants, and the major tocol species in leaves of these plants instead was α-tocopherol. In addition, the tocol content of leaves of transformed plants was increased by as much as 10 to 15-fold relative to leaves of untransformed plants.

Leaves of T1 plants were found to accumulate large amounts of tocotrienols, which were absent from leaves of non-transformed plants. The

content of Vitamin E antioxidants in leaves of segregating T2 plants from one of the selected lines was examined in detail. Leaves from plants displaying a null phenotype accumulated tocopherols (almost exclusively as α -tocopherol) to amounts of 40 to 60 mg/g dry weight. (Similar levels of tocopherols were detected in non-transformed plants.) In contrast, the total content of tocopherols and tocotrienols in leaves of phenotype-positive plants ranged from 700 to 900 mg/g dry weight. In leaves of these plants, γ -tocotrienol accounted for approximately 85% of the total Vitamin E content.

Example 12: Identification of Protein Sequences Specific to HGGT Homologs

HGGT polypeptides described in the instant invention are members of the UbiA prenyltransferase family, which includes a number of functionally diverse enzymes such as chlorophyll synthase and 4-hydroxybenzoate octaprenyltransferase. Members of this family are distinguished by the presence of a UbiA consensus motif. Of the known members of this family, HGGTs are most closely related to HPTs. HGGTs described in the instant invention (SEQ ID NOs: 2, 4, 6, 8 and 10) share 40 to 50% identity with previously disclosed HPTs (SEQ ID NOs: 13-16). Using amino acid sequence alignments, one skilled in the art can readily distinguish HGGT polypeptides from HPT polypeptides by the presence of amino acid residues that are uniquely conserved in HGGTs. Such residues include (using SEQ ID NO: 2 as the basis for amino acid numbering): arginine 72, glutamine 73, cysteine 85, cysteine 118, phenylalanine 124, isoleucine 127, isoleucine 128, glycine 129, threonine 131, proline 137, aspartate 142, phenylalanine 144, threonine 145, cysteine 161, isoleucine 213, methionine 270, glutamine 272, leucine 279, alanine 280, isoleucine 333, threonine 338, threonine 351, glutamine 355, glycine 364, leucine 365, asparagine 381 and phenylalanine 401.

Protein motifs can be defined as short regions of conserved amino acid sequences that comprise part of a longer sequence. One skilled in the art can discern several HGGT-specific protein motifs. Using the barley HGGT amino acid sequence as the basis for numbering (SEQ ID 2), HGGT-specific motifs include "FXXIIGXT" which encompasses amino acids 124 through 131 and "(K/R)XXXDXFT" which encompasses amino acids 139 through 145. (Note: "X"

indicates that a residue is not conserved in HGGTs or is not uniquely conserved in HGGTs.)

One skilled in the art can use amino acid sequence alignments such as those described above to identify "new" HGGTs that correspond to an isolated polypeptide or are deduced from an isolated nucleic acid fragment. An amino acid sequence of an HGGT polypeptide would be expected to be related to UbiA prenyltransferases and of highest relation to known HPT and HGGT polypeptides. In addition, an HGGT polypeptide would be expected to contain one or more of the protein motifs described above or one or more of the amino acid residues that are uniquely conserved in HGGT sequences disclosed in the instant invention as detailed above. Isolation of nucleic acid fragments encoding HGGTs can be achieved through a variety of techniques including hybridization with nucleic acid fragments encoding portions HGGT-related polypeptides or PCR-based strategies such as those described in the Examples herein. The biological source of the isolated nucleic acid fragments would preferably be a plant, plant tissue, or microbe that is known to produce tocotrienols. An isolated nucleic acid fragment can then be expressed in a tissue or cell, preferably a plant tissue or cell, that does not contain tocotrienol to determine whether it encodes a functional HGGT. Methods of expression in a host tissue or cell can include those described in Examples 2, 3, 4, 5, 6, 8, 9, and 11. Expression of a functional HGGT would be expected to confer the ability to synthesize tocotrienols to the tissue or cell. Accumulation of tocotrienols can be determined using analytical methods described in Example 2.

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 individually indicated to be incorporated by reference.

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

SEQUENCE LISTING

<110> E.I. duPont de Nemours

<120> COMPOSITIONS AND METHODS FOR ALTERING
TOCOTRIENOL CONTENT

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<150> 60/366,757

<151> 2002-03-22

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Asn	Ala	Asn	Arg	Leu	Glu	Glu	Ile	Arg	Gly	Asp	Val	Leu	Lys	Lys	Leu	
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Ser Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile			
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Leu Leu Thr Ala Tyr Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr			
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Arg Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu			
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Tyr Phe Leu Ile Pro Phe Val Gln *			
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Arg Pro Arg Arg Asn Thr Lys Arg Gln Cys Ser Asp Asp Tyr Pro Ala			
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Phe Tyr Gln Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly			
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 Val Arg Cys Tyr Ala Ile Ala Gly Asp Gln His Glu Ser Ile Ala Thr
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tat cgg ttt tgc cga ccc cac aca att ttt ggc act ata ata gga atc 386
 Tyr Arg Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly Ile
 115 120 125

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Leu Lys Arg Pro Leu Ala Pro Thr Lys Ser Val Val Phe Ala Thr Leu			
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ttc atg tgt tgc ttt tct tca gtt ata gct tta ttc aag gat att cca			914
Phe Met Cys Cys Phe Ser Ser Val Ile Ala Leu Phe Lys Asp Ile Pro			
290	295	300	
gat att gat ggt gac aga cat ttt ggc gtc gaa tcc ctg agc gta cgt			962
Asp Ile Asp Gly Asp Arg His Phe Gly Val Glu Ser Leu Ser Val Arg			
305	310	315	
ttg ggt cca gaa aga gtg tat tgg ctc tgc ata aac ata cta tta aca			1010
Leu Gly Pro Glu Arg Val Tyr Trp Leu Cys Ile Asn Ile Leu Leu Thr			
320	325	330	335
gca tat ggg gct gcc att ttg gct gga gca tca tct aca aat cta tgt			1058
Ala Tyr Gly Ala Ala Ile Leu Ala Gly Ala Ser Ser Thr Asn Leu Cys			
340	345	350	
caa atg att atc acc gtt ttc ggc cat ggc ctg ctt gcc ttt gca ctt			1106
Gln Met Ile Ile Thr Val Phe Gly His Gly Leu Leu Ala Phe Ala Leu			
355	360	365	
tgg cag aga gca cag cac tgt gac gtt gaa aac aag gcg tgg atc aca			1154
Trp Gln Arg Ala Gln His Cys Asp Val Glu Asn Lys Ala Trp Ile Thr			
370	375	380	
tca ttt tac atg ttc att tgg aag ttg ttc tac gct gag tat ttc ctt			1202
Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu			
385	390	395	
ata cca ttt gtg cag tga gcactatata cacaaggcca ag			1242

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Ile Pro Phe Val Gln
400

<210> 6

<211> 404

<212> PRT

<213> Oryza sativa

<400> 6

Met	Gln	Ala	Ser	Ser	Ala	Ala	Ala	Ala	Ala	Ala	Cys	Ser	Ala	Ile	Lys
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Pro	Ala	Ala	His	Gln	His	Thr	Val	Gln	Val	Gln	Glu	Asp	Lys	Arg	Gly
			20					25					30		
Ser	Glu	Phe	Arg	Ala	Arg	Phe	Gly	Thr	Arg	Lys	Leu	Ser	Trp	Gly	Gly
		35					40					45			
Lys	Leu	Ser	Val	Glu	Asn	Ser	Ala	Leu	His	Gln	Cys	Gln	Ser	Leu	Thr
	50					55					60				
Arg	Ser	Ile	Arg	Arg	Gln	Lys	Arg	Gln	His	Ser	Pro	Val	Leu	Gln	Val
65					70					75					80
Arg	Cys	Tyr	Ala	Ile	Ala	Gly	Asp	Gln	His	Glu	Ser	Ile	Ala	Thr	Glu
			85						90					95	
Phe	Glu	Glu	Ile	Cys	Lys	Glu	Val	Pro	Gln	Lys	Leu	Gly	Ala	Phe	Tyr
			100					105					110		
Arg	Phe	Cys	Arg	Pro	His	Thr	Ile	Phe	Gly	Thr	Ile	Ile	Gly	Ile	Thr
		115					120					125			
Ser	Val	Ser	Leu	Leu	Pro	Met	Arg	Ser	Leu	Asp	Asp	Phe	Thr	Met	Lys
	130					135				140					
Ala	Leu	Trp	Gly	Phe	Leu	Glu	Ala	Leu	Ser	Ser	Ser	Leu	Cys	Met	Asn
145					150					155					160
Ile	Tyr	Val	Val	Gly	Leu	Asn	Gln	Leu	Tyr	Asp	Ile	Gln	Ile	Asp	Lys
				165				170					175		
Val	Asn	Lys	Pro	Ser	Leu	Pro	Leu	Ala	Ser	Gly	Glu	Phe	Ser	Val	Ala
			180					185					190		
Thr	Gly	Ala	Val	Leu	Val	Leu	Thr	Ser	Leu	Ile	Met	Ser	Ile	Ala	Ile
		195					200					205			
Gly	Ile	Arg	Ser	Lys	Ser	Ala	Pro	Leu	Leu	Cys	Ala	Leu	Phe	Ile	Ser
	210					215					220				
Phe	Phe	Leu	Gly	Ser	Ala	Tyr	Ser	Val	Asp	Ala	Pro	Leu	Leu	Arg	Trp
225					230					235					240
Lys	Arg	Asn	Ala	Phe	Leu	Ala	Ala	Ser	Cys	Ile	Leu	Phe	Val	Arg	Ala
				245					250					255	
Val	Leu	Val	Gln	Leu	Ala	Phe	Phe	Ala	His	Met	Gln	Gln	His	Val	Leu
			260					265					270		
Lys	Arg	Pro	Leu	Ala	Pro	Thr	Lys	Ser	Val	Val	Phe	Ala	Thr	Leu	Phe
		275					280					285			
Met	Cys	Cys	Phe	Ser	Ser	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro	Asp
	290					295					300				
Ile	Asp	Gly	Asp	Arg	His	Phe	Gly	Val	Glu	Ser	Leu	Ser	Val	Arg	Leu
305					310					315					320
Gly	Pro	Glu	Arg	Val	Tyr	Trp	Leu	Cys	Ile	Asn	Ile	Leu	Leu	Thr	Ala
				325					330					335	
Tyr	Gly	Ala	Ala	Ile	Leu	Ala	Gly	Ala	Ser	Ser	Thr	Asn	Leu	Cys	Gln
			340					345					350		
Met	Ile	Ile	Thr	Val	Phe	Gly	His	Gly	Leu	Leu	Ala	Phe	Ala	Leu	Trp

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	355		360		365										
Gln	Arg	Ala	Gln	His	Cys	Asp	Val	Glu	Asn	Lys	Ala	Trp	Ile	Thr	Ser
	370					375					380				
Phe	Tyr	Met	Phe	Ile	Trp	Lys	Leu	Phe	Tyr	Ala	Glu	Tyr	Phe	Leu	Ile
385					390					395					400
Pro	Phe	Val	Gln												

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<211> 1730

<212> DNA

<213> Zea mays

<220>

<221> CDS

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gatggccaag cggctcgccg gtgcccacaa agaggtgctc gtcgaggtgg tgaggttcac 180
gcataagagc ggactgaggg gctgtgacgg cgg ctg gaa gga ttt cct ggc cca 234
Arg Leu Glu Gly Phe Pro Gly Pro
1 5

gaa cga cag gaa gtt tgg cgc gtc ggt gag cga ccc gag gaa gcg ctc 282
Glu Arg Gln Glu Val Trp Arg Val Gly Glu Arg Pro Glu Glu Ala Leu
10 15 20

cag gga cgt gct gtt cgc ctt cct gca gac ctt ccc caa gga ttt cca 330
Gln Gly Arg Ala Val Arg Leu Pro Ala Asp Leu Pro Gln Gly Phe Pro
25 30 35 40

gaa gaa aca ctt gat gcc act agt ccg acg aga gcc acc gga aga caa 378
Glu Glu Thr Leu Asp Ala Thr Ser Pro Thr Arg Ala Thr Gly Arg Gln
45 50 55

cgc agg cat tcc tca gtc ccc aaa gtg agc tgc tgg gca gct gct cat 426
Arg Arg His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His
60 65 70

cac caa cac aat tct aac ccc cag cag ttt cag gcg att ggc ata cga 474
His Gln His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg
75 80 85

atc gca aag acg ctg cat gcc ttc tat cag ttc tgc cga cca cac aca 522
Ile Ala Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr
90 95 100

ata ttt gga acc ata ata ggc att act tcg gtg tct atc ctg cca gtg 570
Ile Phe Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Ile Leu Pro Val
105 110 115 120

aag agc ctg gac gat ttt acg ttg ata gct ata tgg gga ttt ctc gag 618

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Lys	Ser	Leu	Asp	Asp	Phe	Thr	Leu	Ile	Ala	Ile	Trp	Gly	Phe	Leu	Glu		
				125					130					135			
gct	ttg	gcc	gcc	gca	tta	tgt	atg	aac	gtt	tat	gta	gta	ggg	ctg	aac	666	
Ala	Leu	Ala	Ala	Ala	Leu	Cys	Met	Asn	Val	Tyr	Val	Val	Gly	Leu	Asn		
			140					145					150				
aag	gtc	aat	aag	cca	acc	ctc	cca	tta	tcg	ttc	gga	gag	ttt	tca	atg	714	
Lys	Val	Asn	Lys	Pro	Thr	Leu	Pro	Leu	Ser	Phe	Gly	Glu	Phe	Ser	Met		
		155					160					165					
cca	act	gca	gta	ttg	tta	gta	gtg	gca	ttc	ttg	gtc	atg	agc	att	agc	762	
Pro	Thr	Ala	Val	Leu	Leu	Val	Val	Ala	Phe	Leu	Val	Met	Ser	Ile	Ser		
	170					175					180						
atc	gga	ata	aga	tca	aag	tct	gct	cca	ttg	atg	tgt	gct	ttg	ctt	gtt	810	
Ile	Gly	Ile	Arg	Ser	Lys	Ser	Ala	Pro	Leu	Met	Cys	Ala	Leu	Leu	Val		
185					190					195					200		
tgc	ttc	ctt	ctt	gga	agc	gca	tac	ccc	att	gac	gtc	cca	tta	ctc	cgg	858	
Cys	Phe	Leu	Leu	Gly	Ser	Ala	Tyr	Pro	Ile	Asp	Val	Pro	Leu	Leu	Arg		
				205				210						215			
tgg	aag	cga	cat	gct	ttt	cta	gct	gca	ttc	tgc	ata	atc	ttt	gtg	agg	906	
Trp	Lys	Arg	His	Ala	Phe	Leu	Ala	Ala	Phe	Cys	Ile	Ile	Phe	Val	Arg		
			220					225					230				
cct	gta	gtg	gtc	cag	tta	gct	ttc	ttt	gca	cac	atg	cag	caa	cat	gtt	954	
Pro	Val	Val	Val	Gln	Leu	Ala	Phe	Phe	Ala	His	Met	Gln	Gln	His	Val		
		235					240					245					
ctg	aag	agg	ccc	ttg	gca	cct	aca	agg	tcg	gtg	gtc	ttt	gca	aca	tgt	1002	
Leu	Lys	Arg	Pro	Leu	Ala	Pro	Thr	Arg	Ser	Val	Val	Phe	Ala	Thr	Cys		
	250					255					260						
ttc	atg	tgt	tgc	ttc	gct	gca	gta	ata	gcg	cta	ttc	aag	gat	att	cct	1050	
Phe	Met	Cys	Cys	Phe	Ala	Ala	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro		
265					270				275						280		
gat	gtc	gat	gga	gat	aga	gat	ttc	ggc	att	cag	tcc	atg	act	gta	cga	1098	
Asp	Val	Asp	Gly	Asp	Arg	Asp	Phe	Gly	Ile	Gln	Ser	Met	Thr	Val	Arg		
				285				290						295			
tta	ggc	caa	cag	aga	gtg	cat	agg	ctc	tgc	att	aat	att	ctc	atg	aca	1146	
Leu	Gly	Gln	Gln	Arg	Val	His	Arg	Leu	Cys	Ile	Asn	Ile	Leu	Met	Thr		
			300					305					310				
gca	tac	gca	gcc	gca	att	ttg	gta	ggc	gcg	tca	tct	acg	aac	ctg	tat	1194	
Ala	Tyr	Ala	Ala	Ala	Ile	Leu	Val	Gly	Ala	Ser	Ser	Thr	Asn	Leu	Tyr		
		315					320					325					
cag	aag	att	gtc	att	gtg	tct	ggc	cat	ggc	ttg	ctt	gcc	tcc	aca	ctc	1242	
Gln	Lys	Ile	Val	Ile	Val	Ser	Gly	His	Gly	Leu	Leu	Ala	Ser	Thr	Leu		
	330					335					340						

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tgg caa aga gca caa caa ttt gac att gag aat aag gat tgt atc aca 1290
 Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn Lys Asp Cys Ile Thr
 345 350 355 360

caa ttt tat atg ttc att tgg aag tta ttc tac gcc gag tat ttt ctt 1338
 Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu
 365 370 375

ata cca ttt gtg tag taaagaatca tgcgaagaac aacacccctg ctatagacat 1393
 Ile Pro Phe Val *
 380

gtgaagggttt attgctaattg ttactctacc ccctgctata gacatgtgaa ggtttatttgc 1453
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 <212> PRT
 <213> Zea mays

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

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cat tcc tca gtc ccc aaa gtg agc tgc tgg gca gct gct cat cac caa	502
His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His His Gln	
35 40 45	
cac aat tct aac ccc cag cag ttt cag gcg att ggc ata cga atc gca	550
His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg Ile Ala	
50 55 60 65	
aag acg ctg cat gcc ttt tat cag ttc tgc cga cca cac aca ata ttt	598
Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr Ile Phe	
70 75 80	
gga acc ata ata ggc att act tcg gtg tct ctc ctg cca gtg aag agc	646
Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Val Lys Ser	
85 90 95	
ctg gac gat ttt acg ttg ata gct ata tgg gga ttt ctc gag gct ttg	694
Leu Asp Asp Phe Thr Leu Ile Ala Ile Trp Gly Phe Leu Glu Ala Leu	
100 105 110	
gcc gcc gca tta tgt atg aac gtt tat gta gta ggg ctg aac cag cta	742
Ala Ala Ala Leu Cys Met Asn Val Tyr Val Val Gly Leu Asn Gln Leu	
115 120 125	
ttt gac att gag att gac aag gtc aat aag cca acc ctc cca tta gcg	790
Phe Asp Ile Glu Ile Asp Lys Val Asn Lys Pro Thr Leu Pro Leu Ala	
130 135 140 145	
tcc gga gag ttt tca gtg cca act gca gta ttg tta gta gtg gca ttc	838
Ser Gly Glu Phe Ser Val Pro Thr Ala Val Leu Leu Val Val Ala Phe	
150 155 160	
ttg gtc atg agc att agc atc gga ata aga tca aag tgt gcg cca ttg	886
Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Cys Ala Pro Leu	
165 170 175	
atg tgt gct ttg ctt gtt agc ttc ctt ctt gga agc gca tac tcc att	934
Met Cys Ala Leu Leu Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile	
180 185 190	
gac gtt cca tta ctc cga tgg aag cga cat gct ttt cta gct gca ttc	982
Asp Val Pro Leu Leu Arg Trp Lys Arg His Ala Phe Leu Ala Ala Phe	
195 200 205	
tgc ata atc ttt gtg agg gct gta gtg gtc cgg tta gct ttc ttt gca	1030
Cys Ile Ile Phe Val Arg Ala Val Val Val Arg Leu Ala Phe Phe Ala	
210 215 220 225	
cac atg cag caa cat gtt ctg aag agg ccc ttg gca cct aca agg tcg	1078
His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro Thr Arg Ser	
230 235 240	
gtg gtc ttt gca aca tgt ttc atg tgt tgc ttc gct gca gta ata gcg	1126
Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala Val Ile Ala	
245 250 255	

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cta ttc aag gat att cct gat gtc gat gga gat aga gat ttc ggc att 1174
 Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile
 260 265 270

cag tcc atg act gta cga tta ggc caa cag ann gag ctc tgc att aat 1222
 Gln Ser Met Thr Val Arg Leu Gly Gln Gln Xaa Glu Leu Cys Ile Asn
 275 280 285

att ctc atg aca gca tac gca gtc aca att ttg gta gga gcg ttg tct 1270
 Ile Leu Met Thr Ala Tyr Ala Val Thr Ile Leu Val Gly Ala Leu Ser
 290 295 300 305

acg aac ctg tat cag aag att gtc att gtg tct ggt cat ggc ttg ctt 1318
 Thr Asn Leu Tyr Gln Lys Ile Val Ile Val Ser Gly His Gly Leu Leu
 310 315 320

gcc tcc aca ctc tgg caa aga gca caa caa ttt gac att gag aat aag 1366
 Ala Ser Thr Leu Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn Lys
 325 330 335

gat tgt atc aca caa ttt tat atg ttc att tgg aag tta ttc tat gcc 1414
 Asp Cys Ile Thr Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala
 340 345 350

gag tat ttt ctt ata cca ttt gtg tag taaagaatca tgcgaagaac 1461
 Glu Tyr Phe Leu Ile Pro Phe Val *
 355 360

atcacccttg ctatagacat gtgaagggttc attgctaattg ttactctacc gaatgggtctg 1521
 aatgtctatg cgtcatttgt atgtaatatg actttgttgt atcagggttaa caactggagc 1581
 aaatgtacca tgtatattaa gcattaattt agctgtgtca tttgtaccat gtatatattg 1641
 actatgtatg agatattgtc tcttattagt actagatgtg atgtgtctta ttatgactat 1701
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 20 25 30

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Arg His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His His
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Gln His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg Ile
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Ala Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr Ile
65                      70                      75                      80
Phe Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Val Lys
      85                      90                      95
Ser Leu Asp Asp Phe Thr Leu Ile Ala Ile Trp Gly Phe Leu Glu Ala
      100                     105                     110
Leu Ala Ala Ala Leu Cys Met Asn Val Tyr Val Val Gly Leu Asn Gln
      115                     120                     125
Leu Phe Asp Ile Glu Ile Asp Lys Val Asn Lys Pro Thr Leu Pro Leu
      130                     135                     140
Ala Ser Gly Glu Phe Ser Val Pro Thr Ala Val Leu Leu Val Val Ala
145                     150                     155                     160
Phe Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Cys Ala Pro
      165                     170                     175
Leu Met Cys Ala Leu Leu Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser
      180                     185                     190
Ile Asp Val Pro Leu Leu Arg Trp Lys Arg His Ala Phe Leu Ala Ala
      195                     200                     205
Phe Cys Ile Ile Phe Val Arg Ala Val Val Val Arg Leu Ala Phe Phe
      210                     215                     220
Ala His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro Thr Arg
225                     230                     235                     240
Ser Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala Val Ile
      245                     250                     255
Ala Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly
      260                     265                     270
Ile Gln Ser Met Thr Val Arg Leu Gly Gln Gln Xaa Glu Leu Cys Ile
      275                     280                     285
Asn Ile Leu Met Thr Ala Tyr Ala Val Thr Ile Leu Val Gly Ala Leu
      290                     295                     300
Ser Thr Asn Leu Tyr Gln Lys Ile Val Ile Val Ser Gly His Gly Leu
305                     310                     315                     320
Leu Ala Ser Thr Leu Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn
      325                     330                     335
Lys Asp Cys Ile Thr Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr
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Ala Glu Tyr Phe Leu Ile Pro Phe Val
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<211> 702

<212> DNA

<213> Hordeum vulgare

<220>

<221> CDS

<222> (1)...(702)

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1				5					10					15		
aac	aag	cca	ggt	ctt	cca	ttg	gca	tct	ggg	gaa	ttt	tca	gta	gca	act	96
Asn	Lys	Pro	Gly	Leu	Pro	Leu	Ala	Ser	Gly	Glu	Phe	Ser	Val	Ala	Thr	
			20					25					30			
gga	ggt	ttc	tta	gta	ctc	gca	ttc	ctg	atc	atg	agc	ttt	agc	ata	gga	144
Gly	Val	Phe	Leu	Val	Leu	Ala	Phe	Leu	Ile	Met	Ser	Phe	Ser	Ile	Gly	
		35					40					45				
ata	cgt	tcc	gga	tcg	gcg	cca	ctg	atg	tgt	gct	tta	att	gtc	agc	ttc	192
Ile	Arg	Ser	Gly	Ser	Ala	Pro	Leu	Met	Cys	Ala	Leu	Ile	Val	Ser	Phe	
	50					55					60					
ctt	ctt	gga	agt	gcg	tac	tcc	att	gag	gct	ccg	ttc	ctc	cgg	tgg	aaa	240
Leu	Leu	Gly	Ser	Ala	Tyr	Ser	Ile	Glu	Ala	Pro	Phe	Leu	Arg	Trp	Lys	
65					70					75					80	
cgg	cac	gcg	ctc	ctc	gct	gca	tca	tgt	atc	cta	ttt	gtg	agg	gct	atc	288
Arg	His	Ala	Leu	Leu	Ala	Ala	Ser	Cys	Ile	Leu	Phe	Val	Arg	Ala	Ile	
				85				90						95		
ttg	gtc	cag	ttg	gct	ttc	ttt	gca	cat	atg	cag	caa	cat	gtt	ctg	aaa	336
Leu	Val	Gln	Leu	Ala	Phe	Phe	Ala	His	Met	Gln	Gln	His	Val	Leu	Lys	
			100					105					110			
agg	cca	ttg	gca	gca	acc	aaa	tcg	ctg	gtg	ttt	gca	aca	ttg	ttt	atg	384
Arg	Pro	Leu	Ala	Ala	Thr	Lys	Ser	Leu	Val	Phe	Ala	Thr	Leu	Phe	Met	
		115					120					125				
tgt	tgc	ttc	tct	gcc	gtc	ata	gca	cta	ttc	aag	gat	att	cca	gat	gtt	432
Cys	Cys	Phe	Ser	Ala	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro	Asp	Val	
	130					135					140					
gat	gga	gat	cga	gac	ttt	ggt	atc	caa	tcc	ttg	agt	gtg	aga	ttg	ggg	480
Asp	Gly	Asp	Arg	Asp	Phe	Gly	Ile	Gln	Ser	Leu	Ser	Val	Arg	Leu	Gly	
145					150					155					160	
cct	caa	aga	gtg	tat	cag	ctc	tgc	ata	agc	ata	ttg	ttg	aca	gcc	tat	528
Pro	Gln	Arg	Val	Tyr	Gln	Leu	Cys	Ile	Ser	Ile	Leu	Leu	Thr	Ala	Tyr	
				165					170					175		
ggc	gct	gcc	act	cta	gta	gga	gct	tca	tcc	aca	aac	cta	ttt	caa	aag	576
Gly	Ala	Ala	Thr	Leu	Val	Gly	Ala	Ser	Ser	Thr	Asn	Leu	Phe	Gln	Lys	
			180					185					190			
atc	atc	act	gtg	tct	ggt	cat	ggc	ctg	ctt	gct	ttg	aca	ctt	tgg	cag	624
Ile	Ile	Thr	Val	Ser	Gly	His	Gly	Leu	Leu	Ala	Leu	Thr	Leu	Trp	Gln	
		195					200					205				
aga	gcg	cag	cac	ttt	gag	gtt	gaa	aac	caa	gcg	cgt	gtc	aca	tca	ttt	672
Arg	Ala	Gln	His	Phe	Glu	Val	Glu	Asn	Gln	Ala	Arg	Val	Thr	Ser	Phe	
	210						215				220					

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tac atg ttc atc tgg aac ttg ttt tac gcg
 Tyr Met Phe Ile Trp Asn Leu Phe Tyr Ala
 225 230

<210> 12
 <211> 234
 <212> PRT
 <213> Hordeum vulgare

<400> 12
 Tyr Leu Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile Asp Lys Ile
 1 5 10 15
 Asn Lys Pro Gly Leu Pro Leu Ala Ser Gly Glu Phe Ser Val Ala Thr
 20 25 30
 Gly Val Phe Leu Val Leu Ala Phe Leu Ile Met Ser Phe Ser Ile Gly
 35 40 45
 Ile Arg Ser Gly Ser Ala Pro Leu Met Cys Ala Leu Ile Val Ser Phe
 50 55 60
 Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro Phe Leu Arg Trp Lys
 65 70 75 80
 Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu Phe Val Arg Ala Ile
 85 90 95
 Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val Leu Lys
 100 105 110
 Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe Ala Thr Leu Phe Met
 115 120 125
 Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Val
 130 135 140
 Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu Ser Val Arg Leu Gly
 145 150 155 160
 Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile Leu Leu Thr Ala Tyr
 165 170 175
 Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr Asn Leu Phe Gln Lys
 180 185 190
 Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala Leu Thr Leu Trp Gln
 195 200 205
 Arg Ala Gln His Phe Glu Val Glu Asn Gln Ala Arg Val Thr Ser Phe
 210 215 220
 Tyr Met Phe Ile Trp Asn Leu Phe Tyr Ala
 225 230

<210> 13
 <211> 393
 <212> PRT
 <213> Arabidopsis thaliana

<400> 13
 Met Glu Ser Leu Leu Ser Ser Ser Ser Leu Val Ser Ala Ala Gly Gly
 1 5 10 15
 Phe Cys Trp Lys Lys Gln Asn Leu Lys Leu His Ser Leu Ser Glu Ile
 20 25 30
 Arg Val Leu Arg Cys Asp Ser Ser Lys Val Val Ala Lys Pro Lys Phe
 35 40 45

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Arg	Asn	Asn	Leu	Val	Arg	Pro	Asp	Gly	Gln	Gly	Ser	Ser	Leu	Leu	Leu
50						55					60				
Tyr	Pro	Lys	His	Lys	Ser	Arg	Phe	Arg	Val	Asn	Ala	Thr	Ala	Gly	Gln
65					70					75					80
Pro	Glu	Ala	Phe	Asp	Ser	Asn	Ser	Lys	Gln	Lys	Ser	Phe	Arg	Asp	Ser
				85					90					95	
Leu	Asp	Ala	Phe	Tyr	Arg	Phe	Ser	Arg	Pro	His	Thr	Val	Ile	Gly	Thr
			100					105					110		
Val	Leu	Ser	Ile	Leu	Ser	Val	Ser	Phe	Leu	Ala	Val	Glu	Lys	Val	Ser
		115				120					125				
Asp	Ile	Ser	Pro	Leu	Leu	Phe	Thr	Gly	Ile	Leu	Glu	Ala	Val	Val	Ala
	130					135					140				
Ala	Leu	Met	Met	Asn	Ile	Tyr	Ile	Val	Gly	Leu	Asn	Gln	Leu	Ser	Asp
145					150					155					160
Val	Glu	Ile	Asp	Lys	Val	Asn	Lys	Pro	Tyr	Leu	Pro	Leu	Ala	Ser	Gly
			165					170						175	
Glu	Tyr	Ser	Val	Asn	Thr	Gly	Ile	Ala	Ile	Val	Ala	Ser	Phe	Ser	Ile
			180					185					190		
Met	Ser	Phe	Trp	Leu	Gly	Trp	Ile	Val	Gly	Ser	Trp	Pro	Leu	Phe	Trp
		195				200						205			
Ala	Leu	Phe	Val	Ser	Phe	Met	Leu	Gly	Thr	Ala	Tyr	Ser	Ile	Asn	Leu
	210					215					220				
Pro	Leu	Leu	Arg	Trp	Lys	Arg	Phe	Ala	Leu	Val	Ala	Ala	Met	Cys	Ile
225					230					235					240
Leu	Ala	Val	Arg	Ala	Ile	Ile	Val	Gln	Ile	Ala	Phe	Tyr	Leu	His	Ile
				245				250						255	
Gln	Thr	His	Val	Phe	Gly	Arg	Pro	Ile	Leu	Phe	Thr	Arg	Pro	Leu	Ile
			260					265					270		
Phe	Ala	Thr	Ala	Phe	Met	Ser	Phe	Phe	Ser	Val	Val	Ile	Ala	Leu	Phe
		275					280					285			
Lys	Asp	Ile	Pro	Asp	Ile	Glu	Gly	Asp	Lys	Ile	Phe	Gly	Ile	Arg	Ser
	290					295				300					
Phe	Ser	Val	Thr	Leu	Gly	Gln	Lys	Arg	Val	Phe	Trp	Thr	Cys	Val	Thr
305					310					315					320
Leu	Leu	Gln	Met	Ala	Tyr	Ala	Val	Ala	Ile	Leu	Val	Gly	Ala	Thr	Ser
			325					330						335	
Pro	Phe	Ile	Trp	Ser	Lys	Val	Ile	Ser	Val	Val	Gly	His	Val	Ile	Leu
			340					345				350			
Ala	Thr	Thr	Leu	Trp	Ala	Arg	Ala	Lys	Ser	Val	Asp	Leu	Ser	Ser	Lys
		355					360				365				
Thr	Glu	Ile	Thr	Ser	Cys	Tyr	Met	Phe	Ile	Trp	Lys	Leu	Phe	Tyr	Ala
	370					375					380				
Glu	Tyr	Leu	Leu	Leu	Pro	Phe	Leu	Lys							
385					390										

<210> 14

<211> 395

<212> PRT

<213> Glycine max

<400> 14

Met	Asp	Ser	Met	Leu	Leu	Arg	Ser	Phe	Pro	Asn	Ile	Asn	Asn	Ala	Ser
1				5					10					15	
Ser	Leu	Ala	Thr	Thr	Gly	Ser	Tyr	Leu	Pro	Asn	Ala	Ser	Trp	His	Asn

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			20					25					30				
Arg	Lys	Ile	Gln	Lys	Glu	Tyr	Asn	Phe	Leu	Arg	Phe	Arg	Trp	Pro	Ser		
		35					40					45					
Leu	Asn	His	His	Tyr	Lys	Ser	Ile	Glu	Gly	Gly	Cys	Thr	Cys	Lys	Lys		
	50					55					60						
Cys	Asn	Ile	Lys	Phe	Val	Val	Lys	Ala	Thr	Ser	Glu	Lys	Ser	Phe	Glu		
65					70					75					80		
Ser	Glu	Pro	Gln	Ala	Phe	Asp	Pro	Lys	Ser	Ile	Leu	Asp	Ser	Val	Lys		
				85					90					95			
Asn	Ser	Leu	Asp	Ala	Phe	Tyr	Arg	Phe	Ser	Arg	Pro	His	Thr	Val	Ile		
			100					105					110				
Gly	Thr	Ala	Leu	Ser	Ile	Ile	Ser	Val	Ser	Leu	Leu	Ala	Val	Glu	Lys		
		115					120					125					
Ile	Ser	Asp	Ile	Ser	Pro	Leu	Phe	Phe	Thr	Gly	Val	Leu	Glu	Ala	Val		
	130					135					140						
Val	Ala	Ala	Leu	Phe	Met	Asn	Ile	Tyr	Ile	Val	Gly	Leu	Asn	Gln	Leu		
145					150					155					160		
Ser	Asp	Val	Glu	Ile	Asp	Lys	Ile	Asn	Lys	Pro	Tyr	Leu	Pro	Leu	Ala		
				165					170					175			
Ser	Gly	Glu	Tyr	Ser	Phe	Glu	Thr	Gly	Val	Thr	Ile	Val	Ala	Ser	Phe		
			180					185					190				
Ser	Ile	Leu	Ser	Phe	Trp	Leu	Gly	Trp	Val	Val	Gly	Ser	Trp	Pro	Leu		
	195						200				205						
Phe	Trp	Ala	Leu	Phe	Val	Ser	Phe	Val	Leu	Gly	Thr	Ala	Tyr	Ser	Ile		
	210					215					220						
Asn	Val	Pro	Leu	Leu	Arg	Trp	Lys	Arg	Phe	Ala	Val	Leu	Ala	Ala	Met		
225					230					235					240		
Cys	Ile	Leu	Ala	Val	Arg	Ala	Val	Ile	Val	Gln	Leu	Ala	Phe	Phe	Leu		
				245					250					255			
His	Ile	Gln	Thr	His	Val	Tyr	Lys	Arg	Pro	Pro	Val	Phe	Ser	Arg	Ser		
		260						265					270				
Leu	Ile	Phe	Ala	Thr	Ala	Phe	Met	Ser	Phe	Phe	Ser	Val	Val	Ile	Ala		
		275					280					285					
Leu	Phe	Lys	Asp	Ile	Pro	Asp	Ile	Glu	Gly	Asp	Lys	Val	Phe	Gly	Ile		
	290					295					300						
Gln	Ser	Phe	Ser	Val	Arg	Leu	Gly	Gln	Lys	Pro	Val	Phe	Trp	Thr	Cys		
305					310					315					320		
Val	Ile	Leu	Leu	Glu	Ile	Ala	Tyr	Gly	Val	Ala	Leu	Leu	Val	Gly	Ala		
				325					330					335			
Ala	Ser	Pro	Cys	Leu	Trp	Ser	Lys	Ile	Val	Thr	Gly	Leu	Gly	His	Ala		
			340					345					350				
Val	Leu	Ala	Ser	Ile	Leu	Trp	Phe	His	Ala	Lys	Ser	Val	Asp	Leu	Lys		
		355					360					365					
Ser	Lys	Ala	Ser	Ile	Thr	Ser	Phe	Tyr	Met	Phe	Ile	Trp	Lys	Leu	Phe		
	370					375					380						
Tyr	Ala	Glu	Tyr	Leu	Leu	Ile	Pro	Phe	Val	Arg							
385					390					395							

<210> 15

<211> 404

<212> PRT

<213> Oryza sativa

<400> 15

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Met	Asp	Ser	Leu	Arg	Leu	Arg	Pro	Ser	Leu	Leu	Ala	Ala	Arg	Ala	Pro
1				5					10					15	
Gly	Ala	Ala	Ser	Leu	Pro	Pro	Leu	Arg	Arg	Asp	His	Phe	Leu	Pro	Pro
			20					25					30		
Leu	Cys	Ser	Ile	His	Arg	Asn	Gly	Lys	Arg	Pro	Val	Ser	Leu	Ser	Ser
		35					40					45			
Gln	Arg	Thr	Gln	Gly	Pro	Ser	Phe	Asp	Gln	Cys	Gln	Lys	Phe	Phe	Gly
	50					55					60				
Trp	Lys	Ser	Ser	His	His	Arg	Ile	Pro	His	Arg	Pro	Thr	Ser	Ser	Ser
65					70					75					80
Ala	Asp	Ala	Ser	Gly	Gln	Pro	Leu	Gln	Ser	Ser	Ala	Glu	Ala	His	Asp
				85					90					95	
Ser	Ser	Ser	Ile	Trp	Lys	Pro	Ile	Ser	Ser	Ser	Pro	Asp	Ala	Phe	Tyr
			100					105					110		
Arg	Phe	Ser	Arg	Pro	His	Thr	Val	Ile	Gly	Thr	Ala	Leu	Ser	Ile	Val
		115					120					125			
Ser	Val	Ser	Leu	Leu	Ala	Val	Glu	Asn	Leu	Ser	Asp	Val	Ser	Pro	Leu
	130					135					140				
Phe	Leu	Thr	Gly	Leu	Leu	Glu	Ala	Val	Val	Ala	Ala	Leu	Phe	Met	Asn
145					150					155					160
Ile	Tyr	Ile	Val	Gly	Leu	Asn	Gln	Leu	Phe	Asp	Ile	Glu	Ile	Asp	Lys
			165						170					175	
Val	Asn	Lys	Pro	Thr	Leu	Pro	Leu	Ala	Ser	Gly	Glu	Tyr	Ser	Pro	Ala
			180					185					190		
Thr	Gly	Val	Ala	Leu	Val	Ser	Ala	Phe	Ala	Ala	Met	Ser	Phe	Gly	Leu
		195					200					205			
Gly	Trp	Ala	Val	Gly	Ser	Gln	Pro	Leu	Phe	Leu	Ala	Leu	Phe	Ile	Ser
	210					215					220				
Phe	Ile	Leu	Gly	Thr	Ala	Tyr	Ser	Ile	Asn	Leu	Pro	Phe	Leu	Arg	Trp
225					230					235					240
Lys	Arg	Ser	Ala	Val	Val	Ala	Ala	Leu	Cys	Ile	Leu	Ala	Val	Arg	Ala
			245						250					255	
Val	Ile	Val	Gln	Leu	Ala	Phe	Phe	Leu	His	Ile	Gln	Thr	Phe	Val	Phe
			260					265					270		
Arg	Arg	Pro	Ala	Val	Phe	Thr	Arg	Pro	Leu	Ile	Phe	Ala	Thr	Ala	Phe
		275					280					285			
Met	Thr	Phe	Phe	Ser	Val	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro	Asp
	290					295					300				
Ile	Glu	Gly	Asp	Arg	Ile	Phe	Gly	Ile	Lys	Ser	Phe	Ser	Val	Arg	Leu
305					310					315					320
Gly	Gln	Lys	Lys	Val	Phe	Trp	Ile	Cys	Val	Gly	Leu	Leu	Glu	Met	Ala
			325						330					335	
Tyr	Cys	Val	Ala	Ile	Leu	Met	Gly	Ala	Thr	Ser	Ala	Cys	Leu	Trp	Ser
		340						345					350		
Lys	Tyr	Ala	Thr	Val	Val	Gly	His	Ala	Ile	Leu	Ala	Ala	Ile	Leu	Trp
		355					360					365			
Asn	Arg	Ser	Arg	Ser	Ile	Asp	Leu	Thr	Ser	Lys	Thr	Ala	Ile	Thr	Ser
	370					375						380			
Phe	Tyr	Met	Phe	Ile	Trp	Lys	Leu	Phe	Tyr	Ala	Glu	Tyr	Leu	Leu	Ile
385					390					395					400
Pro	Leu	Val	Arg												

<210> 16

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<211> 399

<212> PRT

<213> Zea mays

<400> 16

Met	Asp	Ala	Leu	Arg	Leu	Arg	Pro	Ser	Leu	Leu	Pro	Val	Arg	Pro	Gly
1				5					10					15	
Ala	Ala	Arg	Pro	Arg	Asp	His	Phe	Leu	Pro	Pro	Cys	Cys	Ser	Ile	Gln
			20					25					30		
Arg	Asn	Gly	Glu	Gly	Arg	Ile	Cys	Phe	Ser	Ser	Gln	Arg	Thr	Gln	Gly
		35					40					45			
Pro	Thr	Leu	His	His	His	Gln	Lys	Phe	Phe	Glu	Trp	Lys	Ser	Ser	Tyr
	50					55					60				
Cys	Arg	Ile	Ser	His	Arg	Ser	Leu	Asn	Thr	Ser	Val	Asn	Ala	Ser	Gly
65					70					75					80
Gln	Gln	Leu	Gln	Ser	Glu	Pro	Glu	Thr	His	Asp	Ser	Thr	Thr	Ile	Trp
				85					90					95	
Arg	Ala	Ile	Ser	Ser	Ser	Leu	Asp	Ala	Phe	Tyr	Arg	Phe	Ser	Arg	Pro
			100					105					110		
His	Thr	Val	Ile	Gly	Thr	Ala	Leu	Ser	Ile	Val	Ser	Val	Ser	Leu	Leu
		115					120					125			
Ala	Val	Gln	Ser	Leu	Ser	Asp	Ile	Ser	Pro	Leu	Phe	Leu	Thr	Gly	Leu
	130					135					140				
Leu	Glu	Ala	Val	Val	Ala	Ala	Leu	Phe	Met	Asn	Ile	Tyr	Ile	Val	Gly
145					150					155					160
Leu	Asn	Gln	Leu	Phe	Asp	Ile	Glu	Ile	Asp	Lys	Val	Asn	Lys	Pro	Thr
				165					170					175	
Leu	Pro	Leu	Ala	Ser	Gly	Glu	Tyr	Thr	Leu	Ala	Thr	Gly	Val	Ala	Ile
			180					185					190		
Val	Ser	Val	Phe	Ala	Ala	Met	Ser	Phe	Gly	Leu	Gly	Trp	Ala	Val	Gly
		195					200					205			
Ser	Gln	Pro	Leu	Phe	Trp	Ala	Leu	Phe	Ile	Ser	Phe	Val	Leu	Gly	Thr
	210					215					220				
Ala	Tyr	Ser	Ile	Asn	Leu	Pro	Tyr	Leu	Arg	Trp	Lys	Arg	Phe	Ala	Val
225				230						235					240
Val	Ala	Ala	Leu	Cys	Ile	Leu	Ala	Val	Arg	Ala	Val	Ile	Val	Gln	Leu
				245					250					255	
Ala	Phe	Phe	Leu	His	Ile	Gln	Thr	Phe	Val	Phe	Arg	Arg	Pro	Ala	Val
			260					265					270		
Phe	Ser	Arg	Pro	Leu	Leu	Phe	Ala	Thr	Gly	Phe	Met	Thr	Phe	Phe	Ser
		275					280					285			
Val	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro	Asp	Ile	Glu	Gly	Asp	Arg
	290					295					300				
Ile	Phe	Gly	Ile	Arg	Ser	Phe	Ser	Val	Arg	Leu	Gly	Gln	Lys	Lys	Val
305				310						315					320
Phe	Trp	Ile	Cys	Val	Gly	Leu	Leu	Glu	Met	Ala	Tyr	Ser	Val	Ala	Ile
			325					330						335	
Leu	Met	Gly	Ala	Thr	Ser	Ser	Cys	Leu	Trp	Ser	Lys	Thr	Ala	Thr	Ile
		340						345					350		
Ala	Gly	His	Ser	Ile	Leu	Ala	Ala	Ile	Leu	Trp	Ser	Cys	Ala	Arg	Ser
		355					360					365			
Val	Asp	Leu	Thr	Ser	Lys	Ala	Ala	Ile	Thr	Ser	Phe	Tyr	Met	Phe	Ile
	370					375					380				
Trp	Lys	Leu	Phe	Tyr	Ala	Glu	Tyr	Leu	Leu	Ile	Pro	Leu	Val	Arg	
385					390					395					

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<210> 17
 <211> 308
 <212> PRT
 <213> Synechocystis PCC6803

<400> 17
 Met Ala Thr Ile Gln Ala Phe Trp Arg Phe Ser Arg Pro His Thr Ile
 1 5 10 15
 Ile Gly Thr Thr Leu Ser Val Trp Ala Val Tyr Leu Leu Thr Ile Leu
 20 25 30
 Gly Asp Gly Asn Ser Val Asn Ser Pro Ala Ser Leu Asp Leu Val Phe
 35 40 45
 Gly Ala Trp Leu Ala Cys Leu Leu Gly Asn Val Tyr Ile Val Gly Leu
 50 55 60
 Asn Gln Leu Trp Asp Val Asp Ile Asp Arg Ile Asn Lys Pro Asn Leu
 65 70 75 80
 Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val
 85 90 95
 Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu
 100 105 110
 Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr
 115 120 125
 Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu
 130 135 140
 Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu
 145 150 155 160
 Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile
 165 170 175
 Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile
 180 185 190
 Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln
 195 200 205
 Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu
 210 215 220
 Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp
 225 230 235 240
 Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys
 245 250 255
 Leu Leu Ala Leu Leu Trp Trp Arg Ser Arg Asp Val His Leu Glu Ser
 260 265 270
 Lys Thr Glu Ile Ala Ser Phe Tyr Gln Phe Ile Trp Lys Leu Phe Phe
 275 280 285
 Leu Glu Tyr Leu Leu Tyr Pro Leu Ala Leu Trp Leu Pro Asn Phe Ser
 290 295 300
 Asn Thr Ile Phe
 305

<210> 18
 <211> 290
 <212> PRT
 <213> Escherichia coli K12

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<400> 18

Met Glu Trp Ser Leu Thr Gln Asn Lys Leu Leu Ala Phe His Arg Leu
 1 5 10 15
 Met Arg Thr Asp Lys Pro Ile Gly Ala Leu Leu Leu Leu Trp Pro Thr
 20 25 30
 Leu Trp Ala Leu Trp Val Ala Thr Pro Gly Val Pro Gln Leu Trp Ile
 35 40 45
 Leu Ala Val Phe Val Ala Gly Val Trp Leu Met Arg Ala Ala Gly Cys
 50 55 60
 Val Val Asn Asp Tyr Ala Asp Arg Lys Phe Asp Gly His Val Lys Arg
 65 70 75 80
 Thr Ala Asn Arg Pro Leu Pro Ser Gly Ala Val Thr Glu Lys Glu Ala
 85 90 95
 Arg Ala Leu Phe Val Val Leu Val Leu Ile Ser Phe Leu Leu Val Leu
 100 105 110
 Thr Leu Asn Thr Met Thr Ile Leu Leu Ser Ile Ala Ala Leu Ala Leu
 115 120 125
 Ala Trp Val Tyr Pro Phe Met Lys Arg Tyr Thr His Leu Pro Gln Val
 130 135 140
 Val Leu Gly Ala Ala Phe Gly Trp Ser Ile Pro Met Ala Phe Ala Ala
 145 150 155 160
 Val Ser Glu Ser Val Pro Leu Ser Cys Trp Leu Met Phe Leu Ala Asn
 165 170 175
 Ile Leu Trp Ala Val Ala Tyr Asp Thr Gln Tyr Ala Met Val Asp Arg
 180 185 190
 Asp Asp Asp Val Lys Ile Gly Ile Lys Ser Thr Ala Ile Leu Phe Gly
 195 200 205
 Gln Tyr Asp Lys Leu Ile Ile Gly Ile Leu Gln Ile Gly Val Leu Ala
 210 215 220
 Leu Met Ala Ile Ile Gly Glu Leu Asn Gly Leu Gly Trp Gly Tyr Tyr
 225 230 235 240
 Trp Ser Ile Leu Val Ala Gly Ala Leu Phe Val Tyr Gln Gln Lys Leu
 245 250 255
 Ile Ala Asn Arg Glu Arg Glu Ala Cys Phe Lys Ala Phe Met Asn Asn
 260 265 270
 Asn Tyr Val Gly Leu Val Leu Phe Leu Gly Leu Ala Met Ser Tyr Trp
 275 280 285
 His Phe
 290

<210> 19

<211> 378

<212> PRT

<213> Avena sativa

<400> 19

Met Ala Thr Ser His Pro Leu Ala Ala Ala Ala Ala Thr Ser Ser Ser
 1 5 10 15
 Ser Ala Thr Phe Arg Pro Pro Leu Arg Phe Leu Ser Ser Pro Pro Ser
 20 25 30
 Ser Leu Thr Leu Asn Arg Arg Arg Ser Phe Pro Val Val Cys Ala Ala
 35 40 45
 Asp Ala Asp Ala Lys Glu Thr Thr Lys Lys Pro Thr Ile Pro Asp Lys
 50 55 60

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Ala Pro Ala Ala Gly Ser Ser Phe Asn Gln Leu Leu Gly Ile Lys Gly
 65 70 75 80
 Ala Lys Gln Glu Thr Asn Ile Trp Lys Ile Arg Leu Gln Leu Thr Lys
 85 90 95
 Pro Val Thr Trp Pro Pro Leu Val Trp Gly Val Leu Cys Gly Ala Ala
 100 105 110
 Ala Ser Gly Asn Phe His Trp Thr Val Glu Asp Val Thr Lys Ser Ile
 115 120 125
 Val Cys Met Leu Met Ser Gly Pro Cys Leu Thr Gly Tyr Thr Gln Thr
 130 135 140
 Ile Asn Asp Trp Tyr Asp Arg Asp Ile Asp Ala Ile Asn Glu Pro Tyr
 145 150 155 160
 Arg Pro Ile Pro Ser Gly Ala Ile Ser Glu Asn Glu Val Ile Thr Gln
 165 170 175
 Ile Trp Val Leu Leu Leu Gly Gly Leu Gly Leu Gly Ala Leu Leu Asp
 180 185 190
 Ile Trp Ala Gly His Asp Phe Pro Ile Ile Phe Tyr Leu Ala Leu Gly
 195 200 205
 Gly Ser Leu Leu Ser Tyr Ile Tyr Ser Ala Pro Pro Leu Lys Leu Lys
 210 215 220
 Gln Asn Gly Trp Ile Gly Asn Phe Ala Leu Gly Ala Ser Tyr Ile Gly
 225 230 235 240
 Leu Pro Trp Trp Ala Gly Gln Ala Leu Phe Gly Thr Leu Thr Pro Asp
 245 250 255
 Ile Val Val Leu Thr Cys Leu Tyr Ser Ile Ala Gly Leu Gly Ile Ala
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 Ile Val Asn Asp Phe Lys Ser Ile Glu Gly Asp Arg Thr Leu Gly Leu
 275 280 285
 Gln Ser Leu Pro Val Ala Phe Gly Met Glu Thr Ala Lys Trp Ile Cys
 290 295 300
 Val Gly Ala Ile Asp Ile Thr Gln Leu Ser Val Ala Ala Tyr Leu Leu
 305 310 315 320
 Ser Thr Gly Lys Leu Tyr Tyr Ala Leu Ala Leu Leu Gly Leu Thr Ile
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 Pro Gln Val Ile Leu Gln Phe Gln Tyr Phe Leu Lys Asp Pro Val Lys
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 acaaagagtg tatcagctct gcataagcat actgttaaca gcctatgggg ctgccactgt 180
 agtaggagct tcatccacac acctacttca aaagatcatc actgtgtctg gccatggcct 240
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 attctctaag ccattaaact ttggctatat atgcctaata attatttgca cttattgtgt 420
 cactttcatg cagctattct atgcagagta tttccttata ccgtttgtgc agtaaaattt 480
 gtacaagggc cagcagatgt gaactatata tacatgtaaa acaaattata ttactgatga 540
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38

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1

5

10

15

Val Ser

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Ala

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23

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising:
 - a) a nucleotide sequence encoding a polypeptide that alters tocol
 - 5 content, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:2, 4, 6, 8 or 10 have at least 70% sequence identity, or
 - b) a nucleotide sequence that is complementary to the nucleotide sequence of a), wherein the complement and the nucleotide sequence contain the same number of nucleotides and are 100% complementary.
- 10 2. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 75% identity.
- 15 3. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 80% identity.
- 20 4. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 85% identity.
- 25 5. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 90% identity.
- 30 6. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 95% identity.
7. An expression cassette comprising the polynucleotide of claim 1, wherein the polynucleotide is operably linked to at least one regulatory sequence.

8. The expression cassette of claim 7, wherein the regulatory sequence is a promoter.

9. A non-human host cell transformed with the expression cassette of claim 7.

5

10. The host cell of claim 9, wherein the host cell is a plant cell.

11. A transformed plant comprising in its genome at least one stably incorporated polynucleotide of claim 1 operably linked to a promoter that drives expression in a plant cell.

10

12. The plant of claim 11, wherein the promoter is operably linked to the nucleotide sequence for the production of antisense transcripts.

13. The plant of claim 11, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred, and developmentally regulated promoters.

15

14. The plant of claim 11, wherein the plant is a monocot.

20

15. The plant of claim 14, wherein the monocot is selected from the group consisting of maize, wheat, rice, sorghum, barley, millet and rye.

16. The plant of claim 11, wherein the plant is a dicot.

25

17. The plant of claim 16, wherein the dicot is selected from the group consisting of soybean, *Brassica sp.*, alfalfa, safflower, sunflower, cotton, peanut and potato.

18. Transformed seed of the plant of claim 11.

30

19. The seed of claim 18 wherein the tocol content is increased.

20. The seed of claim 19 wherein the tocol content is increased to between 70 and 575ppm.

21. The seed of claim 20 wherein the tocol content is increased to between 150
5 and 350 ppm.

22. The seed of claim 21 wherein the tocol content is increased to between 200 and 300 ppm.

10 23. The seed of claim 19 wherein the tocols are tocotrienols.

24. The seed of claim 23 wherein the tocotrienols are increased to between 19 and 461 ppm.

15 25. The seed of claim 24 wherein the tocotrienols are increased to between 100 and 400 ppm.

26. The seed of claim 25 wherein the tocotrienols are increased to between 250 and 350 ppm.

20

27. An isolated nucleotide molecule comprising a nucleotide sequence selected from the group consisting of:

- a) a nucleotide sequence set forth in SEQ ID NO: 1, 3, 5, 7 or 9;
- b) a nucleotide sequence encoding the amino acid sequence set forth
25 in SEQ ID NO: 2, 4, 6, 8 or 10;
- c) a nucleotide sequence having at least 80% sequence identity to the entire coding sequence of at least one of the nucleotide sequences set forth in a), wherein the nucleotide sequence encodes a polypeptide that alters tocol content; and
- 30 d) a nucleotide sequence that is complementary to the nucleotide sequence of a), b), c) or d), wherein the nucleotide sequence is capable of the production of antisense transcripts in a cell.

28. An expression cassette comprising the nucleotide molecule of claim 27, wherein the nucleotide sequence is operably linked to at least one regulatory sequence.

5 29. The expression cassette of claim 28, wherein the regulatory sequence is a promoter.

30. A non-human host cell transformed with the expression cassette of claim 28.

10

31. The host cell of claim 30, wherein the host cell is a plant cell.

15

32. A transformed plant comprising in its genome at least one stably incorporated nucleotide sequence of claim 27 operably linked to a promoter that drives expression in a plant cell.

33. The plant of claim 32, wherein the promoter is operably linked to the nucleotide sequence for the production of antisense transcripts.

20

34. The plant of claim 32, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred, and developmentally regulated promoters.

25

35. The plant of claim 32, wherein the plant is a monocot.

36. The plant of claim 35, wherein the monocot is selected from the group consisting of maize, wheat, rice, sorghum, barley, millet and rye.

37. The plant of claim 32, wherein the plant is a dicot.

30

38. The plant of claim 37, wherein the dicot is selected from the group consisting of soybean, *Brassica sp.*, alfalfa, safflower, sunflower, cotton, peanut and potato.

39. Transformed seed of the plant of claim 32.

40. A method for altering tocotrienol as compared to wild-type, in a plant or part thereof, the method comprising transforming the plant with a nucleotide construct
5 comprising a nucleotide sequence of claim 1.

41. The method of claim 40 wherein the tocotrienol is increased.

42. The method of claim 43, wherein the part is seed or grain.

10

43. The method of claim 43, wherein the nucleotide construct further comprises an operably linked promoter that drives expression in a plant cell.

15

44. The method of claim 43, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred, and developmentally regulated promoters.

20

45. The method of claim 43, wherein the promoter is operably linked to the nucleotide sequence for the production of antisense transcripts.

46. The method of claim 41 wherein antioxidant activity in the plant or plant part is increased.

25

47. A method for altering tocols as compared to wild-type, in a non-human host cell, the method comprising transforming the host cell with a nucleotide construct comprising a nucleotide sequence of claim 1.

48. The method of claim 47 wherein the tocol content is increased.

30

49. The method of claim 48, wherein the host cell is a microbe.

50. The method of claim 49 wherein the microbe is a photosynthetic microbe.

51. The method of claim 48, wherein the nucleotide construct further comprises an operably linked promoter that drives expression in the host cell.

52. The method of claim 48, wherein the host cell comprises a cell from green
5 plant tissue, root tissue, tuber tissue, flower tissue or grain tissue.

53. The method of claim 48, where the host cell has increased antioxidant activity.

10 54. An isolated polypeptide that alters tocol content , wherein the polypeptide amino acid sequence comprises at least four conserved motifs selected from the group consisting of SEQ ID NOs: 56-66.

15 55. An isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide of claim 53.

56. An isolated polynucleotide that is complementary to the polynucleotide of claim 55.

20 57. An isolated polypeptide that alters tocol content, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, 4, 6, 8 or 10 have at least 70% identity.