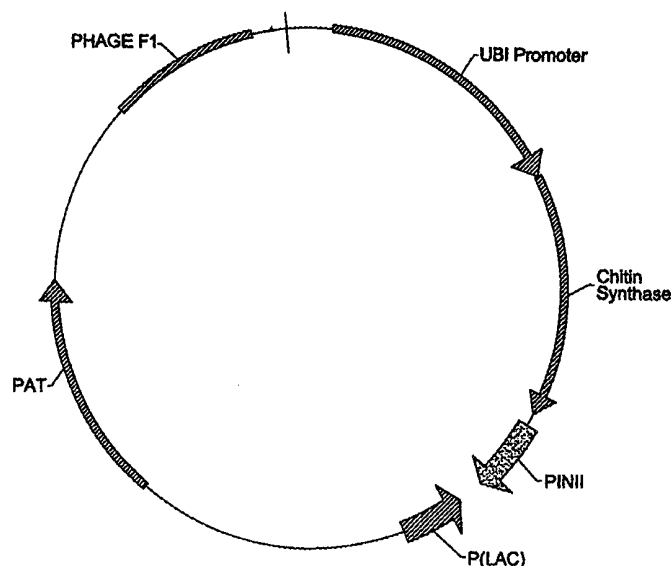




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(54) Title: EXPRESSION OF CHITIN SYNTHASE AND CHITIN DEACETYLASE GENES IN PLANTS TO ALTER THE CELL WALL FOR INDUSTRIAL USES AND IMPROVED DISEASE RESISTANCE

**(57) Abstract**

Compositions and methods for producing chitin and chitosan in plants are provided. The compositions comprise plant expression cassettes for chitin synthase, chitin deacetylase, glutamine:fructose-6-phosphate amidotransferase and/or biologically active fragments and variants thereof. Such expression vectors find use in increasing the tensile strength of plant tissues, providing pathogen-resistant plants, and in producing chitin, chitosan, chitin:cellulose blends and chitosan:cellulose blends for industrial uses. Additionally provided are transformed plant cells, plants and seeds.

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EXPRESSION OF CHITIN SYNTHASE AND
CHITIN DEACETYLASE GENES IN PLANTS TO ALTER THE CELL WALL
FOR INDUSTRIAL USES AND IMPROVED DISEASE RESISTANCE

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FIELD OF THE INVENTION

The field of the invention relates to the genetic manipulation of plants,
particularly to the expression of chitin and chitosan in transgenic plants.

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BACKGROUND OF THE INVENTION

Chitin, a β -1,4-linked polymer of N-acetylglucosamine (GlcNAc), is a
major structural component of fungal cell walls, crustacean shells and insect
15 exoskeleton. Chitin and chitosan, a deacetylated chitin derivative, have numerous
applications in the food, medical, cosmetic, agricultural and textile industries.

Recently, there has been interest in the paper and textile industries in
developing chitin:cellulose and chitosan:cellulose blends. Accordingly, it would
be advantageous to produce chitin and chitosan in plants in order to provide a
20 single source of cellulose, chitin and chitosan. Of particular interest would be the
production of chitin and chitosan in corn. At an annual corn production of 8
billion bushels, and given a harvest index of ~45%, 200 million metric tons of
above ground biomass is discarded for lack of use. The production of chitin and
chitosan in the cell walls may make corn suitable for use in the paper industry, thus
25 improving the value of corn considerably. Undesirable components, such as
lignin, which are already present at a lower level in corn than in tree pulp, could be
further reduced by genetic manipulation, facilitating the use of corn stalks in paper
production.

Chitin is made at the plasma membrane of fungi, crustaceans, insects and
30 other animals by the enzyme chitin synthase. Chitin deacetylase, as the name
indicates, deacetylates GlcNAc, imparting a net positive charge. The resulting
product is chitosan. Because chitosan is positively charged, it is more soluble than
chitosan, and thus favored in a number of industrial applications. Genes encoding
chitin synthase have been cloned from a number of fungal organisms. The gene

for chitin deacetylase has been cloned from *Mucor rouxii*. However, chitin synthase and chitin deacetylase genes have not been expressed in plants.

SUMMARY OF THE INVENTION

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Compositions and methods for producing chitin and chitosan in plants are provided. The compositions comprise plant expression cassettes for chitin synthase, chitin deacetylase, glutamine:fructose-6-phosphate amidotransferase, mutase, N-acetyltransferase and/or biologically active fragments and variants thereof.

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Such expression vectors find use in increasing the tensile strength of plant tissues, providing pathogen-resistant plants, and in producing chitin, chitosan, chitin:cellulose blends and chitosan:cellulose blends for industrial uses. Additionally provided are transformed plant cells, plants and seeds.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth the nucleotide sequence for the maize glutamine:fructose-6-phosphate amidotransferase cDNA. The translational start and stop codons are underlined.

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Figure 2 schematically illustrates the plasmid vector comprising the chitin synthase gene operably linked to the ubiquitin promoter.

DETAILED DESCRIPTION OF THE INVENTION

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The formation of chitin, a linear homopolymer composed of N-acetylglucosamine residues (GlcNAc), is catalyzed by the enzyme chitin synthase. Chitosan is formed through deacetylation of chitin by the enzyme chitin deacetylase. The present invention discloses compositions and methods for the synthesis of chitin and chitosan in plants, plant cells and specific tissues. Expression of chitin and chitosan in plants would provide chitin:cellulose and chitin:chitosan blends suitable for use in the paper and textile industries. The expression of chitin and chitosan in plants would have additional advantages. For

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example, the treatment of plant tissues with chitosan elicits anti-fungal and anti-viral defense responses that protect the plant from the further spread of pathogen. Thus, expression of chitosan in plants may result in plants with increased resistance to fungal and viral pathogens. In addition, the presence of these
5 polymers in food plants may reduce the growth of mycotoxin-producing fungi. Furthermore, expression of chitin and chitosan in plants would increase tensile strength. Improved tensile strength would be of particular importance in plants subject to brittle snap.

The compositions of the invention comprise expression cassettes containing
10 a plant promoter driving a gene encoding chitin synthase, chitin deacetylase, or biologically active fragments or variants thereof, and recombinant plants, plant cells and seeds containing such expression cassettes. Such expression cassettes have use in the transformation of plant cells of interest.

The methods of the invention involve expression of enzymes for chitin and
15 chitosan production in plants. In one embodiment, a method for producing chitin in a plant comprises the steps of: (a) transforming a plant cell with at least one nucleotide sequence encoding a chitin synthase, or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell; (b) screening the plant cells
20 transformed in step (a) for stable expression of chitin synthase to obtain a clonal cell line; (c) regenerating plants from said clonal cell line; and (d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

In another embodiment, a method for producing chitosan in a plant
25 comprises the steps of: (a) transforming a plant cell that stably expresses a chitin synthase with at least one nucleotide sequence encoding a chitin deacetylase or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell; (b) screening the plant cells transformed in step (a) for stable expression of chitin deacetylase to
30 obtain a clonal cell line; (c) regenerating plants from said clonal cell line; and (d) growing said plants from step (c) under conditions appropriate for the synthesis of chitosan.

A key step in production of chitin is providing an adequate supply of the

substrate UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is normally present in the plant cell cytoplasm where it is utilized by the endoplasmic reticulum and Golgi protein glycosyl transferases for protein glycosylation. In those instances where the levels of UDP-GlcNAc are low in the plant cytosol and might not support enough chitin synthesis to have a tangible effect on the properties of plant fiber for paper production. The invention encompasses methods for overexpressing the levels of one or more enzymes of a UDP-GlcNAc biosynthetic pathway in plants, plant cells, and specific plant tissues in order to prime chitin production. The enzyme glutamine:fructose-6-phosphate amidotransferase (GFA) catalyzes the first committed step toward the formation of UDP-GlcNAc by converting fructose-6-phosphate to glucosamine-6-phosphate. Two other enzymes in the UDP-GlcNAc biosynthetic pathway are mutase and N-acetyltransferase. Mutase transfers the phosphate group from position 6 to 1. N-acetyltransferase transfers an acetyl group to the amino group on position 2. Thus, overexpression of GFA, mutase, or N-acetyltransferase, and/or other enzymes in a UDP-GlcNAc biosynthetic pathway in transgenic plants containing chitin synthase may result in increased levels of chitin production.

Accordingly, the present invention is further drawn to compositions and methods for expression of enzymes of a UDP-GlcNAc biosynthetic pathway in plants. Compositions are expression cassettes containing nucleic acids encoding enzymes of a UDP-GlcNAc biosynthetic pathway in plants, preferably to GFA or GFA-like genes. Thus in one embodiment, a method for producing chitin in a plant comprises the steps of: (a) transforming a plant cell expressing chitin synthase or a biologically active fragment or variant thereof, with a nucleotide sequence encoding an enzyme in a chitin biosynthetic pathway, or a biologically active fragment or variant of an enzyme in a chitin biosynthetic pathway; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant; (b) screening the plant cells transformed in step (a) for stable expression of said enzyme to obtain a clonal cell line; (c) regenerating plants from said clonal cell line; and (d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

The nucleotide sequence encoding GFA or another enzyme of a UDP-GlcNAc biosynthetic pathway may be derived from any organism. Nucleic acid

sequences encoding plant GFAs are disclosed in the commonly owned co-pending U.S. patent application 60/097,881, filed 25 August 1998, the contents of which are incorporated herein by reference. The human GFA cDNA sequence is disclosed in European patent application EP 08424149 A2, the contents of which are incorporated by reference.

Preferably, the nucleotide sequence encoding GFA or another enzyme of a UDP-GlcNAc biosynthetic pathway is native to maize or soybean. By native to maize or soybean is meant that the enzyme may be present in a naturally occurring or cultivated species of maize or soybean. The nucleotide sequence for the maize GFA gene is shown in Figure 1.

By overexpression is meant causing an increase of at least 0.2-200 fold in the level of an RNA, enzyme or substrate in a transformed plant, as compared with the non-transformed plant. Preferably, the increase is at least 5-200 fold, more preferably at least 10-200 fold, and most preferably more than 100 fold.

By "enzymes of the UDP-GlcNAc biosynthetic pathway" is meant GFA, mutase and N-acetyltransferase and fragments and variants thereof. It will be recognized that as the UDP-GlcNAc pathway is further elucidated, expression cassettes containing nucleic acid sequences encoding newly discovered UDP-GlcNAc biosynthetic enzymes are included in the methods of the invention.

By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of a native chitin synthase, chitin deacetylase, GFA or other enzyme of the UDP-GlcNAc biosynthetic pathway.

By "variant" protein is intended a protein derived from the native protein by deletion or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Preferably, amino acid substitutions will be conservative, as shown in Table 1. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Conservative substitutions are likely to be phenotypically

silent. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic
5 residues Lys and Arg and replacements among the aromatic residues Phe, Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found in Bowie *et al.*, *Science* 247:1306-1310 (1990).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine
	Tryptophan
	Tyrosine
Hydrophobic	Leucine
	Isoleucine
	Valine
Polar	Glutamine
	Asparagine
Basic	Arginine
	Lysine
	Histidine
Acidic	Aspartic Acid
	Glutamic Acid
Small	Alanine
	Serine
	Threonine
	Methionine
	Glycine

The variant proteins will either comprise at least 15 consecutive amino acids of the native protein, or have at least 50% amino acid sequence identity with the native protein. Preferably, the variant will have at least about 80%, more preferably at least about 90%, and most preferably at least about 95% sequence identity with the native protein. Sequence identity is determined according to the algorithm of Myers and Miller, CABIOS (1989). This algorithm is incorporated into the ALIGN program (version 2.0) which is part of the CGC sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12,

and a gap penalty of 4 are used.

The variant proteins will be functionally equivalent to the native proteins. By "functionally equivalent" is intended that the sequence of the variant defines a chain that produces a protein having substantially the same biological effect as the native protein of interest. Thus, for purposes of the present invention, a functionally equivalent variant of chitin synthase or chitin deacetylase will catalyze the formation of chitin or chitosan, respectively. Functionally equivalent variants that comprise substantial sequence variations are also encompassed by the invention. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the cloned DNA sequence encoding the native protein of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, Eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the chitin synthase, chitin deacetylase, GFA, or other UDP-GlcNAc biosynthetic enzymes of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

The nucleotide sequences encoding the chitin synthase or chitin deacetylase

proteins of interest may be naturally occurring sequences cloned from any organism having chitin synthase or chitin deacetylase genes. The nucleotide sequence encoding chitin synthase is preferably from fungal, crustacean, or insect, and most preferably from yeast, *Neurospora crassa* or *Aspergillus nidulans*. The
5 nucleotide sequence encoding chitin deacetylase is preferably from *Mucor rouxii*. Alternatively, the nucleotide sequences encoding chitin synthase or chitin deacetylase may be synthetically derived sequences. The nucleotide sequences encoding the GlcNAc biosynthetic enzymes may be naturally occurring sequences cloned from any organism, preferably from a plant, most preferably from maize or
10 soybean, or they may be synthetically derived sequences.

The sequences of known chitin synthase, chitin deacetylase, GFA, mutase, and N-acetyltransferase genes can be used to isolate corresponding sequences from a variety of organisms, preferably from fungi, crustaceans, insects or plants. Chitin has been found in members of at least 19 animal phyla. However, the majority of
15 information about genes encoding chitin producing enzymes is from fungi. Multiple genes (up to 4 in *Neurospora crassa*) encoding chitin synthase have been cloned from a number of fungal organisms. See Barney *et al.* (1996) *Molecular Microbiology* 19:443-453; Cooper (1996) *Abstracts Of The General Meeting Of The American Society For Microbiology* 96:93; Din *et al.* (1996) *Molecular &*
20 *General Genetics* 250:214-222; Karuppayil *et al.* (1996) *Journal Of Medical & Veterinary Mycology* 34:117-125; Mellado *et al.* (1995) *Molecular & General Genetics* 246:353-359; Miyazaki *et al.* (1993) *Gene* 134:129-134; Namgung *et al.* (1996) *Fems Microbiology Letters* 145:71-76; Silverman *et al.* (1988) *Proceedings Of The National Academy Of Sciences Of The United States Of America* 85:4735-
25 4739; Valdivieso *et al.* (1991) *Journal Of Cell Biology* 114:101-110; Wang *et al.* (1996) *Abstracts of The General Meeting Of The American Society For Microbiology* 96:95; Weiss *et al.* (1996) *Gene* 168:99-102; Yanai *et al.* (1994) *Bioscience Biotechnology And Biochemistry* 58:1828-1835; Yarden *et al.*, (1991) *Genes & Development* 5:2420-2430; and Au-Young *et al.* (1990) *Molecular*
30 *Microbiology* 4:197-208, the contents of which are incorporated herein by reference.

In yeast, there are at least three different chitin synthases. Chitin synthase I and II are zymogens, requiring protease activation. Chitin synthase III does not

require proteolysis for activation. The structural genes for yeast chitin synthase 1 and 2 (CHS1 and CHS2) have been cloned and sequenced. *See* Bulawa *et al.* (1986) *Cell* 46:213-225; Silverman *et al.* (1989) *Yeast* 5:459-567, the contents of which are incorporated herein by reference. The conserved amino acid regions
5 between chitin synthase I and II are limited to the catalytic and membrane-spanning domains. Three genes are required for the synthesis of chitin by chitin synthase III (CSD2, CSD3 and CSD4). It is believed that CSD2 encodes the catalytic activity, while CSD3 and CSD4 encode activator or transport proteins. *See* Bulawa *et al.* (1992) *Mol. Cell. Biol.* 12:1764-1776, the contents of which are
10 incorporated herein by reference. The CHS1 gene has also been cloned from *Candida albicans*. *See* Au-Young *et al.* (1990) *Mol. Microbiol* 4:197-207; Yarden *et al.* (1991) *Gene Dev.* 5:2420-2430, the contents of which are incorporated herein by reference.

Various forms of chitin deacetylase from a number of organisms with
15 molecular masses of 27, 75 or 150 kDa have been characterized and genes for some of them have been cloned. *See* Alfonso *et al.* (1995) *Current Microbiology* 30:49-54; Christodoulidou *et al.* (1996) *Journal Of Biological Chemistry* 271:31420-31425; Kafetzopoulos *et al.* (1993) *Proceedings Of The National Academy Of Sciences Of The United States Of America* 90:2564-2568; and Tsigos
20 *et al.* (1995) *Journal Of Biological Chemistry* 270:26286-26291, the contents of which are incorporated by reference.

Methods such as PCR, hybridization, and the like can be used to identify sequences having substantial similarity to known chitin synthase, chitin deacetylase, and GFA genes. *See*, for example, Sambrook *et al.* (1989) *Molecular Cloning: A
25 Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) and Innis *et al.* (1990), *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York). In a PCR method, nucleotide primers can be designed based on any 12 to 50 nucleotide stretch, preferably any 12 to 30 nucleotide stretch of contiguous sequence. Pairs of primers can be used in PCR
30 reactions for amplification of DNA sequences from cDNA or genomic DNA extracted from plants of interest. In addition, a single specific primer with a sequence corresponding to one of the nucleotide sequences disclosed herein can be paired with a primer having a sequence of the DNA vector in the cDNA or

genomic libraries for PCR amplification of the sequences 5' or 3' to the nucleotide sequences disclosed herein. Similarly, nested primers may be used instead of a single specific primer. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989)

- 5 *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Ignis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York).

- Thus, PCR may be used to amplify chitin synthase, chitin deacetylase or GFA coding sequences from a desired organism or as a diagnostic assay to
10 determine the presence of these coding sequences in an organism. For example, degenerate primers for completely conserved regions in yeast CHS1, CHS2 and the *Candida albicans* CHS1 polypeptides have been used to amplify CHS gene fragments of about 600 bp from 13 fungal species. See Bowen *et al.* (1992) *Proc. Natl. Acad. Sci.* 89:519-523, the contents of which are incorporated by reference.
15 These primers did not amplify the yeast CSD2 gene.

- In a hybridization method, 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 ³²P, or any other detectable marker. Probes for hybridization can be made by labeling synthetic oligonucleotides based
20 on known chitin synthase, chitin deacetylase and GFA genes. Preparation of probes for hybridization is generally known in the art and is disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York), hereby incorporated by reference. The labeled probes can be used to screen cDNA or genomic libraries made from
25 plants of interest. Methods for construction of such 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).

- In hybridization techniques, all or part of the known coding sequence is
30 used as a probe that selectively hybridizes to other possible chitin synthase, chitin deacetylase or GFA coding sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*i.e.*, genomic or cDNA libraries) from a chosen organism. To achieve specific hybridization under a variety of conditions,

such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, *e.g.*, Innis *et al.*, eds. (1990) *PCR Protocols: A*
5 *Guide to Methods and Applications* (Academic Press, New York)).

The genes encoding chitin synthase, chitin deacetylase, GFA or other UDP-GlcNAc biosynthetic enzymes can be optimized for enhanced expression in plants of interest. See, for example, EPA0359472; WO91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:3324-3328; and Murray *et al.* (1989) *Nucleic Acids*
10 *Res.* 17:477-498. In this manner, the genes can be synthesized utilizing plant-preferred codons. See, for example, Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, the disclosure of which is incorporated herein by reference. In this manner, synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid. Thus, the nucleotide sequences can
15 be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

The expression cassettes of the invention may be used alone or in combination, to transform any plant of interest in order to produce chitin and/or
20 chitosan, increase tensile strength and provide disease resistance. The expression cassettes comprise a promoter region linked to the coding sequence of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable
25 marker genes.

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 promoter is not found in the native plant into which the promoter is introduced. As used herein, a chimeric
30 gene comprises a coding sequence operably linked to a promoter that is heterologous to the coding sequence.

The expression cassettes of the invention may contain either constitutive or tissue-specific promoters. Constitutive promoters would provide a constant supply

of chitin synthase, chitin deacetylase, GFA or other enzyme of the UDP-GlcNAc biosynthetic pathway throughout the plant. Such constitutive promoters include, for example, the core promoter of the Rsyn7 (copending U.S. Patent Application Serial No. 08/661,601), the CaMV 19S and 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 Application Serial No. 08/409,297), the Sep1 promoter, the promoter for the small subunit of ribulose-1,5-bis-phosphate carboxylase, promoters from tumor-inducing plasmids of *Agrobacterium tumefaciens*, such as the nopaline synthase and octopine synthase, the figwort mosaic virus 35S promoter 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; and 5,608,142.

Alternatively, it may be desirable to produce chitin or chitosan in a tissue-specific manner. Thus tissue-specific promoters, particularly stalk-specific and tree trunk-specific promoters may be used. Examples of tissue-specific promoters include stalk-specific, seed-preferred, pericarp-specific, leaf-specific, tree trunk specific and bamboo shoot-specific promoters. Seed-preferred promoters include promoters active during seed development and germination as well as embryo-specific and endosperm-specific promoters (See Thompson *et al.* (1989) *BioEssays* 10:108, the contents of which are incorporated by reference). Such seed-preferred promoters include Cim1 (cytokinin-induced message); cZ19B1 (maize 19KDa zein); gama-zein; glob-1 and celA (cellulose synthase). For dicots, particular promoters include phaseolin, napin, β -conglycinin, soybean lectin, and the like. For monocots, particular promoters include maize 15kD zein, 22kD zein, 27kD zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc. Leaf-specific 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.

- 5 Pericarp-specific promoters are preferred when chitin or chitosan is to be used for fiber production. Expression of chitin synthase, chitin deacetylase and/or enzymes of the UDP-GlcNAc biosynthetic pathway from endosperm-specific and embryo-specific promoters is useful for imparting plants with disease resistance.

The transcriptional cassette will include in the 5'-to-3' direction of
10 transcription, a promoter, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native to the transcriptional initiation region or to the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from
15 the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See 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;
20 Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639. The cassette will include 5' and 3' regulatory sequences operably linked to the sequence of interest. The cassette may additionally contain at least one additional sequence to be co-transformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

25 The expression cassette may further contain a nucleotide sequence encoding a signal sequence in frame with the coding region of the enzyme of interest. Such a signal sequence would target the enzyme to a desired cellular location. For example, in a preferred embodiment, chitin deacetylase is targeted to the apoplast or cell wall. Single chain chitin polymers released into the apoplast by
30 the chitin synthase complex are a better substrate for chitin deacetylase than the crystallized chitin microfibrils. Accordingly, targeting of chitin deacetylase to the apoplast or cell wall in plants expressing chitin synthase should result in enough

chitin deacetylation to impart a net positive charge to the chitosan polymer. The positive charge in the cell wall will make the plant fiber more suitable for use in the paper industry. Many plant signal sequences are known in the art. For example, plant signal sequences, include, but not limited to, plant cell wall targeting signals (Steifel *et al.*, (1990) *Plant Cell* 2:785-793; Dehio *et al.*, (1992) *Plant J* 2:117-128; Zheng *et al.*, (1992) *Plant Cell* 4:1147-1156; de Silva *et al.*, (1993) *Plant J* 3:701-711; Heintzen *et al.*, (1994) *Plant Physiol* 106:905-915; Deutch *et al.*, (1995) *Plant Mol Biol* 27:411-418; Harpster *et al.* (1998) *Plant Physiol* 118:1307-1316; Crombie *et al.*, (1998) *Plant J* 15:27-38;); the signal-peptide encoding DNA/RNA sequences which target proteins to the extracellular matrix of the plant cell (Dratewka-Kos *et al.*, (1989) *J. Biol. Chem.* 264:4896-4900), the *Nicotiana plumbaginifolia* extension gene (DeLoose *et al.* (1991) *Gene* 99:95-100), signal peptides which target proteins to the vacuole like the sweet potato sporamin gene (Matsuka *et al.* (1991) *PNAS* 8:834) and the barley lectin gene (Wikins *et al.* (1990) *Plant Cell* 2:301-313), signal peptides which cause proteins to be secreted such as that of PR1b (Lind *et al.* (1992) *Plant Mol. Biol.* 18:47-53), or the barley alpha amylase (BAA) (Rahmatullah *et al.* (1989) *Plant Mol. Biol.* 12:119), or signal peptides which target proteins to the plastids such as that of rapeseed enoyl-Acp reductase (Verwaert *et al.* (1994) *Plant Mol. Biol.* 26:189-202) hereby incorporated by reference are useful in the invention.

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. Other molecular techniques may be employed to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis techniques such as primer repair, fill-in, etc. may be used.

The sequences of the present invention can be used to transform or transfect any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Plants of particular interest include corn, trees, bamboo, Sudan grass, wheat, barley, rice, sorghum, rye, cotton, soybean, safflower, sunflower, Brassica, alfalfa and the like.

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),
- 5 electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921), 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.* (1995) "Direct DNA Transfer into Intact Plant Cells via
- 10 Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Annual 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
- 15 (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); Klein
- 20 *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooydaas-Van Slogteren and Hooykaas (1984) *Nature (London)* 311:763-764; 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);
- 25 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
- 30 *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

Suitable methods of introducing nucleotide sequences into tree cells and subsequent insertion into the genome include *Agrobacterium*-mediated transformation (U.S. Patent No. 4,886,937; Loopstra *et al.* *Plant Mol. Biol.* (1990)

15:1-9 and microprojectile bombardment (U.S. Patent No. 5,122,466; Robertson *et al. Plant Mol. Biol.* (1992) 19:925-935; Ellis *et al. Plant Mol. Biol.* (1991) 17:19-27).

Plant cells expressing chitin synthase, chitin deacetylase, GFA or other enzymes in the UDP-GlcNAc biosynthetic pathway may be detected by a variety of methods known to those skilled in the art. Such assays include Northern assays for the detection of chitin synthase or chitin deacetylase mRNA (See Sambrook *et al.* (1989) *A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.)); enzymatic assays and immunoassays for chitin synthase, chitin deacetylase or GFA activity and assays for levels of chitin, chitosan or UDP-GlcNAc. Methods for the detection of chitin are disclosed by Wagner (EXS (1994) 69:559-577), the contents of which, and the publications cited thereby, are incorporated herein by reference. Methods for the detection of chitin deacetylase activity, and immunoglobulins specifically reactive with chitin deacetylase are disclosed by PCT application WO 93/07262, the contents of which are incorporated herein by reference.

The modified plant 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 the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

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

EXPERIMENTAL

Example 1: Incorporation of Chitin Synthase DNA

Sequences into Expression Vectors

A full-length cDNA sequence encoding a yeast CHS1 chitin synthase gene is isolated by PCR amplification of a yeast cDNA library. The isolated chitin

synthase cDNA is then cloned into a plasmid vector, such as that shown in Figure 2, in the sense orientation so that they are under the transcriptional control of the ubiquitin promoter. A selectable marker gene may reside on this plasmid or may be introduced as part of a second plasmid. The transformation construct is then
5 available for introduction into maize embryos by bombardment methods as described in Example 2.

Example 2: Transformation and Regeneration of Maize Callus

10 Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a chitin synthase cDNA operably linked to the ubiquitin promoter plus a plasmid containing the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37) that confers resistance to the herbicide Bialophos (see Figure 2). Transformation is performed as follows. All media recipes are in the
15 Appendix.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature
20 embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate. These are cultured on 560 L medium for 4 days prior to bombardment, in the dark. The day of bombardment, the embryos are transferred to 560 Y medium for 4 hours, arranged within the 2.5-cm target zone.

25 Preparation of DNA

A plasmid vector comprising a chitin synthase cDNA operably linked to the ubiquitin promoter is constructed. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl_2 precipitation procedure as follows:

30

100 μ l prepared tungsten particles in water
10 μ l (1 μ g) DNA in TrisEDTA buffer (1 μ g total)
100 μ l 2.5 M CaCl_2
10 μ l 0.1 M spermidine

5

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with
10 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 μ l 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 μ l spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

15

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

20

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialophos, and subcultured every 2 weeks. After approximately 10 weeks of selection,
25 selection-resistant callus clones are sampled for PCR and chitin synthase activity. Clonal cell lines are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred
30 to medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2

weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored for expression of chitin synthase activity or chitin.

APPENDIX

Ingredient	Amount	Unit
D-I H ₂ O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolystate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2,4-D 0.5 mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1 mg/ml #	1.200	ml
Silver Nitrate 2 mg/ml #	1.700	ml

Directions:

@= Add after bringing up to volume

#= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H₂O in sequence

- 5 Adjust to pH 5.8

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

##= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H₂O. Then dissolve 4.629 g of Ammonium Sulfate; 4.000 g of Potassium

- 10 Phosphate Monobasic KH₂PO₄; 1.850 g of Magnesium Sulfate 7-H₂O, MgSO₄, 7H₂O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate

Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium

- 15 Iodide in polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H₂O. Bring up to volume with D-I H₂O.

Total Volume (L) = 1.00

604 A

Ingredient	Amount	Unit
D-I H ₂ O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

- 5 Dissolve ingredients in polished D-I H₂O in sequence

Adjust to pH 5.8

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished

- 10 D-I H₂O. Then dissolve 4.629 g of Ammonium Sulfate; 4.000 g of Potassium Phosphate Monobasic KH₂PO₄; 1.850 g of Magnesium Sulfate 7-H₂O, MgSO₄, 7H₂O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate

- 15 Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium Iodide in polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H₂O. Bring up to volume with D-I H₂O.

- 20 Total Volume (L) = 1.00

605 J

Ingredient	Amount	Unit
D-I H ₂ O	900.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X #####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
Sucrose	20.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1 mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H₂O in sequence

5 Adjust to pH 5.8

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H₂O. Then dissolve 4.629 g of Ammonium Sulfate; 4.000 g of Potassium

10 Phosphate Monobasic KH₂PO₄; 1.850 g of Magnesium Sulfate 7-H₂O, MgSO₄, 7H₂O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate

Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium

15 Iodide in polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into D-I H₂O. Bring up to volume with D-I H₂O.

Total Volume (L) = 1.00

604 S

Ingredient	Amount	Unit
D-I H ₂ O	800.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	1.600	g
N6 Macronutrients 10X Stock ##	60.000	ml
Potassium Nitrate	1.680	g
B5H Minor Salts 1000X ###	0.600	ml
B5H Fe Na EDTA 100X ####	6.000	ml
Eriksson's Vitamin Mix (1000X SIGMA-1511)	0.400	ml
S & H Vitamin Mixture 100X Stock (S3766)	6.000	ml
Thiamine.HCL 0.4mg/ml	0.500	ml
L-Proline	1.980	g
Casein Hydrolysate (acid)	0.300	g
Sucrose	120.000	g
Glucose	0.600	g
2, 4-D 0.5mg/ml	1.600	ml
Gelrite @	2.000	g
Dicamba 1mg/ml #	1.200	ml
Silver Nitrate 2mg/ml #	1.700	ml

Directions:

@ = Add after bringing up to volume

= Add after sterilizing and cooling to temp.

Dissolve ingredients in polished D-I H₂O in sequence

5 Adjust to pH 5.8

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 1.660 g of Calcium Chloride Dihydrate in 950.000 ml of polished D-I H₂O. Then dissolve 4.629 g of Ammonium Sulfate; 4.000 g of Potassium

10 Phosphate Monobasic KH₂PO₄; 1.850 g of Magnesium Sulfate 7-H₂O, MgSO₄, 7H₂O; and 28.300 g of Potassium Nitrate into sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.000 g of Boric Acid; 10.000 g of Manganous Sulfate

Monohydrate; 0.250 g of Sodium Molybdate Dihydrate; and 0.750 g of Potassium

15 Iodide in 950.000 ml of polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O.

= Dissolve 3.700 g of Disodium EDTA Dihydrate and 2.790 g of Ferrous Sulfate 7-Hydrate into 950.000 ml of D-I H₂O. Bring up to volume with D-I H₂O.

Total Volume (L) = 1.00

272 V

Ingredient	Amount	Unit
D-I H ₂ O	950.000	ml
MS Salts (GIBCO 11117-074)	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Sucrose	40.000	g
Bacto-Agar @	6.000	g

Directions:

@ = Add after bringing up to volume

- 5 Dissolve ingredients in polished D-I H₂O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of

- 10 Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month, unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

288 J

Ingredient	Amount	Unit
D-I H ₂ O	950.000	ml
MS Salts	4.300	g
Myo-Inositol	0.100	g
MS Vitamins Stock Solution ##	5.000	ml
Zeatin .5mg/ml	1.000	ml
Sucrose	60.000	g
Gelrite @	3.000	g
Indole Acetic Acid 0.5 mg/ml #	2.000	ml
.1mM Absissic Acid	1.000	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

- 5 @ = Add after bringing up to volume

Dissolve ingredients in polished D-I H₂O in sequence

Adjust to pH 5.6

Bring up to volume with polished D-I H₂O after adjusting pH

Sterilize and cool to 60°C.

- 10 Add 3.5g/L of Gelrite for cell biology.

= Dissolve 0.100 g of Nicotinic Acid; 0.020 g of Thiamine.HCL; 0.100 g of Pyridoxine.HCL; and 0.400 g of Glycine in 875.00 ml of polished D-I H₂O in sequence. Bring up to volume with polished D-I H₂O. Make in 400 ml portions. Thiamine.HCL & Pyridoxine.HCL are in Dark Desiccator. Store for one month,

- 15 unless contamination or precipitation occur, then make fresh stock.

Total Volume (L) = 1.00

560 L

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511	0.400	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	20.000	g
2, 4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

- 5 @ = Add after bringing up to volume
= Add after sterilizing and cooling to temp.
Dissolve ingredients in D-I H₂O in sequence
Adjust to pH 5.8 with KOH
Bring up to volume with D-I H₂O
10 Sterilize and cool to room temp.
Total Volume (L) = 1.00

560 R

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511)	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	30.000	g
2, 4-D 0.5mg/ml	4.000	ml
Gelrite @	3.000	g
Silver Nitrate 2mg/ml #	0.425	ml
Bialaphos 1mg/ml #	3.000	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H₂O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H₂O

Sterilize and cool to room temp.

10 Total Volume (L) = 1.00

560 Y

Ingredient	Amount	Unit
D-I Water, Filtered	950.000	ml
CHU (N6) Basal Salts (SIGMA C-1416)	4.000	g
Eriksson's Vitamin Mix (1000X SIGMA-1511	1.000	ml
Thiamine.HCL 0.4mg/ml	1.250	ml
Sucrose	120.000	g
2,4-D 0.5mg/ml	2.000	ml
L-Proline	2.880	g
Gelrite @	2.000	g
Silver Nitrate 2mg/ml #	4.250	ml

Directions:

@ = Add after bringing up to volume

5 # = Add after sterilizing and cooling to temp.

Dissolve ingredients in D-I H₂O in sequence

Adjust to pH 5.8 with KOH

Bring up to volume with D-I H₂O

Sterilize and cool to room temp.

10 ** Autoclave less time because of increased sucrose**

Total Volume (L) = 1.00

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
5 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.

THAT WHICH IS CLAIMED:

1. An expression cassette comprising a nucleotide sequence encoding a chitin synthase or a biologically active fragment or variant thereof, wherein said
5 nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.
2. The expression cassette of claim 1, wherein said chitin synthase is a fungal chitin synthase, a crustacean chitin synthase or an insect chitin synthase.
10
3. The expression cassette of claim 2, wherein said fungal chitin synthase is selected from the group consisting of yeast, *Mucor rouxii*, *Neurospora crassa* and *Aspergillus nidulans* chitin synthases.
- 15 4. The expression cassette of claim 1, wherein said promoter is a constitutive promoter or a tissue-specific promoter.
5. The expression cassette of claim 4, wherein said promoter is selected from the group of promoters consisting of: a ubiquitin, a seed-preferred,
20 an embryo-specific, an endosperm-specific, a pericarp-specific, a green-tissue specific, a stalk-specific, a tree trunk-specific and a bamboo shoot-specific promoter.
6. An expression cassette comprising a nucleotide sequence encoding a chitin deacetylase or a biologically active fragment or variant thereof; wherein
25 said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.
7. The expression cassette of claim 6, wherein said nucleotide
30 sequence is further operably linked to a second nucleotide sequence encoding an apoplast or a cell wall targeting signal.
8. The expression cassette of claim 6, wherein said chitin deacetylase

is a fungal chitin deacetylase.

9. The expression cassette of claim 8, wherein said fungal chitin deacetylase is selected from the group consisting of yeast, *Mucor rouxii*,
5 *Neurospora crassa* and *Aspergillus nidulans* chitin deacetylases.

10. The expression cassette of claim 6, wherein said promoter is a constitutive promoter or a tissue-specific promoter.

- 10 11. The expression cassette of claim 10, wherein said tissue specific promoter is selected from the group of promoters consisting of a ubiquitin, a seed-preferred, an embryo-specific, an endosperm-specific, a pericarp-specific, a green-tissue specific, a stalk-specific, a tree trunk-specific and a bamboo shoot-specific promoter.

15

12. An expression cassette comprising a nucleotide sequence encoding a glutamine:fructose-6-phosphate amidotransferase or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a plant promoter selected from the group of promoters consisting of: a ubiquitin, a
20 seed-preferred, an embryo-specific, an endosperm-specific, a pericarp-specific, a green-tissue specific, a stalk-specific, a tree trunk-specific and a bamboo shoot-specific promoter.

13. A method for producing chitin in a plant, comprising the steps of:
25 (a) transforming a plant cell with at least one nucleotide sequence encoding a chitin synthase, or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell;
(b) screening the plant cells transformed in step (a) for stable
30 expression of chitin synthase to obtain a clonal cell line;
(c) regenerating plants from said clonal cell line; and
(d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

14. The method of claim 13, wherein said promoter is a constitutive promoter or a tissue-specific promoter.

5 15. The method of claim 14, wherein said promoter is selected from the group consisting of: a ubiquitin promoter, a seed-preferred promoter, an embryo-specific promoter, an endosperm-specific promoter, a pericarp-specific promoter, a green tissue-specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

10

16. The method of claim 13, wherein said chitin synthase is a fungal chitin synthase, a crustacean chitin synthase or an insect chitin synthase.

17. The method of claim 16, wherein said fungal chitin synthase is
15 selected from consisting of yeast, *Mucor rouxii*, *Neurospora crassa* and *Aspergillus nidulans* chitin synthases.

18. The method of claim 13, wherein said plant is a monocot.

20 19. The method of claim 18, wherein said monocot is maize, wheat, rice, barley, sorghum, rye, bamboo or Sudan grass.

20. The method of claim 13, wherein said plant is a dicot.

25 21. The method of claim 20, wherein said dicot is soybean, *Brassica* sunflower, alfalfa or safflower.

22. The method of claim 13, wherein said plant is a tree.

30 23. A method for producing chitosan in a plant, comprising, the steps of:

(a) transforming a plant cell that stably expresses a chitin synthase with at least one nucleotide sequence encoding a chitin deacetylase or a

biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell;

(b) screening the plant cells transformed in step (a) for stable expression of chitin deacetylase to obtain a clonal cell line;

5 (c) regenerating plants from said clonal cell line; and

(d) growing said plants from step (c) under conditions appropriate for the synthesis of chitosan.

24. The method of claim 23, wherein said nucleotide sequence is further operably linked to a second nucleotide sequence encoding an apoplast targeting signal.

25. The method of claim 23, wherein said promoter is a constitutive promoter or a tissue-specific promoter.

15

26. The method of claim 25, wherein said promoter is selected from the group consisting of: a ubiquitin promoter, a seed-preferred promoter, an embryo-specific promoter, an endosperm-specific promoter, a pericarp-specific promoter, a green tissue-specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

20

27. The method of claim 23, wherein said chitin synthase is a fungal chitin synthase, a crustacean chitin synthase or is an insect chitin synthase.

25 28. The method of claim 27, wherein said fungal chitin synthase is selected from the group consisting of yeast, *Mucor rouxii*, *Neurospora crassa* and *Aspergillus nidulans* chitin synthases.

29. The method of claim 23, wherein said plant is a monocot.

30

30. The method of claim 29, wherein said monocot is maize, wheat, rice, barley, sorghum, rye, bamboo or Sudan grass.

31. The method of claim 23, wherein said plant is a dicot.

32. The method of claim 31, wherein said dicot is soybean, *Brassica*, sunflower, alfalfa or safflower.

5

33. The method of claim 23, wherein said plant is a tree.

34. A method for producing chitin in a plant, comprising the steps of:

10 (a) transforming a plant cell expressing chitin synthase or a biologically active fragment or variant thereof, with a nucleotide sequence encoding an enzyme in a chitin biosynthetic pathway, or a biologically active fragment or variant of an enzyme in a chitin biosynthetic pathway; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant;

15 (b) screening the plant cells transformed in step (a) for stable expression of said enzyme to obtain a clonal cell line;

(c) regenerating plants from said clonal cell line; and

(d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

20

35. The method of claim 34, wherein said enzyme is glutamine:fructose-6-phosphate amidotransferase.

36. The method of claim 34, wherein said plant cell is a monocot.

25

37. The method of claim 36, wherein said monocot is maize, wheat, rice, barley, sorghum, rye, bamboo or Sudan grass.

38. The method of claim 34, wherein said plant cell is a dicot.

30

39. The method of claim 38, wherein said dicot is soybean, *Brassica*, sunflower, alfalfa or safflower.

40. The method of claim 34, wherein said plant is a tree.

41. The method of claim 34, wherein said promoter is a constitutive promoter or a tissue-specific promoter.

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42. The method of claim 41, wherein said promoter is selected from the group consisting of: a ubiquitin promoter, a seed-preferred promoter, an embryo-specific promoter, an endosperm-specific promoter, a pericarp-specific promoter, a green tissue-specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

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43. A transformed plant cell having stably incorporated into its genome at least one nucleotide encoding a chitin synthase or a biologically active fragment or variant thereof, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.

15

44. A transformed plant having stably incorporated into its genome at least one nucleotide sequence encoding a chitin synthase or a biologically active fragment or variant thereof, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.

20

45. The plant of claim 44, wherein said chitin synthase is a fungal chitin synthase, a crustacean chitin synthase or is an insect chitin synthase.

25

46. The plant of claim 45, wherein said fungal chitin synthase is selected from the group consisting of yeast, *Mucor rouxii*, *Neurospora crassa* and *Aspergillus nidulans* chitin synthases.

30

47. The plant of claim 44, wherein said promoter is a constitutive promoter or a tissue-specific promoter.

48. The plant of claim 47, wherein said promoter is selected from the group of promoters consisting of: a ubiquitin promoter, a seed-preferred promoter,

an embryo-specific promoter, an endosperm specific promoter, a pericarp-specific promoter, a green tissue-specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

5 49. The plant of claim 44, wherein said plant is a monocot.

 50. The plant of claim 49, wherein said plant is maize, wheat, rice, barley, sorghum, rye, bamboo or Sudan grass.

10 51. The plant of claim 44, wherein said plant is a dicot.

 52. The plant of claim 51, wherein said dicot is soybean, *Brassica*, sunflower, alfalfa or safflower.

15 53. The plant of claim 44, wherein said plant is a tree.

 54. A transformed plant or plant cell having stably incorporated into its genome:

 at least one of a first nucleotide sequence encoding a chitin synthase
20 or a biologically active fragment or variant thereof; wherein said first nucleotide sequence is operably linked to a first promoter that drives expression in a plant cell; and

 at least one of a second nucleotide sequence encoding a chitin deacetylase, or a biologically active fragment or variant thereof; wherein said
25 second nucleotide sequence is operably linked to a second promoter that drives expression in a plant cell.

 55. The plant of claim 54, wherein said chitin synthase and/or said chitin deacetylase is from a fungus, insect or crustacean.

30

 56. The plant of claim 55, wherein said fungal chitin synthases and/or said chitin deacetylases are selected from the group consisting of yeast, *Mucor rouxii*, *Neurospora crassa* and *Aspergillus nidulans* chitin synthases and chitin

deacetylases.

57. The plant of claim 54, wherein said first promoter and/or second promoter are selected from the group consisting of: a constitutive promoter and a tissue-specific promoter.

58. The plant of claim 57, wherein said first promoter and/or second promoter are selected from the group consisting of: is a ubiquitin promoter, a seed-preferred promoter, an embryo-specific promoter, an endosperm-specific promoter, a pericarp-specific promoter, a green tissue-specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

59. The plant of claim 54, wherein said plant is a monocot.

60. The plant of claim 59, wherein said monocot is maize, wheat, rice, barley, sorghum, rye, bamboo or Sudan grass.

61. The plant of claim 54, wherein said plant is a dicot.

62. The plant of claim 61, wherein said dicot is soybean, *Brassica*, sunflower, alfalfa or safflower.

63. The plant of claim 54, wherein said plant is a tree.

64. The plant or plant cell of claims 54-63, having further incorporated into its genome at least one nucleotide sequence encoding a glutamine:fructose-6-phosphate amidotransferase, or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.

65. The plant or plant cell of claim 64, wherein said glutamine:fructose-6-phosphate amidotransferase is from a fungus or a plant.

66. The plant or plant cell of claim 65, wherein said glutamine:fructose-6-phosphate amidotranferase is selected from the group consisting of: yeast, *Mucor rouxii* *Neurospora crassa*, *Aspergillus nidulans* maize, wheat, rice, barley, sorghum, rye, bamboo, Sudan grass, *Brassica*, sunflower, alfalfa, safflower, and
5 soybean glutamine:fructose-6-phosphate amidotransferases.

67. The plant or plant cell of claim 64, wherein said promoter driving expression of a nucleic acid encoding a glutamine:fructose-6-phosphate amidotransferase, or a biologically active fragment or variant thereof, is a
10 constitutive promoter or a tissue-specific promoter.

68. The plant of claim 67, wherein said promoter is selected from the group consisting of: a ubiquitin promoter, a seed-preferred promoter, an embryo-specific promoter, an endosperm-specific promoter, a pericarp-specific promoter, a
15 green-tissue specific promoter, a stalk-specific promoter, a tree trunk-specific promoter and a bamboo shoot-specific promoter.

69. The seed of the plant of claim 54.

20 70. A method for increasing the tensile strength or pathogen resistance of a plant, comprising the steps of:

(a) transforming a plant cell with at least one nucleotide sequence encoding a chitin synthase, or a biologically active fragment or variant thereof; wherein said nucleotide sequence is operably linked to a promoter that
25 drives expression in a plant cell;

(b) screening the plant cells transformed in step (a) for stable expression of chitin synthase to obtain a clonal cell line;

(c) regenerating plants from said clonal cell line; and

(d) growing said plants from step (c) under conditions
30 appropriate for the synthesis of chitin.

71. A method for increasing the tensile strength or pathogen resistance of a plant of interest, comprising the steps of:

- 5 (a) transforming a plant cell expressing chitin synthase or a biologically active fragment or variant thereof, with a nucleotide sequence encoding an enzyme in a N-acetyl glucosamine chitin biosynthetic pathway, or a biologically active fragment or variant of an enzyme in a N-acetyl glucosamine chitin biosynthetic pathway; wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant;
- (b) screening the plant cells transformed in step (a) for stable expression of said enzyme to obtain a clonal cell line;
- (c) regenerating plants from said clonal cell line; and
- 10 (d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

72. The method of claim 71, wherein said enzyme is glutamine: fructose-6-phosphate amidotransferase.

15

73. The method of claims 70-72, wherein said promoter is a stalk-specific, a tree trunk-specific, a bamboo shoot-specific promoter, an endosperm-specific promoter or an embryo-specific promoter.

20 74. A method for increasing the pathogen-resistance of a plant comprising the steps of:

- (a) transforming a plant cell expressing chitin synthase or a biologically active fragment or variant thereof, with a nucleotide sequence encoding a chitin deacetylase or a biologically active fragment or variant thereof;
- 25 wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell;
- (b) screening the plant cells transformed in step (a) for stable expression of said enzyme to obtain a clonal cell line;
- (c) regenerating plants from said clonal cell line; and
- 30 (d) growing said plants from step (c) under conditions appropriate for the synthesis of chitin.

75. The method of claim 73, wherein said promoter is a constitutive

promoter or a tissue-specific promoter.

76. The expression cassette of claim 1, wherein said nucleotide
sequence is further operably linked to a second nucleotide sequence encoding an
apoplast or a cell wall targeting signal.

77. The expression cassette of claim 12, wherein said nucleotide
sequence is further operably linked to a second nucleotide sequence encoding an
apoplast or a cell wall targeting signal.

10

78. The method of claim 13, wherein said nucleotide sequence is further
operably linked to a second nucleotide sequence encoding an apoplast or a cell
wall targeting signal.

79. The method of claim 23, wherein said nucleotide sequence is further
operably linked to a second nucleotide sequence encoding an apoplast or a cell
wall targeting signal.

80. The method of claim 34, wherein said nucleotide sequence is further
operably linked to a second nucleotide sequence encoding an apoplast or a cell
wall targeting signal.

81. The plant cell of claim 43, wherein said nucleotide sequence is
further operably linked to a second nucleotide sequence encoding an apoplast or a
cell wall targeting signal.

82. The plant or plant cell of claim 54, wherein at least one of said
nucleotide sequences is further operably linked to a third nucleotide sequence
encoding an apoplast or a cell wall targeting signal.

30

83. The method of claim 70, wherein said nucleotide sequence is further
operably linked to a second nucleotide sequence encoding an apoplast or a cell
wall targeting signal.

84. The method of claim 71, wherein said nucleotide sequence is further operably linked to a second nucleotide sequence encoding an apoplast or a cell wall targeting signal.

5

85. The method of claim 74, wherein said nucleotide sequence is further operably linked to a second nucleotide sequence encoding an apoplast or a cell wall targeting signal.

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1 GTCTTCGCCG CTCCTTCCC GGCCTCCCGG GCTGGACGAA ACGAACCCCTC
51 GCTCGCCCTC CTTATAACCG AACGGCCGAA CCCAGCCAAC CCAGCCGTTT
101 CTCTTCGTAC GGCCTCTGCC AGCCAGTGTC CTGCTACTAG GGAAGCATAC
151 CAACTCCCCA TTCTTCTCTT CGCCGCAGCC AGGAAGGAAG GATGTGCGGG
201 ATCTTCGCCT ACCTCAACTA CAACGTCTCG CGGGAGCGCC GCTACATCCT
251 CGAGGTCCCT TCAACGGCC TCCGCCGCCT CGAGTACCGC GGCTACGACT

301 CCGCCGGGAT CGCGCTCGAT GCCGACCGCC AGGTCCCCTC CCCC GCTCCC
351 GCTTCCTCTT CCGACGCGCG GCCGTACGCC GGGGCGCCGC CGCTCGTGTT
401 CCGCCAGGAG GGCAAGATCG AGAACCTCGT GCGATCCGTC TACTCCGAGG
451 TTGATGAGAA GGATGTGAAC CTGGATGCTG CGTTCAGTGT GCATGCTGGG
501 ATCGCACATA NCCAGGTGGGC CACGCACGGT GTGCCTGCTC CAAGGAACAG
551 CCACCCCCAA TCGTCTGGTG CCGGTGATGA GTTCTTGGTT GTCCACAATG

601 GCATTATCAC CAACTATGAG GTCTTGAAAG AGACACTAAC TAGGCACGGC
651 TTCACCTTTG AGTCTGATAC AGACACAGAA GTCATCCCTA AGCTAGCAAA
701 GTTCGTTTTT GATAAATCTC ATGATGAACA AGGTGATGTG ACGTTTAGCCC
751 AAGTTGTTAT GGAAGTCATG AGGCAGCTTG AAGGAGCCTA CGCACTTATC
801 TTTAAAAGCC CGCACTATCC CAATGAATTG ATTGCATGCA AACGAGGCAG
851 CCAACTGATA CTTGGTGTCA ACGAATTGAG TGGTCAACAG AATGGGAAAT

901 CATTTTCATGA TGTCAAAACC TTGACAACAA ATGGAAAGCC CAAAGAATTA
951 TTCTTCTCCA GTGATCTATG TGCTATTGTA GAGCATACGA AGAACTACTT
1001 AGCTCTTGAA GATAATGAAA TTGTTTCATAT TAAGGATGGT AGTGTTTCGA
1051 TCCTCAAGTT TGACCCTCAC AAAGAGAAGC CAGCATCTGT GCAACGAGCA
1101 TTGTCTGTTC TTGAGATGGA AGTTGAGCAA ATAAAGAAAG GAAGTTATGA
1151 TCACTTCATG CAAAAGAAA TCCATGAACA GCCACATTCG TTGAAAACAA

1201 CAATGAGGGG TAGATTGAAG GATGGTGGGG TTGTTCTAGG TGGACTGAAG
1251 GAATATCTCA AGACAATTAG GCGCTGTAGA AGGGTGGTAT TTATTGGTTG
1301 TGGAACAAGT TACAATGCTG CCTTAGCTGC AAGACCTTTT GTGGAAGAAC
1351 TGA CTGGTAT TCCTGTGACT ATGGAGGTTG CAAGTGA CTT GCTGGACAGA
1401 CAAGGTCCCA TCTACAGAGA AGACACTGCA GTTTTGTGTA GTCAATCTGG
1451 GGAGACAGCA GATACCCTCC TTGCTCTAGA TTATGCACTA GAAAATGGAG

TO FIG. 1B.

FIG. 1A.

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FROM FIG. 1A.

1501CTCTCTGTGT TGGCATAACA AATACTGTTG GAAGCACGCT GTCTAGAAAA
1551ACACACTGTG GGGTTCATAT CAATGCTGGT TGTGAGATTG GTGTTGCCAG
1601TACAAAGGCT TATACAAGTC AATAGTAGC CATGGCGATG ATGGCGTTGG
1651CTATTGGGTC CGATCAGATA TCTACTCAAG CTAGGAGGGA CAGTATCATC
1701AGTGGACTGA ACAACCTTTC AAGCAATGTC AGCGAAGTTC TCAAGCTAGA
1751TGCTGGAATG AAGGAGCTTG CCTCTTCGCT GATCGACTCA GAGTCGCTCC

1801TCGTGTTCCG AAGGGGTTAC AACTACGCCA CCGCGCTGGA GGGCGCCCTG
1851AAGGTCAAGG AGGTGGCGCT GATGCACAGC GAGGGCATGC TCGCTGGCGA
1901GATGAAGCAC GGGCCGCTGG CCTCGTGGA CGAGAACCTC CCCATCATTG
1951TCATTGCGAC CCGCGACGCG TGCTTCAGCA AGCAGCAGTC GGTGATCCAA
2001CAGCTCCTCT CGCGCAGGGG GCGCCTGATA GTGATGTGCT CTAGGGGAGA
2051TGCCGCGGCT GTGTGCCCTA GCGGTGGGTC GTGCAGAGTC ATTGAAGTTC

2101CACAGGTTGC AGACTGTCTC CAGCCAGTGA TCAACATAAT TCCATTACAG
2151TTGCTGCGT ACCATCTGAC TGTTCTCCGG GGATTCGACG TGGACCAACC
2201AAGGAATCTG GCGAAGAGCG TGACCACGCA GTAGGGGAGAG GTAGATGAGA
2251TGTTTGTATT GTAGTTAATT GTCCTTGCTC TTGAGGTGGC TAGTACGTAG
2301CATAAATATT ATGGTGCGTT AACTTGTTG TTTTGTGAAC GAAATGTACC
2351TCTCTTTTTT TAATTATGGT ATATTGGTGT CAATAGCAAA AAAAAAAAAA
2401AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

FIG. 1B.

