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NOVEL SYNTHETIC GENES FOR PLANT GUMS

continuation-in-part of application No. 08/897,556, filed on Jul. 21, 1997, now Pat. No. 6,570,062.

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ABSTRACT

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Continuation of application No. 09/119,507, filed on Jul. 20, 1998, now Pat. No. 6,548,642, which is a

A new approach in the field of plant gums is described which presents a new solution to the production of hydroxyproline(Hyp)-rich glycoproteins (HRGPs), repetitive proline-rich proteins (RPRPs) and arabino-galactan proteins (AGPs). The expression of synthetic genes designed from repetitive peptide sequences of such glycoproteins, including the peptide sequences of gum arabic glycoprotein (GAGP), is taught in host cells, including plant host cells.

FIGURE 2

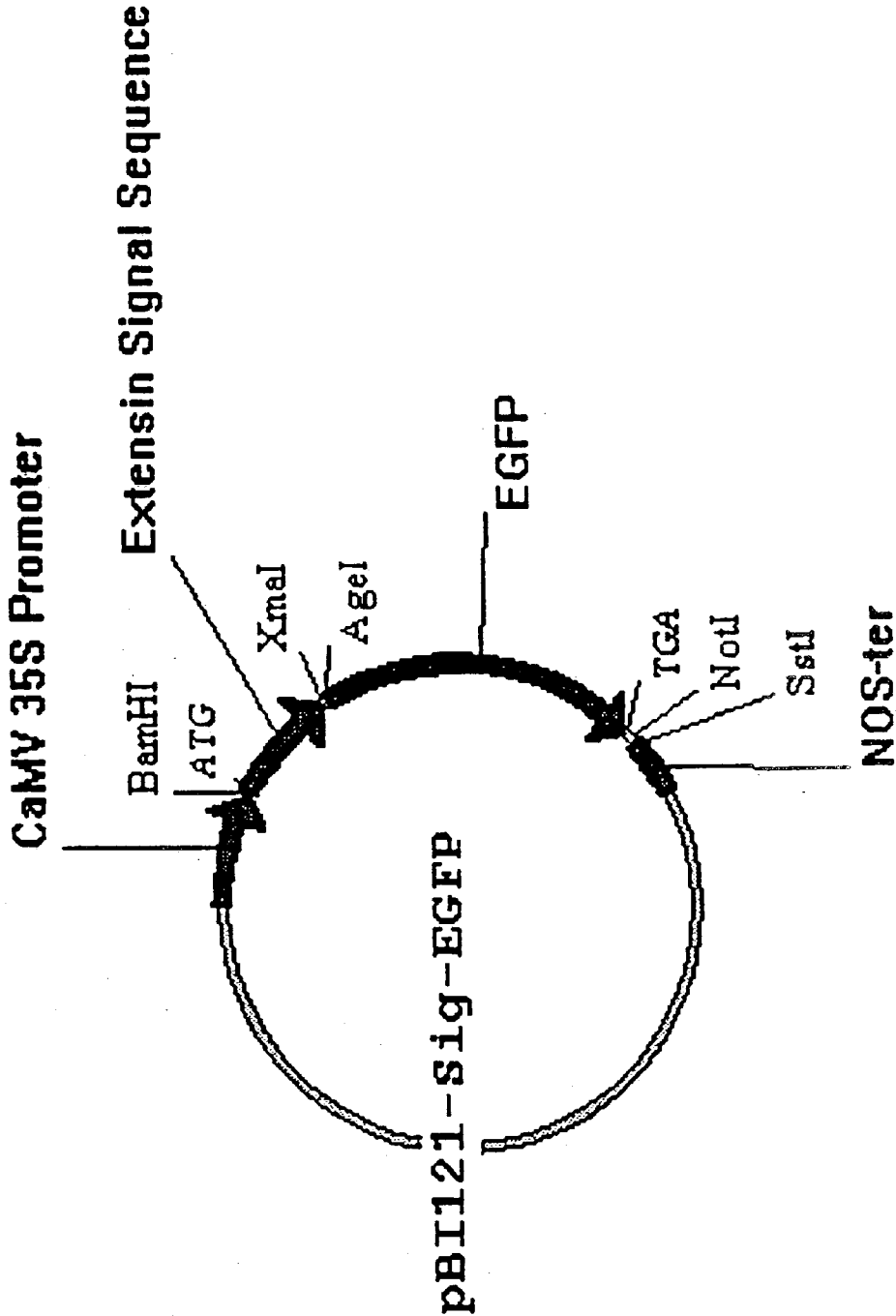


Figure 3

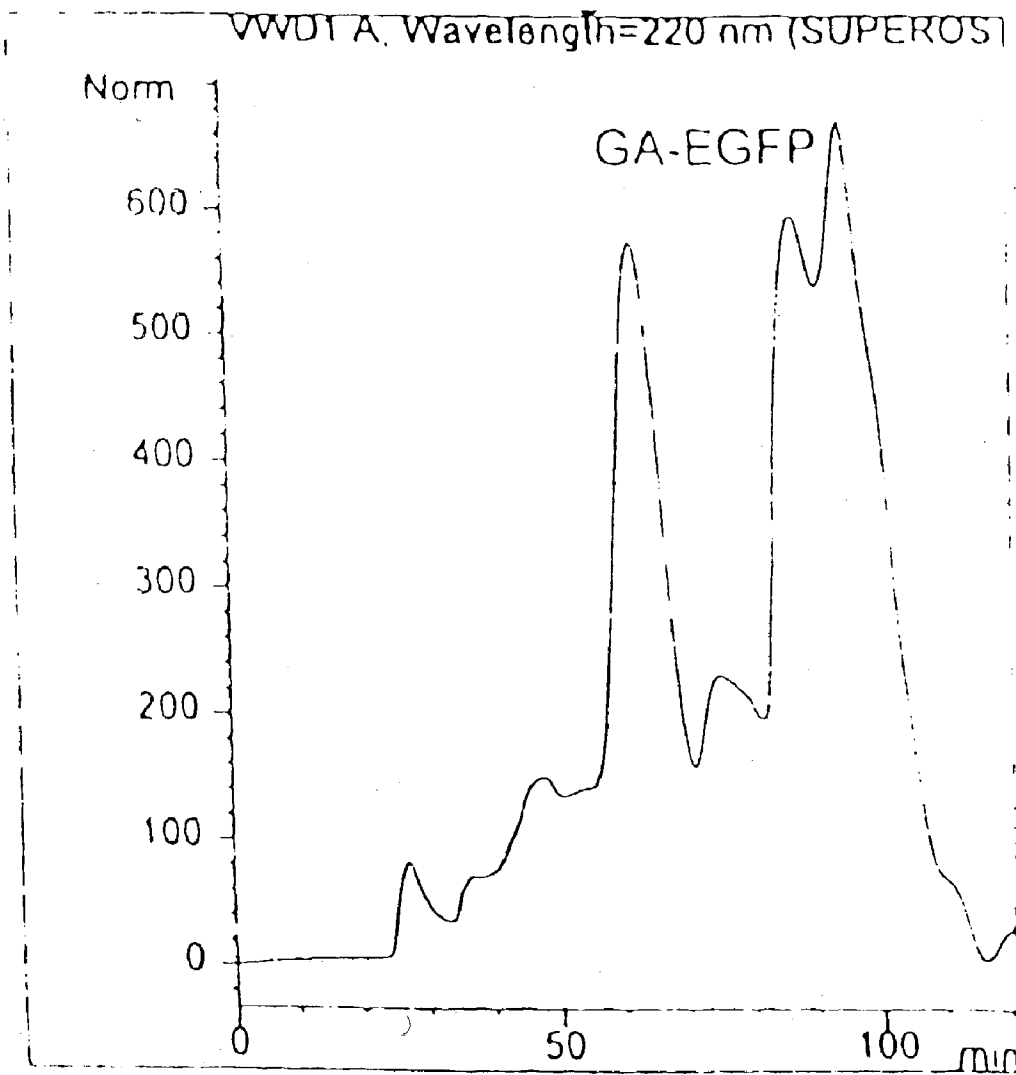
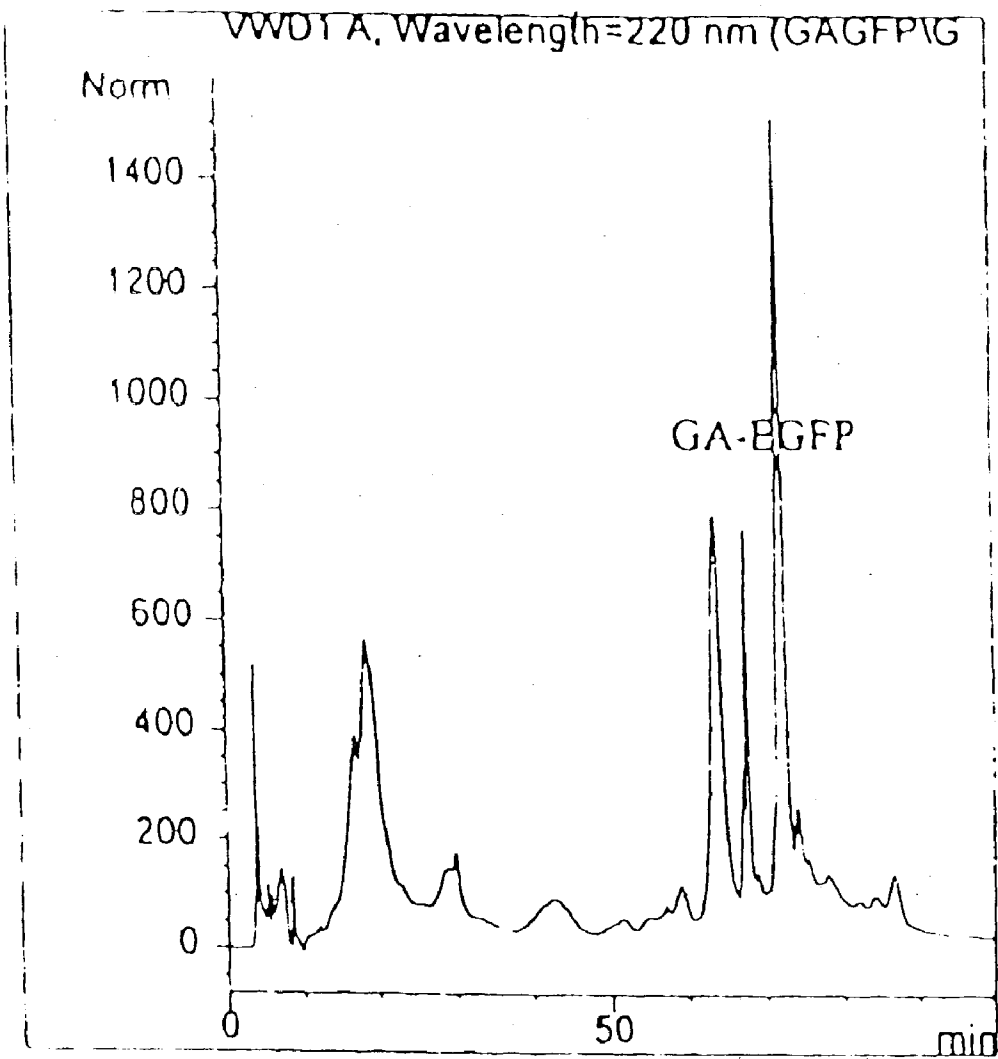


Figure 4



NOVEL SYNTHETIC GENES FOR PLANT GUMS

[0001] The present application is a Continuation-In-Part of U.S. patent application Ser. No. 08/897/556, filed Jul. 21, 1997.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of plant gums and other hydroxyproline-rich glycoproteins, and in particular, to the expression of synthetic genes designed from repetitive peptide sequences.

BACKGROUND

[0003] Gummosis is a common wound response that results in the exudation of a gum sealant at the site of cracks in bark. A. M. Stephen et al., "Exudate Gums", *Methods Plant Biochem.* (1990). Generally the exudate is a composite of polysaccharides and glycoproteins structurally related to cell wall components such as galactans [G. O. Aspinall, "Plant Gums", *The Carbohydrates* 2B:522536 (1970)] and hydroxyproline-rich glycoproteins [Anderson and McDougall, "The chemical characterization of the gum exudates from eight Australian *Acacia* species of the series *Phyllodineae*," *Food Hydrocolloids*, 2: 329 (1988)].

[0004] Gum arabic is probably the best characterized of these exudates (although it has been largely refractory to chemical analysis). It is a natural plant exudate secreted by various species of *Acacia* trees. *Acacia senegal* accounts for approximately 80% of the production of gum arabic with *Acacia seyal*, *Acacia laeta*, *Acacia campylacantha*, and *Acacia drepanolobium* supplying the remaining 20%. The gum is gathered by hand in Africa. It is a tedious process involving piercing and stripping the bark of the trees, then returning later to gather the dried tear drop shaped, spherical balls that form.

[0005] The exact chemical nature of gum arabic has not been elucidated. It is believed to consist of two major components, a microheterogeneous glucurono-arabinorhamnogalactan polysaccharide and a higher molecular weight hydroxyproline-rich glycoprotein. Osman et al., "Characterization of Gum Arabic Fractions Obtained By Anion-Exchange Chromatography" *Phytochemistry* 38:409 (1984) and Qi et al., "Gum Arabic Glycoprotein Is A Twisted Hairy Rope" *Plant Physiol.* 96:848 (1991). While the amino composition of the protein portion has been examined, little is known with regard to the precise amino acid sequence.

[0006] While the precise chemical nature of gum arabic is elusive, the gum is nonetheless particularly useful due to its high solubility and low viscosity compared to other gums. The FDA declared the gum to be a GRAS food additive. Consequently, it is widely used in the food industry as a thickener, emulsifier, stabilizer, surfactant, protective colloid, and flavor fixative or preservative. J. Dziezak, "A Focus on Gums" *Food Technology* (March 1991). It is also used extensively in the cosmetics industry.

[0007] Normally, the world production of gum arabic is over 100,000 tons per year. However, this production depends on the environmental and political stability of the region producing the gum. In the early 1970s, for example, a severe drought reduced gum production to 30,00 tons. Again in 1985, drought brought about shortages of the gum, resulting in a 600% price increase.

[0008] Three approaches have been used to deal with the somewhat precarious supply problem of gum arabic. First, other gums have been sought out in other regions of the world. Second, additives have been investigated to supplement inferior gum arabic. Third, production has been investigated in cultured cells.

[0009] The effort to find other gums in other regions of the world has met with some limited success. However, the solubility of gum arabic from *Acacia* is superior to other gums because it dissolves well in either hot or cold water. Moreover, while other exudates are limited to a 5% solution because of their excessive viscosity, gum arabic can be dissolved readily to make 55% solutions.

[0010] Some additives have been identified to supplement gum arabic. For example, whey proteins can be used to increase the functionality of gum arabic. A. Prakash et al., "The effects of added proteins on the functionality of gum arabic in soft drink emulsion systems," *Food Hydrocolloids* 4:177 (1990). However, this approach has limitations. Only low concentrations of such additives can be used without producing off-flavors in the final food product.

[0011] Attempts to produce gum arabic in cultured *Acacia senegal* cells has been explored. Unfortunately, conditions have not been found which lead to the expression of gum arabic in culture. A. Mollard and J.-P. Joseleau, "*Acacia Senegal* cells cultured in suspension secrete a hydroxyproline-deficient rabinogalactan-protein" *Plant Physiol. Biochem.* 32:703 (1994).

[0012] Clearly, new approaches to improve gum arabic production are needed. Such approaches should not be dependent on environmental or political factors. Ideally, such approaches should simplify production and be relatively inexpensive.

SUMMARY OF THE INVENTION

[0013] The present invention involves a new approach in the field of plant gums and presents a new solution to the production of hydroxyproline(Hyp)-rich glycoproteins (HRGPs), repetitive proline-rich proteins (RPRPs) and arabinogalactan proteins (AGPs). The present invention contemplates the expression of synthetic genes designed from repetitive peptide sequences of such glycoproteins, including the peptide sequences of gum arabic glycoprotein (GAGP).

[0014] With respect to GAGP, the present invention contemplates a substantially purified polypeptide comprising at least a portion of the amino acid sequence Ser-Hyp-Hyp-Hyp-[Hyp/Thr]-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Hyp-Gly-Pro-His (SEQ ID NO:1 and SEQ ID NO:2) or variants thereof. By "variants" it is meant that the sequence need not comprise the exact sequence; up to five (5) amino acid substitutions are contemplated. For example, a Leu or Hyp may be substituted for the Gly; Leu may also be substituted for Ser and one or more Hyp. By "variants" it is also meant that the sequence need not be the entire nineteen (19) amino acids. Illustrative variants are shown in Table 2.

[0015] Indeed, it is not intended that the present invention be limited by the precise length of the purified polypeptide. In one embodiment, the peptide comprises more than twelve (12) amino acids from the nineteen (19) amino acids of the sequence. In another embodiment, a portion of the nineteen

(19) amino acids (see SEQ ID NO:1 and SEQ ID NO:2) is utilized as a repetitive sequence. In yet another embodiment, all nineteen (19) amino acids (see SEQ ID NO:1 and SEQ ID NO:2 with or without amino acid substitutions) are utilized as a repetitive sequence.

[0016] It is not intended that the present invention be limited by the precise number of repeats. The sequence (i.e. SEQ ID NO: 1 and SEQ ID NO:2) or variants thereof may be used as a repeating sequence between one (1) and up to fifty (50) times, more preferably between ten (10) and up to thirty (30) times, and most preferably approximately twenty (20) times. The sequence (i.e. SEQ ID NO:1 and SEQ ID NO:2) or variants thereof may be used as contiguous repeats or may be used as non-contiguous repeats (with other amino acids, or amino acid analogues, placed between the repeating sequences).

[0017] The present invention specifically contemplates fusion proteins comprising a non-gum arabic protein or glycoprotein sequence and a portion of the gum arabic glycoprotein sequence (SEQ ID NO:1 and SEQ ID NO:2). It is not intended that the present invention be limited by the nature of the non-gum arabic glycoprotein sequence. In one embodiment, the non-gum arabic glycoprotein sequence is a green fluorescent protein.

[0018] As noted above, the present invention contemplates synthetic genes encoding such peptides. By "synthetic genes" it is meant that the nucleic acid sequence is derived using the peptide sequence of interest (in contrast to using the nucleic acid sequence from cDNA). In one embodiment, the present invention contemplates an isolated polynucleotide sequence encoding a polypeptide comprising at least a portion of the polypeptide of SEQ ID NO:1 and SEQ ID NO:2 or variants thereof. The present invention specifically contemplates a polynucleotide sequence comprising a nucleotide sequence encoding a polypeptide comprising one or more repeats of SEQ ID NO:1 and SEQ ID NO:2 or variants thereof. Importantly, it is not intended that the present invention be limited to the precise nucleic acid sequence encoding the polypeptide of interest.

[0019] The present invention contemplates synthetic genes encoding portions of HRGPs, wherein the encoded peptides contain one or more of the highly conserved Ser-Hyp₄ (SEQ ID NO:3) motif(s). The present invention also contemplates synthetic genes encoding portions of RPRPs, wherein the encoded peptides contain one or more of the pentapeptide motif: Pro-Hyp-Val-Tyr-Lys (SEQ ID NO:4) and variants of this sequence such as X-Hyp-Val-Tyr-Lys (SEQ ID NO:5) and Pro-Hyp-Val-X-Lys (SEQ ID NO:6) and Pro-Pro-X-Tyr-Lys and Pro-Pro-X-Tyr-X (SEQ ID NO:8), where "X" can be Thr, Glu, Hyp, Pro, His and Ile. The present invention also contemplates synthetic genes encoding portions of AGPs, wherein the encoded peptides contain one or more Xaa-Hyp-Xaa-Hyp (SEQ ID NO:9) repeats. Such peptides can be expressed in a variety of forms, including but not limited to fusion proteins.

[0020] With regard to motifs for HRGPs, the present invention contemplates a polynucleotide sequence comprising the sequence: 5'-CCA CCA CCT TCA CCT CCA CCC CCA TCT CCA-3' (SEQ ID NO:10). With regard to motifs for AGPs, the present invention contemplates a polynucleotide sequence comprising the sequence: 5'-TCA CCA TCA CCA TCT CCT TCG CCA TCA CCC-3' (SEQ ID NO:11).

Of course, it is not intended that the present invention be limited by the particular sequence. Indeed, the present invention specifically contemplates sequences homologous to the sequences of SEQ ID NOS: 10 and 11. The present invention also contemplates sequences that are complementary (including sequences that are only partially complementary) sequences to the sequences of SEQ ID NOS: 10 and 11. Such complementary sequences include sequences that will hybridize to the sequences of SEQ ID NOS: 10 and 11 under low stringency conditions as well as high stringency conditions (see Definitions below).

[0021] The present invention also contemplates the mixing of motifs (i.e. modules) which are not found in wild-type sequences. For example, one might add GAGP modules to extensin and RPRP crosslinking modules to AGP-like molecules.

[0022] The present invention contemplates using the polynucleotides of the present invention for expression of the polypeptides in vitro and in vivo. Therefore, the present invention contemplates polynucleotide sequences encoding two or more repeats of the sequence of SEQ ID NO: 1 and SEQ ID NO:2 or variants thereof, wherein said polynucleotide sequence is contained on a recombinant expression vector. It is also contemplated that such vectors will be introduced into a variety of host cells, both eukaryotic and prokaryotic (e.g. bacteria such as *E. coli*).

[0023] In one embodiment, the vector further comprises a promoter. It is not intended that the present invention be limited to a particular promoter. Any promoter sequence which is capable of directing expression of an operably linked nucleic acid sequence encoding a portion of a plant gum polypeptide (or other hydroxyproline-rich polypeptide of interest as described above) is contemplated to be within the scope of the invention. Promoters include, but are not limited to, promoter sequences of bacterial, viral and plant origins. Promoters of bacterial origin include, but are not limited to, the octopine synthase promoter, the nopaline synthase promoter and other promoters derived from native Ti plasmids. Viral promoters include, but are not limited to, the 35S and 19S RNA promoters of cauliflower mosaic virus (CaMV), and T-DNA promoters from *Agrobacterium*. Plant promoters include, but are not limited to, the ribulose-1,3-bisphosphate carboxylase small subunit promoter, maize ubiquitin promoters, the phaseolin promoter, the E8 promoter, and the Tob7 promoter.

[0024] The invention is not limited to the number of promoters used to control expression of a nucleic acid sequence of interest. Any number of promoters may be used so long as expression of the nucleic acid sequence of interest is controlled in a desired manner. Furthermore, the selection of a promoter may be governed by the desirability that expression be over the whole plant, or localized to selected tissues of the plant, e.g., root, leaves, fruit, etc. For example, promoters active in flowers are known (Benfy et al. (1990) *Plant Cell* 2:849-856).

[0025] The promoter activity of any nucleic acid sequence in host cells may be determined (i.e., measured or assessed) using methods well known in the art and exemplified herein. For example, a candidate promoter sequence may be tested by ligating it in-frame to a reporter gene sequence to generate a reporter construct, introducing the reporter construct into host cells (e.g. tomato or potato cells) using

methods described herein, and detecting the expression of the reporter gene (e.g., detecting the presence of encoded mRNA or encoded protein, or the activity of a protein encoded by the reporter gene). The reporter gene may confer antibiotic or herbicide resistance. Examples of reporter genes include, but are not limited to, dhfr which confers resistance to methotrexate [Wigler M et al., (1980) *Proc Natl Acad Sci* 77:3567-70]; npt, which confers resistance to the aminoglycosides neomycin and G-418 [Colbere-Garapin F et al., (1981) *J. Mol. Biol.* 150:1-14] and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyl transferase, respectively. Recently, the use of a reporter gene system which expresses visible markers has gained popularity with such markers as β -glucuronidase and its substrate (X-Gluc), luciferase and its substrate (luciferin), and β -galactosidase and its substrate (X-Gal) being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system [Rhodes C A et al. (1995) *Methods Mol Biol* 55:121-131].

[0026] In addition to a promoter sequence, the expression construct preferably contains a transcription termination sequence downstream of the nucleic acid sequence of interest to provide for efficient termination. In one embodiment, the termination sequence is the nopaline synthase (NOS) sequence. In another embodiment the termination region comprises different fragments of sugarcane ribulose-1,5-biphosphate carboxylase/oxygenase (rubisco) small subunit (scrbs) gene. The termination sequences of the expression constructs are not critical to the invention. The termination sequence may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0027] If the mRNA encoded by the nucleic acid sequence of interest is to be efficiently translated, polyadenylation sequences are also commonly added to the expression construct. Examples of the polyadenylation sequences include, but are not limited to, the *Agrobacterium* octopine synthase signal, or the nopaline synthase signal.

[0028] The invention is not limited to constructs which express a single nucleic acid sequence of interest. Constructs which contain a plurality of (i.e., two or more) nucleic acid sequences under the transcriptional control of the same promoter sequence are expressly contemplated to be within the scope of the invention. Also included within the scope of this invention are constructs which contain the same or different nucleic acid sequences under the transcriptional control of different promoters. Such constructs may be desirable to, for example, target expression of the same or different nucleic acid sequences of interest to selected plant tissues.

[0029] As noted above, the present invention contemplates using the polynucleotides of the present invention for expression of a portion of plant gum polypeptides in vitro and in vivo. Where expression takes place in vivo, the present invention contemplates transgenic plants. The transgenic plants of the invention are not limited to plants in which each and every cell expresses the nucleic acid sequence of interest. Included within the scope of this invention is any plant (e.g. tobacco, tomato, maize, algae, etc.) which contains at least one cell which expresses the nucleic acid sequence of interest. It is preferred, though not necessary, that the transgenic plant express the nucleic acid

sequence of interest in more than one cell, and more preferably in one or more tissue. It is particularly preferred that expression be followed by proper glycosylation of the plant gum polypeptide fragment or variant thereof, such that the host cell produces functional (e.g. in terms of use in the food or cosmetic industry) plant gum polypeptide.

[0030] The fact that transformation of plant cells has taken place with the nucleic acid sequence of interest may be determined using any number of methods known in the art. Such methods include, but are not limited to, restriction mapping of genomic DNA, PCR analysis, DNA-DNA hybridization, DNA-RNA hybridization, and DNA sequence analysis.

[0031] Expressed polypeptides (or fragments thereof) can be immobilized (covalently or non-covalently) on solid supports or resins for use in isolating HRGP-binding molecules from a variety of sources (e.g., algae, plants, animals, microorganisms). Such polypeptides can also be used to make antibodies.

DESCRIPTION OF THE DRAWINGS

[0032] FIG. 1 shows the nucleic acid sequence (SEQ ID NO:12) of one embodiment of a synthetic gene of the present invention.

[0033] FIG. 2 shows one embodiment of a synthetic gene in one embodiment of an expression vector.

[0034] FIG. 3 is a graph showing size-fractionation of expressed protein from transformed tobacco cells.

[0035] FIG. 4 is a graph showing the isolation of GA-EGFP by reverse phase chromatography.

DEFINITIONS

[0036] The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or its precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence.

[0037] The term "nucleic acid sequence of interest" refers to any nucleic acid sequence the manipulation of which may be deemed desirable for any reason by one of ordinary skill in the art (e.g., confer improved qualities).

[0038] The term "wild-type" when made in reference to a gene refers to a gene which has the characteristics of a gene isolated from a naturally occurring source. The term "wild-type" when made in reference to a gene product refers to a gene product which has the characteristics of a gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" when made in reference to a gene or to a gene product refers, respectively, to a gene or to a gene product which displays modifications in sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

[0039] The term "recombinant" when made in reference to a DNA molecule refers to a DNA molecule which is com-

prised of segments of DNA joined together by means of molecular biological techniques. The term “recombinant” when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant DNA molecule.

[0040] As used herein, the terms “vector” and “vehicle” are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

[0041] The term “expression vector” or “expression cassette” as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

[0042] The terms “targeting vector” or “targeting construct” refer to oligonucleotide sequences comprising a gene of interest flanked on either side by a recognition sequence which is capable of homologous recombination of the DNA sequence located between the flanking recognition sequences.

[0043] The terms “in operable combination”, “in operable order” and “operably linked” as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

[0044] The term “transformation” as used herein refers to the introduction of foreign DNA into cells. Transformation of a plant cell may be accomplished by a variety of means known in the art including particle mediated gene transfer (see, e.g., U.S. Pat. No. 5,584,807 hereby incorporated by reference); infection with an *Agrobacterium* strain containing the foreign DNA for random integration (U.S. Pat. No. 4,940,838 hereby incorporated by reference) or targeted integration (U.S. Pat. No. 5,501,967 hereby incorporated by reference) of the foreign DNA into the plant cell genome; electroinjection (Nan et al. (1995) In “Biotechnology in Agriculture and Forestry,” Ed. Y. P. S. Bajaj, Springer-Verlag Berlin Heidelberg, Vol 34:145-155; Griesbach (1992) HortScience 27:620); fusion with liposomes, lysosomes, cells, minicells or other fusible lipid-surfaced bodies (Fraley et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:1859-1863; polyethylene glycol (Krens et al. (1982) *Nature* 296:72-74); chemicals that increase free DNA uptake; transformation using virus, and the like.

[0045] The terms “infecting” and “infection” with a bacterium refer to co-incubation of a target biological sample, (e.g., cell, tissue, etc.) with the bacterium under conditions such that nucleic acid sequences contained within the bacterium are introduced into one or more cells of the target biological sample.

[0046] The term “*Agrobacterium*” refers to a soil-borne, Gram-negative, rod-shaped phytopathogenic bacterium

which causes crown gall. The term “*Agrobacterium*” includes, but is not limited to, the strains *Agrobacterium tumefaciens*, (which typically causes crown gall in infected plants), and *Agrobacterium rhizogens* (which causes hairy root disease in infected host plants). Infection of a plant cell with *Agrobacterium* generally results in the production of opines (e.g., nopaline, agropine, octopine etc.) by the infected cell. Thus, *Agrobacterium* strains which cause production of nopaline (e.g., strain LBA4301, C58, A208) are referred to as “nopaline-type” *Agrobacteria*; *Agrobacterium* strains which cause production of octopine (e.g., strain LBA4404, Ach5, B6) are referred to as “octopine-type” *Agrobacteria*; and *Agrobacterium* strains which cause production of agropine (e.g., strain EHA105, EHA101, A281) are referred to as “agropine-type” *Agrobacteria*.

[0047] The terms “bombarding”, “bombardment”, and “biolistic bombardment” refer to the process of accelerating particles towards a target biological sample (e.g., cell, tissue, etc.) to effect wounding of the cell membrane of a cell in the target biological sample and/or entry of the particles into the target biological sample. Methods for biolistic bombardment are known in the art (e.g., U.S. Pat. No. 5,584,807, the contents of which are herein incorporated by reference), and are commercially available (e.g., the helium gas-driven microprojectile accelerator (PDS-1000/He) (BioRad)).

[0048] The term “microwounding” when made in reference to plant tissue refers to the introduction of microscopic wounds in that tissue. Microwounding may be achieved by, for example, particle or biolistic bombardment.

[0049] The term “transgenic” when used in reference to a plant cell refers to a plant cell which comprises a transgene, or whose genome has been altered by the introduction of a transgene. The term “transgenic” when used in reference to a plant refers to a plant which comprises one or more cells which contain a transgene, or whose genome has been altered by the introduction of a transgene. These transgenic cells and transgenic plants may be produced by several methods including the introduction of a “transgene” comprising nucleic acid (usually DNA) into a target cell or integration into a chromosome of a target cell by way of human intervention, such as by the methods described herein.

[0050] The term “transgene” as used herein refers to any nucleic acid sequence which is introduced into the genome of a plant cell by experimental manipulations. A transgene may be an “endogenous DNA sequence,” or a “heterologous DNA sequence” (i.e., “foreign DNA”). The term “endogenous DNA sequence” refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (e.g., a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence. The term “heterologous DNA sequence” refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into

which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (e.g., proteins which confer drug resistance), etc.

[0051] As used herein, the term “probe” when made in reference to an oligonucleotide (i.e., a sequence of nucleotides) refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. Oligonucleotide probes may be labelled with a “reporter molecule,” so that the probe is detectable using a detection system. Detection systems include, but are not limited to, enzyme, fluorescent, radioactive, and luminescent systems.

[0052] The term “selectable marker” as used herein, refer to a gene which encodes an enzyme having an activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be “positive” or “negative.” Examples of positive selectable markers include the neomycin phosphotransferase (NPTII) gene which confers resistance to G418 and to kanamycin, and the bacterial hygromycin phosphotransferase gene (hyg), which confers resistance to the antibiotic hygromycin. Negative selectable markers encode an enzymatic activity whose expression is cytotoxic to the cell when grown in an appropriate selective medium. For example, the HSV-tk gene is commonly used as a negative selectable marker. Expression of the HSV-tk gene in cells grown in the presence of gancyclovir or acyclovir is cytotoxic; thus, growth of cells in selective medium containing gancyclovir or acyclovir selects against cells capable of expressing a functional HSV TK enzyme.

[0053] The terms “promoter element,” “promoter,” or “promoter sequence” as used herein, refer to a DNA sequence that is located at the 5' end (i.e. precedes) the protein coding region of a DNA polymer. The location of most promoters known in nature precedes the transcribed region. The promoter functions as a switch, activating the expression of a gene. If the gene is activated, it is said to be transcribed, or participating in transcription. Transcription involves the synthesis of mRNA from the gene. The promoter, therefore, serves as a transcriptional, regulatory element and also provides a site for initiation of transcription of the gene into mRNA.

[0054] The term “amplification” is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art [Dieffenbach CW and GS Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview N.Y.]. As used herein, the term “polymerase chain reaction” (“PCR”) refers to the method of K. B. Mullis disclosed in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target

sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one “cycle”; there can be numerous “cycles”) to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the “polymerase chain reaction” (hereinafter “PCR”). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be “PCR amplified.” With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (e.g., hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; and/or incorporation of ³²P-labeled deoxyribonucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. Amplified target sequences may be used to obtain segments of DNA (e.g., genes) for the construction of targeting vectors, transgenes, etc.

[0055] The present invention contemplates using amplification techniques such as PCR to obtain the cDNA (or portions thereof) of plant genes encoding plant gums and other hydroxyproline-rich polypeptides. In one embodiment, primers are designed using the synthetic gene sequences (e.g. containing sequences encoding particular motifs) described herein and PCR is carried out (using genomic DNA or other source of nucleic acid from any plant capable of producing a gum exudate) under conditions of low stringency. In another embodiment, PCR is carried out under high stringency. The amplified products can be run out on a gel and isolated from the gel.

[0056] The term “hybridization” as used herein refers to any process by which a strand of nucleic acid joins with a complementary strand through base pairing [Coombs J (1994) *Dictionary of Biotechnology*, Stockton Press, New York N.Y.].

[0057] As used herein, the terms “complementary” or “complementarity” when used in reference to polynucleotides refer to polynucleotides which are related by the base-pairing rules. For example, for the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity may be “partial,” in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be “complete” or “total” complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the

efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

[0058] The term “homology” when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term “substantially homologous.” The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a sequence which is completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

[0059] Low stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42° C. in a solution consisting of 5×SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄·H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5× Denhardt’s reagent [50× Denhardt’s contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5×SSPE, 0.1% SDS at 42° C. when a probe of about 500 nucleotides in length is employed.

[0060] High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42° C. in a solution consisting of 5×SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄·H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5× Denhardt’s reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1× SSPE, 1.0% SDS at 42° C. when a probe of about 500 nucleotides in length is employed.

[0061] When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above listed conditions.

[0062] “Stringency” when used in reference to nucleic acid hybridization typically occurs in a range from about

$T_m - 5^\circ \text{C}$. (5°C . below the T_m of the probe) to about 20°C . to 25°C . below T_m . As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under “stringent conditions” a nucleic acid sequence of interest will hybridize to its exact complement and closely related sequences.

[0063] As used herein, the term “fusion protein” refers to a chimeric protein containing the protein of interest (i.e., GAGP and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-GAGP sequence). The fusion partner may provide a detectable moiety, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., GAGP protein or fragments thereof) by a variety of enzymatic or chemical means known to the art.

[0064] As used herein the term “non-gum arabic glycoprotein” or “non-gum arabic glycoprotein sequence” refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a gum arabic glycoprotein.

[0065] The term “protein of interest” as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest (e.g., GAGP) will be joined or fused with another protein or protein domain (e.g., GFP), the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

[0066] As used herein, the term “purified” or “to purify” refers to the removal of contaminants from a sample. For example, recombinant HRGP polypeptides, including HRGP-GFP fusion proteins are purified by the removal of host cell components such as nucleic acids, lipopolysaccharide (e.g., endotoxin).

[0067] The term “recombinant DNA molecule” as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

[0068] The term “recombinant protein” or “recombinant polypeptide” as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

[0069] As used herein the term “portion” when in reference to a protein (as in “a portion of a given protein”) refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

GENERAL DESCRIPTION OF INVENTION

[0070] The present invention relates generally to the field of plant gums and other hydroxyproline-rich glycoproteins, and in particular, to the expression of synthetic genes designed from repetitive peptide sequences. The hydroxyproline-rich glycoprotein (HRGP) superfamily is ubiquitous in the primary cell wall or extracellular matrix throughout the plant kingdom. Family members are diverse in structure and implicated in all aspects of plant growth and

development. This includes plant responses to stress imposed by pathogenesis and mechanical wounding.

[0071] Plant HRGPs have no known animal homologues. Furthermore, hydroxyproline residues are O-glycosylated in plant glycoproteins but never in animals. At the molecular level the function of these unique plant glycoproteins remains largely unexplored.

[0072] HRGPS are, to a lesser or greater extent, extended, repetitive, modular proteins. The modules are small (generally 4-6 residue motifs), usually glycosylated, with most HRGPs being made up of more than one type of repetitive module. For purposes of constructing the synthetic genes of the present invention, it is useful to view the glycosylated polypeptide modules not merely as peptides or oligosaccharides but as small functional units.

[0073] The description of the invention involves A) the design of the polypeptide of interest, B) the production of synthetic genes encoding the polypeptide of interest, C) the construction of the expression vectors, D) selection of the host cells, and E) introduction of the expression construct into a particular cell (whether in vitro or in vivo).

[0074] A. Design Of The Polypeptide Of Interest

[0075] The present invention contemplates polypeptides that are fragments of hydroxyproline-rich glycoproteins (HRGPs), repetitive proline-rich proteins (RPRPs) and arabinogalactan proteins (AGPs). The present invention contemplates portions of HRGPs comprising one or more of the highly conserved Ser-Hyp₄ (SEQ ID NO:3) motif(s). The present invention also contemplates portions of RPRPs comprising one or more of the pentapeptide motif: Pro-Hyp-Val-Tyr-Lys (SEQ ID NO:4). The present invention also contemplates portions of AGPs comprising one or more Xaa-Hyp-Xaa-Hyp (SEQ ID NO:9) repeats.

[0076] While an understanding of the natural mechanism of glycosylation is not required for the successful operation of the present invention, it is believed that in GAGP and other HRGPs, repetitive Xaa-Hyp blocks constitute a Hyp-glycosylation code where Hyp occurring in contiguous blocks (Xaa-Hyp-Hyp) and Hyp occurring in non-contiguous Hyp repeats is recognized by different enzymes: arabinosyltransferases and galactosyltransferases, respectively.

[0077] The RPRPs (and some nodulins) consist of short repetitive blocks (e.g. Soybean RRP1: [POVYK]_n where O=Hyp) containing the least amount of contiguous Hyp. They also exemplify the low end of the glycosylation range with relatively few Hyp residues arabinosylated and no arabinogalactan polysaccharide. For example, in soybean RRP1, L-arabinofuranose is attached to perhaps only a single Hyp residue in the molecule.

[0078] The Extensins occupy an intermediate position in the glycosylation continuum, containing about 50% carbohydrate which occurs mainly as Hyp-arabinosides (1-4 Ara residues), but not as Hyp-arabinogalactan polysaccharide. Extensins contain the repetitive, highly arabinosylated, diagnostic Ser-Hyp₄ (SEQ ID NO:3) glycopeptide module. The precise function of this module is unknown, but earlier work indicates that these blocks of arabinosylated Hyp help stabilize the extended polyproline-II helix of the extensins. Monogalactose also occurs on the Ser residues.

[0079] The classical Ser-Hyp₄ (SEQ ID NO:3) glycopeptide module is of special interest. A tetra-L-arabinofuranosyl oligosaccharide is attached to each Hyp residue in the block. Three uniquely b-linked arabinofuranosyl residues and an a-linked nonreducing terminus comprise the tetraarabinooligosaccharide. While an understanding of the natural mechanism of glycosylation is not required for the successful operation of the present invention, it is believed that the arabinosylated Hyp residues together with the single galactosyl-serine residue undoubtedly form a unique molecular surface topography which interacts with and is recognized by other wall components, possibly including itself. Shorter blocks of Hyp, namely Hyp₃ and Hyp₂, lack the fourth (a-linked) arabinose residue, again suggesting that the fourth Ara unique to the Hyp₄ block, has a special role and is presented for recognition or cleavage.

[0080] At the high end of the glycosylation range (~90% sugar), the arabinogalactan-proteins (AGPs) and the related gum arabic glycoprotein (GAGP) are uniquely glycosylated with arabinogalactan polysaccharides. GAGP and all AGPs so far characterized by Hyp-glycoside profiles contain Hyp-linked arabinosides assigned to contiguous Hyp residues by the Hyp contiguity hypothesis. However these glycoproteins also uniquely contain (Xaa-Hyp-Xaa-Hyp) (SEQ ID NO:9) repeats. These repeats are putative polysaccharide attachment sites.

[0081] The present invention contemplates in particular fragments of gum arabic glycoprotein (GAGP). As noted above, GAGP has been largely refractory to chemical analysis. The largest peptide obtained and sequenced from gum arabic was a peptide of twelve (12) amino acids having the sequence Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Hyp-Gly-Pro (SEQ ID NO:13). C. L. Delonay, "Determination of the Protein Constituent Of Gum Arabic" Master of Science Thesis (1993). The present invention contemplates using this Delonay sequence as well as (heretofore undescribed) larger peptide fragments of GAGP (and variants thereof) for the design of synthetic genes. In this manner, "designer plant gums" can be produced ("designer extensins" are also contemplated).

[0082] In one embodiment, the present invention contemplates a substantially purified polypeptide comprising at least a portion of the amino acid consensus sequence Ser-Hyp-Hyp-Hyp-[Hyp/Thr]-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Hyp-Gly-Pro-His (SEQ ID NO: 1 and SEQ ID NO:2) or variants thereof. While an understanding of the natural mechanism of glycosylation is not required for the successful operation of the present invention, it is believed that this GAGP 19-residue consensus repeat (which contains both contiguous Hyp and non-contiguous Hyp repeats) is glycosylated in native GAGP with both Hyp-arabinosides and Hyp-polysaccharide in molar ratios. It is further believed that the high molecular weight protein component of gum arabic (i.e. GAGP) is responsible for the remarkable emulsifying and stabilizing activity exploited by the food and soft drink industries.

[0083] B. Production Of Synthetic Genes

[0084] The present invention contemplates the use of synthetic genes engineered for the expression of repetitive glycopeptide modules in cells, including but not limited to callus and suspension cultures. It is not intended that the present invention be limited by the precise number of repeats.

[0085] In one embodiment, the present invention contemplates the nucleic acid sequences encoding the consensus sequence for GAGP (i.e. SEQ ID NO:1 and SEQ ID NO:2) or variants thereof may be used as a repeating sequence between two (2) and up to fifty (50) times, more preferably between ten (10) and up to thirty (30) times, and most preferably approximately twenty (20) times. The nucleic acid sequence encoding the consensus sequence (i.e. SEQ ID NO:1 and SEQ ID NO:2) or variants thereof may be used as contiguous repeats or may be used as non-contiguous repeats.

[0086] In designing any HRGP gene cassette the following guidelines are employed. Cassette design reflects the following:

[0087] 1) Minimization of the repetitive nature of the coding sequence while still taking into account the HRGP codon bias of the host plant (e.g., when tomato is the host plant, the codon usage bias of the tomato which favors CCA and CCT [but not CCG] for Pro residues, and TCA and TCC for Ser residues is employed). *Zea mays* (such as corn) and perhaps other graminaceous monocots (e.g. rice barley, wheat and all grasses) prefer CCG and CCC for proline; GTC and CTT for valine,; and AAG for lysine. Dicots (including legumes) prefer CCA and CCT for proline and TCA and TCT for serine.

[0088] 2) Minimization of strict sequence periodicity.

[0089] 3) Non-palindromic ends are used for the monomers and end linkers to assure proper "head-to-tail" polymerization.

[0090] 4) The constructs contain no internal restriction enzyme recognition sites for the restriction enzymes employed for the insertion of these sequences into expression vectors or during subsequent manipulations of such vectors. Typically, the 5' linker contains a XmaI site downstream of the BamHI site used for cloning into the cloning vector (e.g., pBluescript). The XmaI site is used for insertion of the HRGP gene cassette into the expression vector (e.g., pBI121-Sig-EGFP). Typically, the 3' linker contains a AgeI site upstream of the EcoRI site used for cloning into the cloning vector (e.g., pBluescript). The AgeI site is used for insertion of the HRGP gene cassette into the expression vector. (For plasmid pBI121-Sig—which does not contain GFP for the fusion protein—the same signal sequence is used, but the 3' linkers contain an Sst I restriction site for insertion as an Xma I/Sst I fragment behind the signal sequence and before the NOS terminator.

[0091] 5) The oligonucleotides used are high quality (e.g., from GibcoBRL, Operon) and have been purified away from unwanted products of the synthesis.

[0092] 6) The T_M of correctly aligned oligomers is greater than the T_M of possible dimers, hairpins or crossdimers.

[0093] C. Construction of Expression Vectors

[0094] It is not intended that the present invention be limited by the nature of the expression vector. A variety of vectors are contemplated. In one embodiment, two plant transformation vectors are prepared, both derived from pBI121 (Clontech). Both contain an extensin signal sequence for transport of the constructs through the ER/Golgi for posttranslational modification. A first plasmid

construct contained Green Fluorescent Protein (GFP) as a reporter protein instead of GUS. A second plasmid does not contain GFP.

[0095] pBI121 is the Jefferson vector in which the BamHI and SstI sites can be used to insert foreign DNA between the ³⁵S CaMV promoter and the termination/polyadenylation signal from the nopaline synthase gene (NOS-ter) of the Agrobacterium Ti plasmid; it also contains an RK2 origin of replication, a kanamycin resistance gene, and the GUS reporter gene.

[0096] Signal Sequences. As noted above, the GUS sequence is replaced (via BamHI/SstI) with a synthetic DNA sequence encoding a peptide signal sequence based on the extensin signal sequences of *Nicotiana plumbaginifolia* and *N. tabacum*

[0097] MGKMASLFATFLVVLVLSLSLAQTTRVVP-VASSAP (SEQ ID NO:14) The DNA sequence also contains 15 bp of the 5' untranslated region, and restriction sites for Bam HI in its 5' terminus and Sst I in its extreme 3' terminus for insertion into pBI121 in place of GUS. An XmaI restriction site occurs 16 bp upstream from the Sst I site to allow subsequent insertion of EGFP into the plasmid as a Xma I/Sst I fragment.

[0098] The sequence underlined above is known to target *N. plumbaginifolia* extensin fusion proteins through the ER and Golgi for post-translational modifications, and finally to the wall. The signal sequence proposed also involves transport of extensins and extensin modules in the same plant family (Solanaceae). Alternatively, one can use the signal sequence from tomato P1 extensin itself.

TABLE 1

GFP MUTANTS		
WAVELENGTH (nm)		
MUTANT	Excitation	Emitting
mGFPX10; F99S, M153T, V163a	Excites at 395	
mGFPX10-5	Excites at 489	Emits at 508
GFP A2; I167T	Excites at 471	
GFP B7; Y66H	Excites at 382	Emits at 440 (blue fluorescence)
GFPX10-C7; F99S, M153T, V163A, I167T, S175G	Excites at 395 and 473	
GFPX10-D3; F99S, M153T, V163A, Y661H	Excites at 382	Emits at 440
	Excites at 382	Emits at 440

[0099] Addition of GFP. The repetitive HRGP-modules can be expressed as GFP fusion products rather than GUS fusions, and can also be expressed as modules without GFP. Fusion with a green fluorescent protein reporter gene appropriately red-shifted for plant use, e.g. EGFP (an S65T variant recommended for plants by Clontech) or other suitable mutants (see Table I above) allows the detection of <700 GFP molecules at the cell surface. GFP requires aerobic conditions for oxidative formation of the fluorophore. It works well at the lower temperatures used for plant cell cultures and normally it does not adversely affect protein function although it may allow the regeneration of plants only when targeted to the ER. Promoters. As noted above, it is not intended that the present invention be limited by the nature of the promoter(s) used in the expression constructs.

The CaMV35S promoter is preferred, although it is not entirely constitutive and expression is "moderate". In some embodiments, higher expression of the constructs is desired to enhance the yield of HRGP modules; in such cases a plasmid with "double" CaMV35S promoters is employed.

[0100] D. Selection Of Host Cells

[0101] A variety of host cells are contemplated (both eukaryotic and prokaryotic). It is not intended that the present invention be limited by the host cells used for expression of the synthetic genes of the present invention. Plant host cells are preferred, including but not limited to legumes (e.g. soy beans) and solanaceous plants (e.g. tobacco).

[0102] The present invention is not limited by the nature of the plant cells. All sources of plant tissue are contemplated, including but not limited to seeds. Seeds of flowering plants consist of an embryo, a seed coat, and stored food. When fully formed, the embryo consists basically of a hypocotyl-root axis bearing either one or two cotyledons and an apical meristem at the shoot apex and at the root apex. The cotyledons of most dicots are fleshy and contain the stored food of the seed. In other dicots and most monocots, food is stored in the endosperm and the cotyledons function to absorb the simpler compounds resulting from the digestion of the food.

[0103] It is also not intended that the present invention be limited to only certain types of plants. Both monocotyledons and dicotyledons are contemplated. Monocotyledons include grasses, lilies, irises, orchids, cattails, palms. Dicotyledons include almost all the familiar trees and shrubs (other than conifers) and many of the herbs (non-woody plants).

[0104] Tomato cultures are the ideal recipients for repetitive HRGP modules to be hydroxylated and glycosylated: Tomato is readily transformed. The cultures produce cell surface HRGPs in high yields easily eluted from the cell surface of intact cells and they possess the required post-translational enzymes unique to plants—HRGP prolyl hydroxylases, hydroxyproline O-glycosyltransferases and other specific glycosyltransferases for building complex polysaccharide side chains. Furthermore, tomato genetics, and tomato leaf disc transformation/plantlet regeneration are well worked out.

[0105] E. Introduction of Nucleic Acid

[0106] Expression constructs of the present invention may be introduced into host cells (e.g. plant cells) using methods known in the art. In one embodiment, the expression constructs are introduced into plant cells by particle mediated gene transfer. Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument described in McCabe, U.S. Pat. No. 5,584,807, the entire contents of which are herein incorporated by reference. This method involves coating the nucleic acid sequence of interest onto heavy metal particles, and accelerating the coated particles under the pressure of compressed gas for delivery to the target tissue.

[0107] Other particle bombardment methods are also available for the introduction of heterologous nucleic acid sequences into plant cells. Generally, these methods involve

depositing the nucleic acid sequence of interest upon the surface of small, dense particles of a material such as gold, platinum, or tungsten. The coated particles are themselves then coated onto either a rigid surface, such as a metal plate, or onto a carrier sheet made of a fragile material such as mylar. The coated sheet is then accelerated toward the target biological tissue. The use of the flat sheet generates a uniform spread of accelerated particles which maximizes the number of cells receiving particles under uniform conditions, resulting in the introduction of the nucleic acid sample into the target tissue.

[0108] Alternatively, an expression construct may be inserted into the genome of plant cells by infecting them with a bacterium, including but not limited to an *Agrobacterium* strain previously transformed with the nucleic acid sequence of interest. Generally, disarmed *Agrobacterium* cells are transformed with recombinant Ti plasmids of *Agrobacterium tumefaciens* or Ri plasmids of *Agrobacterium rhizogenes* (such as those described in U.S. Pat. No. 4,940,838, the entire contents of which are herein incorporated by reference) which are constructed to contain the nucleic acid sequence of interest using methods well known in the art (Sambrook, J. et al., (1989) supra). The nucleic acid sequence of interest is then stably integrated into the plant genome by infection with the transformed *Agrobacterium* strain. For example, heterologous nucleic acid sequences have been introduced into plant tissues using the natural DNA transfer system of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* bacteria (for review, see Klee et al. (1987) Ann. Rev. Plant Phys. 38:467-486).

[0109] One of skill in the art knows that the efficiency of transformation by *Agrobacterium* may be enhanced by using a number of methods known in the art. For example, the inclusion of a natural wound response molecule such as acetosyringone (AS) to the *Agrobacterium* culture has been shown to enhance transformation efficiency with *Agrobacterium tumefaciens* [Shahla et al. (1987) Plant Molec. Biol. 8:291-298]. Alternatively, transformation efficiency may be enhanced by wounding the target tissue to be transformed. Wounding of plant tissue may be achieved, for example, by punching, maceration, bombardment with microprojectiles, etc. [see, e.g., Bidney et al. (1992) Plant Molec. Biol. 18:301-313].

[0110] It may be desirable to target the nucleic acid sequence of interest to a particular locus on the plant genome. Site-directed integration of the nucleic acid sequence of interest into the plant cell genome may be achieved by, for example, homologous recombination using *Agrobacterium*-derived sequences. Generally, plant cells are incubated with a strain of *Agrobacterium* which contains a targeting vector in which sequences that are homologous to a DNA sequence inside the target locus are flanked by *Agrobacterium* transfer-DNA (T-DNA) sequences, as previously described (Offringa et al., (1996), U.S. Pat. No. 5,501,967, the entire contents of which are herein incorporated by reference). One of skill in the art knows that homologous recombination may be achieved using targeting vectors which contain sequences that are homologous to any part of the targeted plant gene, whether belonging to the regulatory elements of the gene, or the coding regions of the gene. Homologous recombination may be achieved at any region of a plant gene so long as the nucleic acid sequence of regions flanking the site to be targeted is known.

[0111] Where homologous recombination is desired, the targeting vector used may be of the replacement- or insertion-type (Offringa et al. (1996), supra). Replacement-type vectors generally contain two regions which are homologous with the targeted genomic sequence and which flank a heterologous nucleic acid sequence, e.g., a selectable marker gene sequence. Replacement type vectors result in the insertion of the selectable marker gene which thereby disrupts the targeted gene. Insertion-type vectors contain a single region of homology with the targeted gene and result in the insertion of the entire targeting vector into the targeted gene.

[0112] Other methods are also available for the introduction of expression constructs into plant tissue, e.g., electroinjection (Nan et al. (1995) In "Biotechnology in Agriculture and Forestry," Ed. Y. P. S. Bajaj, Springer-Verlag Berlin Heidelberg, Vol 34:145-155; Griesbach (1992) Hort-Science 27:620); fusion with liposomes, lysosomes, cells, minicells or other fusible lipid-surfaced bodies (Fraley et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:1859-1863; polyethylene glycol (Krens et al. (1982) *Nature* 296:72-74); chemicals that increase free DNA uptake; transformation using virus, and the like.

[0113] In one embodiment, the present invention contemplates introducing nucleic acid via the leaf disc transformation method. Horsch et al. *Science* 227:1229-1231 (1985). Briefly, disks are punched from the surface of sterilized leaves and submerged with gentle shaking into a culture of *A. tumefaciens* that had been grown overnight in luria broth at 28° C. The disks are then blotted dry and placed upside-down onto nurse culture plates to induce the regeneration of shoots. Following 2-3 days, the leaf disks are transferred to petri plates containing the same media without feeder cells or filter papers, but in the presence of carbenicillin (500 µg/ml) and kanamycin (300 µg/ml) to select for antibiotic resistance. 2-4 weeks later, the shoots that developed are removed from calli and placed into root-inducing media with the appropriate antibiotic. These shoots were then further transplanted into soil following the presence of root formation.

[0114] Experimental

[0115] The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

[0116] In the experimental disclosure which follows, the following abbreviations apply: g (gram); mg (milligrams); µg (microgram); M (molar); mM (milliMolar); µM (microMolar); nm (nanometers); L (liter); ml (milliliter); µl (microliters); ° C. (degrees Centigrade); m (meter); sec. (second); DNA (deoxyribonucleic acid); cDNA (complementary DNA); RNA (ribonucleic acid); mRNA (messenger ribonucleic acid); X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); LB (Luria Broth), PAGE (polyacrylamide gel electrophoresis); NAA (α-naphtaleneacetic acid); BAP (6-benzyl aminopurine); Tris (tris(hydroxymethyl)-aminomethane); PBS (phosphate buffered saline); 2×SSC (0.3

M NaCl, 0.03 M Na₃ citrate, pH 7.0); Agri-Bio Inc. (North Miami, Fla.); Analytical Scientific Instruments (Alameda, Calif.); BioRad (Richmond, Calif.); Clontech (Palo Alto Calif.); Delmonte Fresh Produce (Kunia, Hi.); Difco Laboratories (Detroit, Mich.); Dole Fresh Fruit (Wahiawa, Hi.); Dynatech Laboratory Inc. (Chantilly Va.); Gibco BRL (Gaithersburg, Md.); Gold Bio Technology, Inc. (St. Louis, Mo.); GTE Corp. (Danvers, Mass.); MSI Corp. (Micron Separations, Inc., Westboro, Mass.); Operon (Operon Technologies, Alameda, Calif.); Pioneer Hi-Bred International, Inc. (Johnston, Iowa); 5 Prime 3 Prime (Boulder, Colo.); Sigma (St. Louis, Mo.); Promega (Promega Corp., Madison, Wis.); Stratagene (Stratagene Cloning Systems, La Jolla, Calif.); USB (U.S. Biochemical, Cleveland, Ohio).

Example 1

Determination of the Peptide Sequence of Acacia Gum Arabic Glycoproteins

[0117] In this example, GAGP (SEQ ID NO:15) was isolated and (by using chymotrypsin) the deglycosylated polypeptide backbone was prepared. Although GAGP does not contain the usual chymotryptic cleavage sites, it does contain leucyl and histidyl residues which are occasionally cleaved. Chymotrypsin cleaved sufficient of these "occasionally cleaved" sites to produce a peptide map of closely related peptides.

[0118] Purification and Deglycosylation of GAGP (SEQ ID NO: 15). GAGP was isolated via preparative Superose-6 gel filtration. Anhydrous hydrogen fluoride deglycosylated it (20 mg powder/mL HF at 4° C., repeating the procedure twice to ensure complete deglycosylation), yielding dGAGP which gave a single symmetrical peak (data not shown) after rechromatography on Superose-6. Further purification of dGAGP by reverse phase chromatography also gave a single major peak, showing a highly biased but constant amino acid composition in fractions sampled across the peak. These data indicated that dGAGP was a single polypeptide component sufficiently pure for sequence analysis.

[0119] Sequence Analysis. An incomplete pronase digest gave a large peptide PRP3 which yielded a partial sequence (Table 2) containing all the amino acids present in the suggested dGAGP repeat motif. In view of the limitations of pronase, for further peptide mapping and to obtain more definitive sequence information, dGAGP was digested with chymotrypsin, followed by a two-stage HPLC fractionation scheme. Initial separation of the chymotryptides on a PolySULFOETHYL Atm (designated PSA, PolyLC, Inc. Ellicott City, Md.) cation exchanger yielded three major fractions: S1 and S2 increased with digestion time while S3 showed a concomitant decrease. Further chromatography on PRP-1 resolved PSA fractions S1 and S2 into several peptides.

TABLE 2

Amino Acid Sequences of the Gum Arabic Glycoprotein Polypeptide Backbone	
Peptide	Sequence
S1P5	Ser-Hyp-Hyp-Hyp-Hyp-Leu-Ser-Hyp-Ser-Leu-Thr-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro- (Pro) (SEQ ID NO:16)
S1P3	Ser-Hyp-Hyp-Hyp-Hyp-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro- (Pro) (SEQ ID NO:17)
S3	Ser-Hyp-Hyp-Hyp-Thr-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Gly-Pro-His-Ser-Hyp-Hyp-Hyp- (Hyp) (SEQ ID NO:18)
S1P2	Ser-Hyp-Hyp-Hyp-Ser-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Thr-Gly-Pro-His (SEQ ID NO:19)
S2P1	Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Gly-Pro-His (SEQ ID NO:20)
S2P2a	Ser-Hyp-Ser-Hyp-Ala-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro-His (SEQ ID NO:21)
S2P2b	Ser-Hyp-Leu-Pro-Thr-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro-His (SEQ ID NO:22)
S2P3a	Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro-His (SEQ ID NO:23)
S2P4	Ser-Hyp-Hyp-Leu-Thr-Hyp-Thr-Hyp-Hyp-Leu-Leu-Pro-His (SEQ ID NO:24)
S1P4	Ser-Hyp-Leu-Pro-Thr-Leu-Ser-Hyp-Leu-Pro-Ala/Thr-Hyp-Thr-Hyp-Hyp-Hyp-Gly-Pro-His (SEQ ID NOS:25 and 26)
Consensus	Ser-Hyp-Hyp-Hyp-Thr/Hyp-Leu-Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Leu-Gly-Pro-His (SEQ ID NOS:27 and 28)
	(Leu) (Pro) (Ser) (Leu) (Leu) (Ala) (Hyp) (Pro)
	(Pro)

[0120] Edman degradation showed that these chymotrypsins were closely related to each other, to the partial sequence of the large pronase peptide (Table 2), and to the major pronase peptide of GAGP isolated earlier by Delon-

[0123] a) Ser-Hyp₄ (SEQ ID NO:3) Gene Cassette

[0124] A synthetic gene encoding the extensin-like Ser-Hyp₄ module is constructed using the following partially overlapping sets of oligonucleotide pairs.

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                    5'-Linker:
Amino Acid:      A   G   S   S   T   R   A   S   P   (P   P   P)      (SEQ ID NO:29)
                  5'-GCT GGA TCC TCA ACC CGG GCC TCA CCA      (SEQ ID NO:30)

                  CGA CCT AGG ACT TGG CCC CCG AGT GGT GGT GGT GGA-5'      (SEQ ID NO:31)

                    3' Linker (for pBI121-Sig-EGFP):
Amino Acid:      P   P   P   S   P   V   A   R   N   S   P   P      (SEQ ID NO:32)
                  5'-CCA CCA CCT TCA CCC GTC GCC CGG AAT TCA CCA CCC      (SEQ ID NO:33)

                  AGT GGC CAG CGG GCC TTA AGT GGT GGG-5'      (SEQ ID NO:34)

                    3' Linker (for pBI121-Sig):
Amino Acid:      5'-CCA CCA CCT TAA TAG AGC TCC CCC      (SEQ ID NO:35)

                  ATT ATC TCG AGG GGG-5'      (SEQ ID NO:36)

                    Internal Repeat
Amino Acid:      P   P   P   S   P   P   P   P   S   P      (SEQ ID NO:37)
                  5'-CCA CCA CCT TCA CCT CCA CCC CCA TCT CCA      (SEQ ID NO:38)

                  AGT GGA GGT GGG GGT AGA GGT GGT GGT GGA-5'      (SEQ ID NO:39)

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may (see above). Indeed, all can be related to a single 19-residue consensus sequence with minor variation in some positions (Table 2). These peptides also reflect the overall amino acid composition and are therefore evidence of a highly repetitive polypeptide backbone with minor variations in the repetitive motif; these include occasional substitution of Leu for Hyp and Ser. Remarkably, fifteen residues of the consensus sequence are “quasi-palindromic” i.e. the side chain sequence is almost the same whether read from the N-terminus or C-terminus.

Example 2

Construction of Synthetic HRGP Gene Cassettes

[0121] Synthetic gene cassettes encoding contiguous and noncontiguous Hyp modules are constructed using partially overlapping sets consisting of oligonucleotide pairs, “internal repeat pairs” and “external 3'- and 5'-linker pairs” respectively, all with complementary “sticky” ends. The design strategy for the repetitive HRGP modules combines proven approaches described earlier for the production in *E. coli* of novel repetitive polypeptide polymers (McGrath et al. [1990] Biotechnol. Prog. 6:188), of a repetitious synthetic analog of the bioadhesive precursor protein of the mussel *Mytilus edulis*, of a repetitive spider silk protein (Lewis et al. [1996] Protein Express. Purif. 7:400), and of a highly repetitive elastin-like polymer in tobacco [Zhang, X., Urry, D. W., and Daniell, H. “Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plants,” *Plant Cell Reports*, 16: 174 (1996)].

[0122] The basic design strategy for synthetic HRGP gene cassettes is illustrated by the following illustrative constructs.

[0125] Conversion of the “internal” and 5' & 3“external” gene cassettes to long duplex DNA is accomplished using the following steps:

[0126] 1. Heat each pair of complementary oligonucleotides to 90° and then anneal by cooling slowly to 60° thereby forming short duplex internal and external DNAs.

[0127] 2. Combine the 5' external linker duplex with the internal repeat duplexes in an approximately 1:20 molar ratio and anneal by further cooling to yield long duplex DNA capped by the 5' linker. The 5' linker is covalently joined to the internal repeat duplex by ligation using T4 DNA ligase. (Preferably up to 50, more preferably up to 30, repeats of the internal repeat duplex can be used).

[0128] 3. In molar excess, combine the 3' external linker duplex with the above 5' linker-internal repeat duplex, anneal and ligate as described above.

[0129] 4. Digest the 5' linker-internal repeat-3' linker duplex with BamHI (cuts within the 5'-linker) and EcoRI (cuts within the 3'-linker).

[0130] 5. Size fractionate the reaction products using Sephacryl gel permeation chromatography to select constructs greater than 90 bp.

[0131] 6. Insert the sized, digested synthetic gene cassette into a plasmid having a polylinker containing BamHI and EcoRI sites (e.g., pBluescript SK⁺ or KS⁺[Stratagene]).

[0132] 7. Transform *E. coli* cells (e.g., by electroporation or the use of competent cells) with the plasmid into which the synthetic gene construct has been ligated.

[0133] 8. Following *E. coli* transformation, the internal repeat oligonucleotides are used to screen and identify Ampicillin-resistant colonies carrying the synthetic gene construct.

[0134] 9. The insert contained on the plasmids within the Ampicillin-resistant colonies are sequenced to confirm the fidelity of the synthtic gene construct.

[0135] b) GAGP (SEQ ID NO:15) Consensus Sequence Cassette A synthetic gene cassette encoding the GAGP consensus sequence is generated as described above using the following 5' linker, internal repeat and 3' linker duplexes.

Example 3

Isolation of Tomato P1 Extensin cDNA Clones

[0138] In order to obtain the tomato P1 extensin signal sequence (i.e., signal peptide), P1 extensin cDNA clones were isolated using oligonucleotides designed after the P1-unique protein sequence: Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys (SEQ ID NO: 51). When present at the N-terminus of a protein sequence, the P1 extensin signal sequence directs the nascent peptide chain to the ER.

5'-Linker	
Amino Acid:	
A A G S S T R A (S P S)	(SEQ ID NO:40)
5'-GCT GCC GGA TCC TCA ACC CGG GCC-3'	(SEQ ID NO:41)
3'-CGA CGG CCT AGG AGT TGG GCC CGG AGT GGC AGT-5'	(SEQ ID NO:42)
3'-Linker (for pBI121-Sig-EGFP)	
Amino Acid:	
S P S P V A R N S P P	(SEQ ID NO:43)
5'-TCA CCC TCA CCG GTC GCC CGG AAT TCA CCA CCC-3'	(SEQ ID NO:44)
3'GGC CAG CGG GCC TTA AGT GGT GGG-5'	(SEQ ID NO:45)
3'-Linker (for pBI121-Sig)	
Amino Acid:	
5'-TCA CCC TCA TAA TAG AGC TCC CCC-3'	(SEQ ID NO:46)
3'ATT ATC TCG AGG GGG-5'	(SEQ ID NO:47)
Internal Repeat	
Amino Acid:	
S P S P T P T P P P G P H S P P P T L	(SEQ ID NO:48)
5'-TCA CCC TCA CCA ACT CCT ACC CCA CCA CCT GGT CCA CAC TCA CCA CCA CCA ACA TTG-3'	(SEQ ID NO:49)
3'-GGT TGA GGA TGG GGT GGT GGA CCA GGT GTG AGT GGT GGT GGT TGT AAC AGT GGG AGT-5'	(SEQ ID NO:50)

[0136] Conversion of the “internal” AGP-like motif and 5' & 3’“external” gene cassettes to long duplex DNA is accomplished using the steps described in section a) above. Up to fifty (50) repeats of the internal repeat duplex are desirable (more preferably up to thirty (30) repeats, and more preferably approximately twenty (20) repeats) (i.e., the wild-type protein contains 20 of these repeats).

[0137] Since the above GAGP internal repeat is a consensus sequence, it is also desirable to have repeats that comprise a repeat sequence that varies from the consensus sequence (see e.g. Table 2 above). In this regard, the variant sequences are likely to be glycosylated in a slightly different manner, which may confer different properties (e.g. more soluble etc.). Other constructs are shown for other illustrative modules in Table 3.

Example 4

Construction of One Embodiment of an Expression Vector

[0139] pBI121 is an expression vector which permits the high level expression and secretion of inserted genes in plant cells (e.g., tomato, tobacco, members of the genus Solanace, members of the family Leguminoseae, non-graminaceous monocots). pBI121 contains the ³⁵S CaMV promoter, the tobacco (*Nicotiana plumbaginifolia*) extensin signal sequence, a EGFP gene, the termination/polyadenylation signal from the nopaline synthetase gene (NOS-ter), a kanamycin-resistance gene (nptII) and the right and left borders of T-DNA to permit transfer into plants by *Agrobacterium*-mediated transformation.

TABLE 3

Illustrative HRGP Synthetic Gene Modules		
1. MODULES FOR AGP-LIKE SEQUENCES		
a. The [SP] _n Module		
[SP] _n Internal Repeat Oligo's:		(SEQ ID NO:52)
5'-TCA CCC TCA CCA TCT CCT TCC CCA TCA CCC		
GGT AGA GGA AGC AGT AGT GGG AGT GGG AGT-5'		(SEQ ID NO:53)
The [SP] _n 3' & 5' External Linkers for both plasmids are the same as for the GAGP module.		
b. The [AP] _n Module		
[AP] _n Internal Repeat Oligo's:		(SEQ ID NO:54)
5'-GCT CCA GCA CCT GCC CCA GCC CCT GCA CCA-3'		
GCA CGG GGT CGG GGA CGT GGT-5'		(SEQ ID NO:55)
[AP] _n External Linker Oligo's for plasmid pBI121-Sig-EGFP:		(SEQ ID NO:56)
5'-Linker: 5'-GCT GCC GGA TCC TCA ACC CGG		
3'-CGA CGG CCT AGG AGT TGG GCC CGA GGT CGT-5'		(SEQ ID NO:57)
3'-Linker: 5'-GCT CCA GCA CCG GTC GCC CGG AAT TCA CCA CCC-3'		(SEQ ID NO:58)
3'- GGC CAG CGG GCC TTA AGT GGT GGG-5'		(SEQ ID NO:59)
[AP] _n External 3' Linker Oligos for plasmid pBI121-Sig:		(SEQ ID NO:60)
5'-GCT CCA GCA TAA TAG AGC TCC CCC		
ATT ATC TCG AGG GGG-5'		(SEQ ID NO:61)
c. The [TP] _n Module		
[TP] _n Internal Repeat Oligo's:		(SEQ ID NO:62)
5'-ACA CCA ACC CCT ACT CCC ACG CCA ACA CCT ACA CCC ACT CCA		
GGA TGA GGG TGC GGT TGT GGA TCT GGG TGA GGT TGT GGT TGG-5'		(SEQ ID NO:63)
[TP] _n External Linker Oligo's for pBI121-Sig-ECFP:		(SEQ ID NO:64)
5'-Linker: 5'-GCT GCC GGA TCC TCA ACC CGG		
3'-CGA CGG CCT AGG AGT TGG GCC TGT GGT TGG-5'		(SEQ ID NO:65)
3'-Linker: 5'-ACA CCA ACC CCG GTC GCC CGG AAT TCA CCA CCC-3'		(SEQ ID NO:66)
GGC CAG CGG GCC TTA AGT GGT GGG-5'		(SEQ ID NO:67)
[TP] _n External 3' Linker Oligos for pBI121-Sig:		(SEQ ID NO:68)
5'-ACA CCA ACC TAA TAG AGC TCC CCC		
ATT ATC TCG AGG GGG-5'		(SEQ ID NO:69)

TABLE 3-continued

Illustrative HRGP Synthetic Gene Modules	
2. MODULES FOR EXTENSIN-LIKE SEQUENCES	
a. The [SPP] _n Module [SPP] _n Internal Repeat Oligo's: 5'-CCA CCA TCA CCC TCT CCT CCA TCA CCC CCA TCC CCA CCA TCA GGT GGG AGA GGA GGT AGT GGG GGT AGG GGT GGT AGT GGT AGT-5' [SPP] _n External Linkers for pBE121-Sig-EGFP: 5'Linker: 5'-GCT GCC GGA TCC TCA ACC CGG GCC 3'-CGA CGG CCT AGG AGT TGG GCC CGG GGT GGT AGT-5' 3'Linker: 5'-CCA CCA TCA CCG GTC GCC CGG AAT TCA CCA CCC-3' GGC CAG CGG GCC TTA AGT GGT GGG-5' [SPP] _n External 3' Linker for pBE121-Sig: 5'-CCA CCA TCA TAA TAG AGC TCC CCC ATT ATC TCG AGG GGG-5'	(SEQ ID NO:70) (SEQ ID NO:71) (SEQ ID NO:72) (SEQ ID NO:73) (SEQ ID NO:74) (SEQ ID NO:75) (SEQ ID NO:76) (SEQ ID NO:77)
b. The [SPPP] _n Module [SPPP] _n Internal Repeat Oligo's: 5'-CCA CCA CCT TCA CCA CCT CCA TCT CCC CCA CCT TCC CCT CCA CCA TCA AGT GGT GGA GGT AGA GGG GGT GGA AGG GGA GGT GGT GGT GGA-5' [SPPP] _n External Linker Oligo's for pBI121-Sig-EGFP: 5'-Linker: 5'-GCT GGA TCC TCA ACC CGG GCC TCA 3'-CGA CCT AGG AGT TGG GCC CGG AGT GGT GGT GGA-5' 3'-Linker: 5'-CCA CCA CCT TCA CCG GTC GCC CGG AAT TCA CCA CCC-3' (SEQ ID NO:83) GGC CAG CGG GCC TTA AGT GGT GGG-5' [SPPP] _n External 3' Linker Oligos for pBI121-Sig: 5'-CCA CCA CCT TAA TAG AGC TCC CCC ATT ATC TCG AGG GGG-5'	(SEQ ID NO:78) (SEQ ID NO:79) (SEQ ID NO:80) (SEQ ID NO:81) (SEQ ID NO:82) (SEQ ID NO:84) (SEQ ID NO:85)
d. The P3-Type Extensin Palindromic Module: P3-Type Extensin Palindromic Internal Repeat Oligo's: 5'-CCA CCA CCT TCA CCC TCT CCA CCT CCA CCA TCT CCG TCA CCA AGT GGG AGA GGT GGA GGT GGT AGA GGC AGT GGT GGT GGA-5'	(SEQ ID NO:86) (SEQ ID NO:87)

[0140] The presence of the extensin signal sequence at the N-terminus of proteins encoded by genes inserted into the pBI121 expression vector (e.g., HRGPs encoded by synthetic gene constructs). The tobacco signal sequence was demonstrated to target extensin fusion proteins through the ER and Golgi for posttranslational modifications, and finally to the wall. The targeted expression of recombinant HRGPs is not dependent upon the use of the tobacco extensin signal sequence. Signal sequences involved in the transport of extensins and extensin modules in the same plant family (Solanaceae) as tobacco may be employed; alternatively, the signal sequence from tomato P1 extensin may be employed.

[0141] The EGFP gene encodes a green fluorescent protein (GFP) appropriately red-shifted for plant use (the EGFP gene encodes a S65T variant optimized for use in plants and is available from Clontech). Other suitable mutants may be employed (see Table 1). These modified GFPs allow the detection of less than 700 GFP molecules at the cell surface. The use of a GFP gene provides a reporter gene and permits the formation of fusion proteins comprising repetitive HRGP modules. GFPs require aerobic conditions for oxidative formation of the fluorophore. It is functional at the lower temperatures used for plant cell cultures, normally it does not adversely affect protein function.

[0142] Plasmids pBI121-Sig and pBI121-Sig-EGFP are constructed as follows. For both plasmids, the GUS gene present in pBI121 (Clontech) is deleted by digestion with BamHI and SstI and a pair of partially complementary oligonucleotides encoding the tobacco extensin signal sequence is annealed to the BamHI and SstI ends. The oligonucleotides encoding the 21 amino acid extensin signal sequence have the following sequence: 5'-GA TCC GCA ATG GGA AAA ATG GCT TCT CTA TTT GCC ACA TTT TTA GTG GTT TTA GTG TCA CTT AGC TTA GCA CAA ACA ACC CGG GTA CCG GTC GCC ACC ATG GTG TAA AGC GGC CGC GAG CT-3' (SEQ ID NO:98) and 5'-C GCG GCC GCT TTA CAC CAT GGT GGC GAC CGG TAC CCG GGT TGT TTG TGC TAA GCT AAG TGA CAC TAA AAC CAC TAA AAA TGT GGC AAA TAG AGA AGC CAT TTT TCC CAT TGC G-3' (SEQ ID NO:99). In addition to encoding the extensin signal sequence, this pair of oligonucleotides, when inserted into the digested pBI121 vector, provides a BamHI site (5' end) and XmaI and SstI sites (3' end). The XmaI and SstI sites allow the insertion of the GFP gene. The modified pBI121 vector lacking the GUS gene and containing the synthetic extensin signal sequence is termed pBI121-Sig. Proper construction of pBI121 is confirmed by DNA sequencing.

[0143] The GFP gene (e.g., the EGFP gene) is inserted into pBI121-Sig to make pBI121-Sig-EGFP as follows. The EGFP gene is excised from pEGFP (Clontech), as a 1.48 kb XmaI/NotI fragment (base pairs 270 to 1010 in pEGFP). This 1.48 kb XmaI/NotI fragment is then annealed and ligated to a synthetic 3' linker (see above). The EGFP-3' linker is then digested with SstI to produce an XmaI/SstI EGFP fragment which is inserted into the XmaI/SstI site of pBI121-Sig to create pBI121-Sig-EGFP. The AgeI (dis-

cussed below), XmaI and SstI sites provide unique restriction enzyme sites. Proper construction of the plasmids is confirmed by DNA sequencing.

[0144] The EGFP sequences in pBI121-Sig-EGFP contain an AgeI site directly before the translation start codon (i.e., ATG) of EGFP. Synthetic HRGP gene cassettes are inserted into the plasmid between the signal sequence and the EGFP gene sequences as XmaI/AgeI fragments; the HRGP gene cassettes are excised as XmaI/AgeI fragments from the pBluescript constructs described in Ex.2. Proper construction of HRGP-containing expression vectors is confirmed by DNA sequencing and/or restriction enzyme digestion.

[0145] Expression of the synthetic HRGP gene cassettes is not dependent upon the use of the pBI121-Sig and pBI121-Sig-EGFP gene cassette. Analogous expression vectors containing other promoter elements functional in plant cells may be employed (e.g., the CaMV region IV promoter, ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu) promoter, the nopaline promoter, octopine promoter, mannopine promoter, the β -conglycinin promoter, the ADH promoter, heat shock promoters, tissue-specific promoters, e.g., promoters associated with fruit ripening, promoters regulated during seed ripening (e.g., promoters from the napin, phaseolin and glycinin genes). For example, expression vectors containing a promoter that directs high level expression of inserted gene sequences in the seeds of plants (e.g., fruits, legumes and cereals, including but not limited to corn, wheat, rice, tomato, potato, yam, pepper, squash cucumbers, beans, peas, apple, cherry, peach, black locust, pine and maple trees) may be employed. Expression may also be carried out in green algae.

[0146] In addition, alternative reporter genes may be employed in place of the GFP gene. Suitable reporter genes include β -glucuronidase (GUS), neomycin phosphotransferase II gene (nptII), alkaline phosphatase, luciferase, CAT (Chloramphenicol AcetylTransferase). Preferred reporter genes lack Hyp residues. Further, the proteins encoded by the synthetic HRGP genes need not be expressed as fusion proteins. This is readily accomplished using the pBI121-Sig vector.

Example 5

Expression of Recombinant HRGPs In Tomato Cell Suspension Cultures

[0147] The present invention contemplates recombinant HRGPs encoded by expression vectors comprising synthetic HRGP gene modules are expressed in tomato cell suspension cultures. The expression of recombinant HRGPs in tomato cell suspension cultures is illustrated by the discussion provided below for recombinant GAGP expression.

[0148] a) Expression of Recombinant GAGP

[0149] An expression vector containing the synthetic GAGP gene cassette (capable of being expressed as a fusion with GFP or without GFP sequences) is introduced into tomato cell suspension cultures. A variety of means are

known to the art for the transfer of DNA into tomato cell suspension cultures, including Agrobacterium-mediated transfer and biolistic transformation.

[0150] Agrobacterium-mediated transformation: The present invention contemplates transforming both suspension cultured cells (Bonnie Best cultures) and tomato leaf discs by mobilizing the above-described plasmid constructions (and others) from *E. coli* into *Agrobacterium tumefaciens* strain LBA4404 via triparental mating. Positive colonies are used to infect tomato cultures or leaf discs (*Lysopersicon esculentum*). Transformed cells/plants are selected on MSO medium containing 500 mg/mL carbenicillin and 100 mg/mL kanamycin. Expression of GFP fusion products are conveniently monitored by fluorescence microscopy using a high Q FITC filter set (Chroma Technology Corp.). FITC conjugates (e.g. FITC-BSA) can be used along with purified recombinant GFP as controls for microscopy set-up. Cultured tomato cells show only very weak autofluorescence. Thus, one can readily verify the spatiotemporal expression of GFP-Hyp module fusion products.

[0151] Transgenic cells/plants can be examined for transgene copy number and construct fidelity genomic Southern blotting and for the HRGP construct mRNA by northern blotting, using the internal repeat oligonucleotides as probes. Controls include tissue/plants which are untransformed, transformed with the pBI121 alone, pBI121 containing only GFP, and pBI121 having the signal sequence and GFP but no HRGP synthetic gene.

tissue. Controls include: particles without DNA, particles which contain PBI121 only, and particles which contain PBI121 and GFP.

[0153] b) Expression of Other HRGPs of Interest

[0154] As noted above, the present invention contemplates expressing a variety of HRGPs, fragments and variants. Such HRGPs include, but are not limited to, RPRps, extensins, AGPs and other plant gums (e.g. gum Karaya, gum Tragacanth, gum Ghatti, etc.). HRGP chimeras include but are not limited to HRGP plant lectins, including the solanaceous lectins, plant chitinases, and proteins in which the HRGP portion serves as a spacer (such as in sunflower). The present invention specifically contemplates using the HRGP modules (described above) as spacers to link non-HRGP proteins (e.g., enzymes) together.

Example 6

Construction Of A Synthetic HRGP Gene Cassette
Incorporating A GAGP Construct

[0155] Synthetic gene cassettes encoding contiguous and noncontiguous Hyp modules were constructed using partially overlapping sets consisting of oligonucleotide pairs, "internal repeat pairs" and "external 3'- and 5'-linker pairs" respectively, all with complementary "sticky" ends. The following 5'-linker, internal repeat and 3'-linker duplexes were employed:

5'-Linker	
A A G S S T R A (S P S)	(SEQ ID NO:40)
5'-GCT GCC GGA TCC TCA ACC CGG GCC-3'	(SEQ ID NO:41)
3'-CGA CGG CCT AGG AGT TGG GCC CGG AGT GGG AGT-5'	(SEQ ID NO:42)
3'-Linker	
S P S P V A R N S P P	(SEQ ID NO:43)
5'-TCA CCC TCA CCG GTC GCC CGG AAT TCA CCA CCC-3'	(SEQ ID NO:44)
3'-GGC CAG CGG GCC TTA AGT GGT GGG-5'	(SEQ ID NO:45)
Internal Repeat	
S P S P T P T A P P G P H S P P P T L	(SEQ ID NO:100)
[5'-TCA CCC TCA CCA ACT CCT ACC GCA CCA CCT GGT CCA CAC TCT CCA CCA CCA ACA TTG-3'	(SEQ ID NO:101)]
[3'-AGT GGG AGT GGT TGA GGA TGG CGT GGT GGA CCA GGT GTG AGA GGT GGT GGT TGT AAC-5'	(SEQ ID NO:102)] ₂
then:	
S P S P T P T A P P G P H S P P P S L	(SEQ ID NO:103)
5'-TCA CCC TCA CCA ACT CCT ACC GCA CCA CCT GGT CCA CAC TCT CCA CCA CCA TCA TTG-3'	(SEQ ID NO:104)
3'-AGT GGG AGT GGT TGA GGA TGG CGT GGT GGA CCA GGT GTG AGA GGT GGT GGT AGT AAC-5'	(SEQ ID NO:105)

[0152] Microprojectile bombardment: 1.6 M gold particles are coated with each appropriate plasmid construct DNA for use in a Biolistic particle delivery system to transform the tomato suspension cultures/callus or other

[0156] The following synthetic gene (SEQ ID NO:106) was eventually expressed (SEQ ID NO:107) in tobacco and tomato cell cultures and tobacco plants using the above constructs:

M G K M A S L F A T F L V V L V
 5'-GGA TCC GCA ATG GGA AAA ATG GCT TCT CTA TTT GCC ACA TTT TTA GTC GTT TTA GTG
 3'-CCT AGG CGT TAC CCT TTT TAC CGA AGA GAT AAA CGG TGT AAA AAT CAC CAA AAT CAC
 S L S L A Q T T R D S P S P T P T A P
 TCA CTT AGC TTA GCA CAA ACA ACC CGG GAC TCA CCC TCA CCA ACT CCT ACC GCA CCA
 AGT GAA TCG AAT CGT GTT TGT TGG GCC CTG AGT GGG AGT GGT TGA GGA TGG CGT GGT
 P G P H S P P P T L S P S P T P T A P
 CCT GGT CCA CAC TCT CCA CCA CCA ACA TTG TCA CCC TCA CCA ACT CCT ACC GCA CCA
 GGA CCA GGT GTG AGA GGT GGT GGT TGT AAC AGT GGG AGT GGT TGA GGA TGG CGT GGT
 P G P H S P P P T L S P S P T P T A P
 CCT GGT CCA CAC TCA CCA CCA CCA ACA TTG TCA CCC TCA CCA ACT CCT ACC GCA CCA
 GGA CCA GGT GTG AGT GGT GGT GGT TGT AAC AGT GGG AGT GGT TGA GGA TGG CGT GGT
 P G P H S P P P S L S P S P V
 CCT GGT CCA CAC TCA CCA CCA CCA TCA TTG TCA CCC TCA CCG GTC GCC ACC-gfp-3'
 GGA CCA GGT GTG AGT GGT GGT GGT AGT AAC AGT GGG AGT GGC CAG CGG TGG-gfp-5'

[0157] This example involved: (A) Oligonucleotide pair preparation; (B) Oligonucleotide polymerization; (C) Construct precipitation; (D) Restriction of gene 3'-linker and 5'-linker capped ends; (E) Size-fractionation and removal of enzyme contaminants; (F) Gene insertion into SK plasmid vector. All SDS-PAGE purified oligonucleotides were synthesized by Gibco-BRL.

[0158] (A) Oligonucleotide Pair Preparation

[0159] In separate Eppendorf tubes were combined:

[0160] Tube 1) 5.5 μ l GAGP internal repeat sense oligonucleotide (0.5 nmol/ μ l), 5.5 μ l GAGP internal repeat antisense oligonucleotide (0.5 mmol/ μ l), 11 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs);

[0161] Tube 2) 2 μ l 5'-sense linker (0.05 mmol/ μ l), 2 μ l 5'-antisense linker (0.05 nmol/ μ l), 1 μ l H₂O, 5 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs);

[0162] Tube 3) 2 μ l 3'-sense linker (1 nmol/ μ l), 2 μ l 3'-antisense linker (1 nmol/ μ l), 1 μ l water, 5 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs).

[0163] All tubes were heated to 90-95° C. for 5 minutes, then slowly cooled over the next 3 hours to 45° C. The tubes were then incubated at 45° C. for 2 hours.

[0164] (B) Oligonucleotide Polymerization

[0165] 10 μ l of solution from Tube 1 (internal repeat pair) was combined with 10 μ l of solution from Tube 2 (5' linker pair), and incubated at 17° C. for 3 hours. To this mixture was added 80 μ l water and 21 (4000 U) T4 DNA ligase (New England Biolabs), and again incubated at 12-15° C. for 36 hours. The degree of polymerization was verified on 2.2% agarose gel (Fisher).

[0166] The 3'-end of the polymer was then capped by adding 50 μ l of the ligated GAGP 5'-linker mixture from above to 5 μ l of solution from Tube 3 (3'-linker), heating to 30° C., and incubating at 17° C. for 3 hours. 20 μ l water and 2 μ l T4 DNA ligase (New England Biolabs) was then added, and the solution incubated at 12-15° C. for 36 hr. Finally, the solution was heated at 65° C. for 10 minutes to denature the ligase.

[0167] (C) Construct Precipitation

[0168] 10 μ l GAGP construct from (B) above was combined with 25 μ l water and 5 μ l 3 M NaAcetate. 150 μ l EtOH was then added and the solution incubated at 4° C. for 30 minutes. The solution was then centrifuged at 10,000 rpm for 30 minutes. The resultant pellet was washed with 70% EtOH and dried.

[0169] (D) Restriction Of Gene 3'-Linker And 5'-Linker Capped Ends

[0170] The pellet from (C) above was dissolved in 14 μ l water. 2 μ l 10 $\times$ EcoRI restriction buffer (New England Biolabs), 2 μ l EcoRI 10 U/ μ l (New England Biolabs), and 2 μ l BamHI 20 U/ μ l (New England Biolabs) was then added and the mixture incubated at 37° C. overnight.

[0171] (E) Size-Fractionation and Removal of Enzyme Contaminants

[0172] 10 μ l water was added to 20 μ l of the restricted genes from Step (D) above. This mixture was then loaded onto a Sephacryl S-400 (Pharmacia Microspin™) minicolumn and spun to remove small (<90 bp) oligonucleotide fragments. The first effluent from the column (i.e. the large MW material) was collected. Finally, the enzymes were removed using a Qiaquick Nucleotide removal kit (Qiagen). The final volume of mixture was approximately 50 μ l.

[0173] (F) Gene Insertion Into SK Plasmid Vector

[0174] SK plasmid vector (Stratagene) was restricted with BamHI and EcoRI and restricted large plasmid fragments were isolated from agarose gel. To 2-3 μ g restricted SK plasmid in 10 μ l water was added 6 μ l restricted GAGP gene construct from Step (E), 2 μ l T4 DNA ligase buffer (New England Biolabs), and 1 μ l T4 DNA ligase (New England Biolabs). The solution was then kept at 8° C. overnight for ligation. 100 μ l competent XL1-Blue cells (Stratagene) were then transformed with 3 μ l ligation mixture. Clones were selected via Blue/White assay (Promega Corporation), as described by Promega Protocols and Applications Guide, 2 ed. (1991), by hybridization with ³²P-labeled antisense internal oligonucleotide, and by restriction mapping.

Example 7

Construction Of A Synthetic HRGP Gene Cassette
Incorporating An SP Construct

[0175] Synthetic gene cassettes encoding contiguous and noncontiguous Hyp modules were constructed using partially overlapping sets consisting of oligonucleotide pairs, “internal repeat pairs” and “external 3'- and 5'-linker pairs” respectively, all with complementary “sticky” ends. The following 5'-linker, internal repeat and 3'-linker duplexes were employed:

5'-Linker

A A G S S T R A

(S P S)

5'-GCT GCC GGA TCC TCA ACC CGG GCC-3'

(SEQ ID NO:40)

(SEQ ID NO:41)

3'-CGA CGG CCT AGG AGT TGG GCC CGG AGT GGG AGT-5'

(SEQ ID NO:42)

3'-Linker

S P S P V A R N S P P

(SEQ ID NO:43)

5'-TCA CCC TCA CCG GTC GCC CGG AAT TCA CCA CCC-3'

(SEQ ID NO:44)

3'-GGC CAG CGG CGG CCG TTA AGT GGT GGG-5'

(SEQ ID NO:45)

Internal Repeat

S P S P S P S P S P (S P S)

(SEQ ID NO:108)

(SEQ ID NO:109)

5'TCA CCC TCA CCA TCT CCT TCG CCA TCA CCC

(SEQ ID NO:109)

3'-GGT AGA GGA AGC GGT AGT GGG AGT GGG AGT-5'

(SEQ ID NO:110)

[0176] The following synthetic gene (SEQ ID NO: 111) was eventually expressed (SEQ ID NO: 112) in tobacco and tomato cell cultures and tobacco plants using the above constructs:

G S A M G K M A S L F A T F L V V L V

5'-GGA TCC GCA ATG GGA AAA ATG GCT TCT CTA TTT GCC ACA TTT TTA GTG GTT TTA GTG

3'-CCT AGG CGT TAC CCT TTT TAC CGA AGA GAT AAA CGG TGT AAA AAT CAC CAA AAT CAC

S L S L A Q T T R A [S P S P S P S P S

TCA CTT AGC TTA GCA CPA ACA ACC CGG GCC [TCA CCC TCA CCA TCT CCT TCG CCA TCA

AGT GAA TCG AAT CGT GTT TGT TGG GCC CGG [AGT GGG AGT GGT AGA GCA AGC GGT AGT

P] S P S P V A T

CCC]6 TCA CCC TCA CCG GTC GCC ACC-gfp-3'

GGG]6 AGT GGG AGT GGC CAG CGG TGG-gfp-5'

[0177] This example involved: (A) Oligonucleotide pair preparation; (B) Oligonucleotide polymerization; (C) Construct precipitation; (D) Restriction of gene 3'-linker and 5'-linker capped ends; (E) Size-fractionation and removal of enzyme contaminants; (F) Gene insertion into SK plasmid vector. All SDS-PAGE purified oligonucleotides were synthesized by Gibco-BRL.

[0178] (A) Oligonucleotide Pair Preparation

[0179] In separate Eppendorf tubes were combined:

[0180] Tube 1) 5.5 μ l SP internal repeat sense nucleotide (0.5 mmol/ μ l), 5.5 μ l SP internal repeat antisense oligonucleotide (0.5 mmol/ μ l), 11 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs);

[0181] Tube 2) 2 μ l 5'-sense linker (0.05 mmol/ μ l), 2 μ l 5'-antisense linker (0.05 mmol/ μ l), 1 μ l H₂O, 5 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs);

[0182] Tube 3) 2 μ l 3'-sense linker (1 mmol/ μ l), 2 μ l 3'-antisense linker (1 nmol/ μ l), 1 μ l water, 5 μ l T4 ligase 10 $\times$ ligation buffer (New England Biolabs).

[0183] All tubes were heated to 90-95° C. for 5 minutes, then slowly cooled over the next 3 hours to 45° C. The tubes were then incubated at 45° C. for 2 hours.

[0184] (B) Oligonucleotide Polymerization

[0185] 10 μ l of solution from Tube 1 (internal repeat pair) was combined with 10 μ l of solution from Tube 2 (5' linker pair), and incubated at 17° C. for 3 hours. To this mixture

was added 80 μ l water and 2 μ l (4000 U) T4 DNA ligase (New England Biolabs), and again incubated at 12-15° C. for 36 hours. The degree of polymerization was verified on 2.2% agarose gel (Fisher).

[0186] The 3' end of the polymer was then capped by adding 50 μ l of the ligated SP-5' linker mixture from above to 5 μ l of solution from Tube 3 (3' linker), heating to 30° C., and incubating at 17° C. for 3 hours. 20 μ l water and 2 μ l T4 DNA ligase (New England Biolabs) was then added, and the solution was incubated at 12-15° C. for 36 hr. Finally, the solution was heated at 65° C. for 10 minutes to denature the ligase.

[0187] (C) Construct Precipitation

[0188] 10 μ l SP construct from (B) above was combined with 25 μ l water and 5 μ l 3 M NaAcetate. 150 μ l EtOH was then added and the solution incubated at 4° C. for 30 minutes. The solution was then centrifuged at 10,000 rpm for 30 minutes. The resultant pellet was washed with 70% EtOH and dried.

[0189] (D) Restriction Of Gene 3'-Linker And 5'-Linker Capped Ends

[0190] The pellet from (C) above was dissolved in 14 μ l water. 2 μ l 10 $\times$ EcoRI restriction buffer (New England Biolabs), 2 μ l EcoRI 10 U/ μ l (New England Biolabs), and 2 μ l BamHI 20 U/ μ l (New England Biolabs) was then added and the mixture incubated at 37° C. overnight.

[0191] (E) Size-Fractionation and Removal of Enzyme Contaminants

[0192] 10 μ l water was added to 20 μ l of the restricted genes from Step (D) above. This mixture was then loaded onto a Sephacryl S-400 (Pharmacia Microspin™) minicolumn and spun to remove small (<90 bp) oligonucleotide fragments. The first effluent from the column (i.e. the high molecular weight material) was collected. Finally, the enzymes were removed using a Qiaquick Nucleotide removal kit (Qiagen). The final volume of mixture was approximately 50 μ l.

[0193] (F) Gene Insertion Into SK Plasmid Vector

[0194] SK plasmid vector (Stratagene) was restricted with BamHI and EcoRI and restricted large plasmid fragments were isolated from agarose gel. To 2-3 μ g restricted SK plasmid in 10 μ l water was added 6 μ l restricted SP gene construct from Step (E), 2 μ l T4 DNA ligase buffer (New England Biolabs), and 1 μ l T4 DNA ligase (New England Biolabs). The solution was then kept at 8° C. overnight for ligation. 100 μ l competent XL1-Blue cells (Stratagene) were then transformed with 3 μ l ligation mixture. Clones were selected via Blue/White assay (Promega Corporation), as described by Promega Protocols and Applications Guide, 2 ed. (1991), by hybridization with ³²P-labeled antisense internal oligonucleotide, and by restriction mapping.

Example 8

[0195] Gene Subcloning Into pEGFP, pKS, pUC18 and pBI121 and Signal Sequence Synthesis

[0196] The methods of the following example were used to incorporate the synthetic genes of Examples 6 and 7 into the pBI121 plasmid. Restriction digests, ligations, subclonings, and *E. Coli* transformations were performed generally according to F. M. Ausubel, ed., "Current Protocols in Molecular Biology," (1995), Chapter 3: Enzymatic Manipulation of DNA and DNA Restriction Mapping, Subcloning of DNA Fragments. The restriction digests used were 1-2 μ g of plasmid DNA, 5-10 U of restriction enzyme, and 1 $\times$ recommended restriction buffer (starting with the 10 $\times$ buffer provided by the company). Samples were run on 1-2.2% agarose gels in TBE buffers. Plasmid and DNA fragments were isolated from gels using QIAEX II gel extraction kits (Qiagen). The DNA ligase employed was 400 U T4 (New England Biolabs). Vector:fragment ratios employed were 1:2-1:6, and ligation volumes were 20 μ l.

[0197] Transformation of *E. coli* was done in 5-10 μ l ligation reaction volumes with XL-Blue competent cells (Stratagene). Cells were plated on LB plates containing 50 μ g/ml ampicillin or 30 μ g/ml kanamycin.

[0198] Plasmid isolation was performed by growing transformed XL-Blue cells in 3 mL LB-ampicillin or LB-kanamycin medium. The plasmids were then isolated using a Wizard Plus Miniprep DNA Purification System (Promega).

[0199] This example involved: (A) Insertion of the synthetic gene into pEGFP; (B) Insertion of GAGP-EGFP or SP-EGFP fragment into pKS; (C) Construction of the Signal Sequence and cloning into pUC18; (D) Insertion of GAGP-EGFP or SP-EGFP construct into pUC18; (E) Insertion of SS-GAGP-EGFP or SS-SP-EGFP genes into pBI121.

[0200] (A) Insert Synthetic Gene for GAGP or SP into pEGFP

[0201] This step was carried out to allow directional cloning of the gene at the 5' end of EGFP. First, the GAGP or SP gene was isolated from pSK [from Examples 6(F) and 7(F)] as a BamHI (New England Biolabs) and AgeI (New England Biolabs) fragment. The pEGFP (Clontech) was then restricted with BamHI and AgeI. Finally, the BamHI/AgeI-restricted gene was annealed with BamHI/AgeI-restricted pEGFP, and ligated to yield pEGFP containing the synthetic gene inserted at the 5' end of the EGFP.

[0202] (B) Insert GAGP-EGFP Or SP-EGFP Fragment Into pKS

[0203] This step was carried out to obtain an Sst I site at the 3' end of EGFP. The GAGP-EGFP or SP-EGFP construct from (A) above was isolated from pEGFP as an XmaI/NotI fragment. pKS (Stratagene) was then restricted with XmaI and NotI (New England Biolabs). Finally, the GAGP-EGFP or SP-EGFP construct was annealed with cut pKS and ligated to yield pKS containing GAGP-EGFP or SP-EGFP.

[0204] (C) Construct of the Signal Sequence and Cloning Into pUC18

[0205] In order to anneal the partially overlapping sense and antisense oligonucleotides encoding the extensin signal sequence, 2 μ l signal sequence sense oligonucleotide (0.1 mmol/ μ l), 2 μ l signal sequence antisense oligonucleotide (0.1 nmol/ μ l), 2 μ l 10 $\times$ DNA Polymerase Buffer (New England Biolabs), and 14 μ l H₂O was combined and heated to 85° C. for 5 minutes. The mixture was then slowly cooled to 40° C. over 1 hour.

[0206] The annealed oligonucleotides were then extended via primer extension. To the above mixture was added 2 μ l dNTP 2.5 mM (New England Biolabs) and 1 μ l DNA Polymerase 5 U/l (New England Biolabs), and the resultant mixture incubated at 37° C. for 10 minutes. The polymerase was then denatured by heating at 70° C. for 10 minutes. Then 8 μ l Buffer 4 (New England Biolabs), 66 μ l H₂O, 2 μ l BamHI 20 U/ μ l (New England Biolabs), and 2 μ l SstII 14 U/ μ l (Sigma) was added and the mixture incubated at 37° C. overnight. The restriction enzymes were then denatured by heating at 70° C. for 10 minutes.

[0207] The mixture was then precipitated with EtOH/NaAcetate (6 μ l NaAcetate/300 μ l EtOH), and pelletized in a centrifuge. The pellet was washed with 70% EtOH and dried. The pellet was then dissolved in 20 μ l H₂O and 4 μ l was used for ligation into 2 μ g pSK (Stratagene) as a BamHI/SstII fragment. Finally, the signal sequence was subcloned into pUC18 as a BamHI/SstII fragment.

[0208] (D) Insertion Of GAGP-EGFP Or SP-EGFP Construct Into pUC18

[0209] This step was carried out to insert the GAGP-EGFP or SP-EGFP construct "behind" the signal sequence. The GAGP-EGFP or SP-EGFP construct from (B) above was

removed from pKS as an XmaI/SstI fragment. pUC18 containing the signal sequence (SS-pUC18) was restricted with XmaI/SstI. The GAGP-EGFP or SP-EGFP fragment was then annealed with cut SS-pUC18, and ligated. The SS-GAGP-EGFP or SS-SP-EGFP gene sequence was then confirmed through DNA sequencing using the pUC18 17-residue sequencing primer (Stratagene).

[0210] (E) Insertion Of SS-GAGP-EGFP Or SS—SP-EGFP Genes Into pBI121

[0211] The SS-GAGP-EGFP or SS-SP-EGFP gene from (D) above was removed from pUC18 as BamHI/SstI fragments. pBI121 (Clontech) was restricted with BamHI and SstI and the larger plasmid fragments recovered. The smaller fragments, containing the GUS reporter gene, were discarded. The SS-GAGP-EGFP or SS-SP-EGFP fragment was annealed with the restricted pBI121 fragment and ligated.

Example 9

Agrobacterium Transformation with pBI121-Derived Plasmids

[0212] 2 μ g of the pBI121 containing SS-GAGP-EGFP or SS—SP-EGFP from Example 8 above was used to transform *Agrobacterium tumefaciens* (Strain LB4404, from Dr. Ron Sederoff, North Carolina State University) according to An et al., Plant Molecular Biology Manual A3:1-19 (1988).

Example 10

Transformation of Tobacco Cultured Cells with pBI121-Derived Plasmids

[0213] All steps were carried out under sterile conditions. Tobacco cells were grown for 5-7 days in NT-1 medium (pH 5.2, per liter: 1L packet of MS Salts (Sigma #S5524), 30 g sucrose, 3 ml 6% KH_2PO_4 , 100 mg Myo-Inositol, 1 mL Thiamine.HCl (1 mg/ml stock), 20 μ l 2,4-D (10 mg/ml stock)) containing 100 μ g/ml kanamycin. The cells were grown in IL flasks containing 500 mL medium on a rotary shaker (94 rpm, 27° C.) to between 15-40% packed cell volume. Agrobacterium cells transformed with pBI121-derived plasmid (Example 9) were grown overnight in Luria Broth containing 30 μ g/ml kanamycin. The Agrobacterium cell broth was pelletized for 1 minutes at 6000 rpm, and the pellet resuspended in 200 μ l NT-1 medium.

[0214] Excess medium was removed from the tobacco cell broth until the broth had a consistency approximate to applesauce. The tobacco cells were placed in petri dish, and 200 μ l of the Agrobacterium preparation was added. The mixture was then incubated at room temperature, no light, for 48 hours.

[0215] The mixture was then washed 4 times with 20 mL NT-1 to remove the Agrobacterium cells, and the plant cells were plate-washed on NT-1 plates containing 400 μ g/ml timentin and 100 μ g/ml kanamycin. Cells which grew on the antibiotics were selected and checked for green fluorescence through fluorescence microscopy, excitation wavelength 488 nm (see Example 16).

Example 11

Transformation of Tomato Cultured Cells with pBI121-Derived Plasmids

[0216] All steps were carried out under sterile conditions. Tomato cells were grown for 5-7 days in Schenk-Hildebrand

medium (pH 5.8, per liter: 1L packet of S—H basal salt (Sigma #S6765), 34 g sucrose, 1 g Schenk-Hildebrandt vitamin powder (Sigma #S3766), 100 μ l Kinetin 1 mg/ml stock (Sigma #K32532), 44 μ l 2,4-D 10 mg/ml stock, 2.1 ml p-chlorophenoxy acetic acid 1 mg/ml stock (Sigma)) containing 200 μ g/ml kanamycin. The cells were grown in 1L flasks containing 500 mL medium on a rotary shaker (94 rpm, 27° C.) to between 15-40% packed cell volume. Agrobacterium cells transformed with pBI121-derived plasmid (Example 9) were grown overnight in Luria Broth containing 30 μ g/ml kanamycin. The Agrobacterium cell broth was pelletized for 1 minutes at 6000 rpm, and the pellet resuspended in 200 μ l NT-1 medium.

[0217] Excess medium was removed from the tomato cell broth until the broth had a consistency approximate to applesauce. The tomato cells were placed in petri dish, and 200 μ l of the Agrobacterium preparation was added. The mixture was then incubated at room temperature, no light, for 48 hours.

[0218] The mixture was then washed 4 times with 20 mL NT-1 to remove the Agrobacterium cells, and then the plant cells were plate-washed on NT-1 plates containing 400 μ g/ml timentin and 200 μ g/ml kanamycin. Cells which grew on the antibiotics were selected and checked for green fluorescence through fluorescence microscopy, excitation wavelength 488 nm.

Example 12

Isolation of GAGP-EGFP from Tobacco Cell Suspension Culture Medium

[0219] Transformed tobacco cells were grown on rotary shaker as described in Example 11 above. The medium was separated from the cells by filtration on a glass sintered funnel (coarse grade), and the medium concentrated by freeze-drying. The medium was then resuspended in water (~50 ml/500 mL original volume before lyophilization), and dialyzed against cold water for 48 hours (water changed 6 times). The precipitated pectin contaminants were removed by centrifuge, the pellet discarded, and the supernatant freeze-dried. The dried supernatant was then dissolved in Superose Buffer 20 mg/ml (200 mM sodium phosphate buffer, pH 7, containing 0.05% sodium azide), and spun in a centrifuge to pelletize insolubles. 1.5 ml of this preparation (18-30 mg) was then injected into a semi-preparative Superose-12 gel filtration column (Pharmacia), equilibrated in Superose Buffer and eluted at 1 ml/minutes. The UV absorbance was monitored at 220 nm. 2 mL fractions were collected throughout, with GAGP-EGFP expected to elute between 59 and 70 minutes (~2.5 Vo). GAGP-EGFP actually eluted at 65 minutes (see FIG. 3, Example 15 for method used to analyze peaks).

[0220] The Superose peak containing GAGP-EGFP was dialyzed against cold water for 24 hours (4 water changes), and freeze-dried. The dried GAGP-EGFP peak was then dissolved in 250 μ l 0.1% aqueous TFA (Pierce) and loaded onto a PRP-1 column (Polymeric Reverse Phase, Hamilton) equilibrated in Buffer A (0.1% aqueous TFA). The column was then eluted with Buffer B (0.1% TFA/80% acetonitrile in water; gradient=0-70% B/100 min) at a rate of 0.5 mL/minutes. UV absorbance was monitored at 220 nm, and GAGP-EGFP eluted at 63 minutes (see FIG. 4, Example 15

for method used to analyze peaks). Finally, the TFA/acetonitrile was removed through N₂ (g) blowdown.

Example 13

Characterization of GAGP-EGFP by Neutral Sugar Analysis

[0221] 100 μ g of GAGP-EGFP isolated from tobacco cells was aliquoted into a 1 ml glass microvial and dried under N₂ (g). 200 μ l 2N TFA was added and the vial capped. The vial was heated at 121° C. for 1 hour, then blown down under N₂ at 50° C. to rid the sample of acid. 25 μ l of sodium borohydride solution (20 mg/ml in 3 M ammonium hydroxide) was added and the mixture kept at room temperature for 1 hour. 1-3 drops of concentrated acetic acid were added until fizzing stops, and the mixture blown down under N₂ at 40° C. 100 μ l MeOH was added, the mixture vortexed, and blown down under N₂ at 40° C., then this step was repeated. A mixture of 100 μ l MeOH and 100 μ l H₂O was added, vortexed, and blown down under N₂ at 40° C., then the procedure of adding 100 μ l MeOH, vortexing, and N₂ treatment was repeated 3 times. The resultant mixture was then dried under vacuum overnight.

[0222] 50 μ l reagent grade acetic anhydride was added and the mixture heated at 121° C. for 0.5 hour. The sample was then analyzed by gas chromatography as described in Kieliszewski et al., *Plant Physiol.* 98:919 (1992). The sample was shown to contain hydroxyproline and sugar, accounting for ~50% of the fusion product on a dry weight basis. Galactose, arabinose, and rhamnose occur in 3:3:1 molar ratio similar to that of native GAGP's 3.5:4:1 molar ratio. This is consistent with the likely presence of both Hyp-arabinosides and Hyp-arabinogalactan polysaccharide in the expressed construct. The lower ratio of Ara in the GAGP-EGFP fusion glycoprotein is consistent with the Ala for Pro substitution (See Example 6), which removes one arabinosylation site in the peptide.

Example 14

Characterization of GAGP-EGFP by Hydroxyproline Assay

[0223] 100 μ g purified GAGP-EGFP was hydrolyzed with 6N HCl (Pierce) at 110° C. for 18 hours. The excess acid was then removed by blowing down under N₂. Hydroxyproline was then determined following Kivirikko and Liesma, *Scand. J. Clin. Lab. Invest.* 11:128 (1959).

Example 15

Characterization of Tobacco and Tomato Expression Products by Enzyme-Linked Immunosorbent Assay

[0224] GAGP-EGFP and SP-EGFP products from tomato and tobacco cell medium and column peaks (see Example 12) were detected by Enzyme-Linked Immunosorbent Assays (ELISA) using the method of Kieliszewski and Lampert, "Cross-reactivities of polyclonal antibodies against extension precursors determined via ELISA techniques," *Phytochemistry* 25:673-677 (1986). The GAGP-EGFP product was also assayed using anti-EGFP antibodies. Anti-EGFP antibodies (Clontech) were the primary antibody, diluted 1000-fold as recommended by the manufac-

turer. The secondary antibody was Peroxidase conjugated goat-anti-rabbit IGG diluted 5000-fold (Sigma). Recombinant EGFP (Clontech) was used as a control. This assay was used to generate FIGS. 3 and 4 from Example 12 above.

Example 16

Characterization of Tobacco and Tomato Expression Products by Fluorescence

[0225] Culture medium from both tobacco and tomato cells transformed with the GAGP-EGFP and the SP-EGFP genes was collected. The EGFP tag fluoresces when exposed to UV light; the excitation wavelength used here was 488 nm. These media were compared with media which included EGFP expressed behind the signal sequence and secreted into the medium, cells transformed with unaltered pBI121 and medium from untransformed cells. The unmistakable bright green fluorescence (data not shown) allowed visualization of the targeted products during their transit through the ER/Golgi membrane system. As *Agrobacterium* lacks the posttranslational machinery to make HRGPs, the fluorescing proteins must be of plant origin.

Example 17

Tobacco Leaf Disc Transformation

[0226] Sterile tobacco leaves were cut into small pieces and wounded with a needle. 4 ml NT-1 medium without hormones (NT-1 medium of Example 10, omitting 2-4 D) and 150 μ l concentrated overnight culture of *Agrobacterium* (see Example 9) was added to the leaves, and the leaf discs incubated for 48 hours, no light. The leaf discs were then washed with NT-1 medium, no hormones. The discs were then put on NT-1 solid medium plates (NT-1 medium of Example 10 plus 7.5 g Bactoagar (Difco Laboratories)), 400 μ l/ml timentin, and 100 μ g/ml kanamycin.

[0227] After 3 weeks, shoots were transferred from NT-B solid medium without hormones [NT-1 Medium of Example 10, omitting 2-4 D, and adding 300 μ l/L benzyl adenine, made from a 2 mg/ml stock made up in DMSO(N-benzyl-9-(tetrahydropyranyl) adenine (Sigma)] to root. Transformed plants have expressed SP-EGFP and GAGP-EGFP in leaf and root cells, as determined by the fluorescence assay of Example 16 (data not shown).

[0228] From the above, it should be clear that the present invention provides a new approach and solution to the problem of producing plant gums. The approach is not dependent on environmental factors and greatly simplifies production of a variety of naturally-occurring gums, as well as designer gums.

I claim:

1. A fusion protein comprising a non-gum arabic protein or glycoprotein sequence and at least four amino acids of the amino acid sequence selected from the group consisting of SEQ ID NO:1 or SEQ ID NO:2.

2. The fusion protein of claim 1, wherein said non-gum arabic sequence is a green fluorescent protein amino acid sequence.

3. A method for producing at least a portion of a plant gum glycoprotein, comprising:

a) providing;

i) a recombinant expression vector comprising a polynucleotide sequence encoding at least four amino

acids of a polypeptide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, wherein said sequence contains one amino acid substitution; and

ii) a host cell; and

b) introducing said vector into said host cell under conditions suitable for the expression of said polypeptide.

4. The method of claim 3, wherein said host cell is growing in culture.

5. The method of claim 4, further comprising the step of:

c) recovering said polypeptide from the host cell culture.

6. The method of claim 5, wherein said host cell is a plant cell.

7. The method of claim 6, wherein said plant cell is derived from a plant selected from the family Leguminosae.

8. The method of claim 1, wherein said substitution comprises a Leu or Hyp for Gly.

9. The method of claim 1, wherein said substitution comprises a Leu for Hyp.

10. The method of claim 1, wherein said substitution comprises an Ala for a Hyp.

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