

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 November 2010 (04.11.2010)

PCT

(10) International Publication Number
WO 2010/124324 A1

(51) International Patent Classification:
C12N 15/82 (2006.01) *C12N 9/10* (2006.01)
A01H 1/00 (2006.01)

(21) International Application Number:
PCT/AU2010/000481

(22) International Filing Date:
27 April 2010 (27.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/173,272 28 April 2009 (28.04.2009) US

(71) Applicant (for all designated States except US): **AGRI-CULTURE VICTORIA SERVICES PTY LTD** [AU/AU]; 475 Mickleham Road, Attwood, Victoria 3049 (AU).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SPANGENBERG, German** [AU/AU]; 56 Arthur Street, Bundoora, Victoria 3083 (AU). **MOURADOV, Aidyn** [AU/AU]; 8 Francis Walton Street, Mill Park, Victoria 3082 (AU). **SAW-BRIDGE, Timothy Ivor** [AU/AU]; 174 Keale Street, Collingwood, Victoria 3066 (AU).

(74) Agent: **FREEHILLS PATENT & TRADE MARK ATTORNEYS**; Level 43 101 Collins Street, Melbourne, Victoria 3000 (AU).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: MANIPULATING FRUCTAN BIOSYNTHESIS AND ENHANCING PLANT BIOMASS

(57) Abstract: The present invention relates to genetic constructs capable of manipulating fructan biosynthesis in photosynthetic cells of a plant, said genetic constructs including a promoter, or functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof. The present invention also relates to the modification of fructan biosynthesis in plants and, more particularly, to methods of manipulating fructan biosynthesis in photosynthetic cells. The present invention also relates to increasing plant biomass and, more particularly, to methods of enhancing biomass yield and/or yield stability, including shoot and/or root growth in a plant. The present invention also relates to methods of enhancing the productivity of biochemical pathways.



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Manipulating fructan biosynthesis and enhancing plant biomass

Field of the invention

The present invention relates to the modification of fructan biosynthesis in plants and, more particularly, to methods of manipulating fructan biosynthesis in photosynthetic
5 cells, and to related nucleic acids and constructs.

The present invention also relates to increasing plant biomass and, more particularly, to methods of enhancing biomass yield and/or yield stability, including shoot and/or root growth in a plant, and to related nucleic acids and constructs.

Background of the invention

- 10 Fructans are a type of water-soluble carbohydrate whose primary function is to provide a readily accessible energy reserve for plant growth. Fructans are associated with various advantageous characters in grasses, such as cold and drought tolerance, increased tiller survival, enhanced persistence, good regrowth after cutting or grazing, improved recovery from stress, early spring growth and increased nutritional quality.
- 15 Fructan synthesis and metabolism in grasses and cereals is complex. Fructans consist of linear or branched fructose chains attached to sucrose. The chain length of plant fructans ranges from three up to a few hundred fructose units. Different types of fructans can be distinguished based on the linkage types present. In perennial ryegrass three types of fructans have been identified: inulins, inulin neoseries and levan
20 neoseries, with four fructosyltransferase (FT) enzymes involved in this fructan profile. The enzyme 1-SST (sucrose: sucrose 1-fructosyltransferase) catalyses the first step in fructan biosynthesis while the remaining enzymes elongate the growing fructose chain (1-FFT: fructan: fructan 1-fructosyltransferase, 6G-FFT: 6-glucose fructosyltransferase, and 6-SFT: sucrose: fructose 6-fructosyltransferase). The enzymes 1-FEH or 6-FEH
25 (fructoexohydrolase) reduce fructan chain length by releasing fructose molecules.

Bacteria use only one FT enzyme which contains both 1-SST and 1-FFT activities to synthesize high molecular weight fructan polymers. Most bacterial fructosyltransferases

produce levan type fructan (levansucrases), which is characterized by β -2,6 linkages of fructose molecules, although inulosucrases that produce fructans of the inulin type (β -2,1 linkage) have been isolated from a few bacteria.

At least 3 bacterial levansucrases have been expressed in transgenic plants including,
5 the *SacB* gene from *Bacillus subtilis*, the *SacB* gene from *Bacillus amyloliquefaciens*, and the *FTF* gene from *Streptococcus mutans*. Expression of these bacterial levansucrases in plants leads to the synthesis of very high molecular weight fructans of a DP of several thousands (for review see Cairns, 2003).

Fructans represent the major non-structural carbohydrate in 15% of plant species and
10 play a key role in forage quality. Ruminant livestock grazing on high fructan diets show improved animal performance.

In grasses the level and composition of fructans has been increased in stems and leaf sheaths through the engineered expression of FT genes.

However, manipulating biochemical pathways by manipulating the activity of enzymes in
15 the pathways may be difficult because of the ways in which the various enzymes and their substrates may interact.

Thus, it would be desirable to have improved methods of manipulating biochemical pathways, particularly in plants. For example, it would be desirable to have methods of manipulating fructan biosynthesis in plants, including forage grass species such as
20 *Lolium*, *Festuca*, and *Brachiaria*; sugarcane and other grasses; and sorghum and other cereals such as wheat and maize; thereby facilitating the production of, for example:

- forage grasses with improved herbage quality and/or yield, leading to improved pasture production, improved animal production and/or reduced environmental pollution;
- 25 • bioenergy grasses and crops such as switchgrass, *Miscanthus*, sorghum (grain, forage and energy sorghum), sugarcane and energy cane with enhanced biomass yield, such as for bioethanol production; and

- cereals such as wheat, rice, maize, barley with increased grain and/or biomass yield.

Nucleic acid sequences encoding some of the enzymes involved in the fructan biosynthetic pathway have been isolated for certain species of plants. For example,
5 PCT/AU01/00705 to the present applicants, describes fructosyltransferase homologues from *Lolium* and *Festuca*. However, there remains a need for materials useful in the modification of fructan biosynthesis in plants, and also to engineer fructan accumulation in different parts of the plant.

International patent application PCT/AU2009/001211 describes constructs useful for
10 modifying fructan biosynthesis in plants. However, those constructs preferably include a fusion gene encoding a translational fusion of two or more fructan biosynthetic enzymes, the genes making up the fusion preferably corresponding to plant fructan biosynthetic genes.

It is an object of the present invention to overcome, or at least alleviate, one or more of
15 the difficulties or deficiencies associated with the prior art.

Summary of the invention

Applicants have found that it is possible to nutritionally enhance plants eg. forage grasses and/or to increase plant biomass by spatial reprogramming of the fructan-biosynthesis pathway in photosynthetic cells using a construct including a promoter or a
20 functionally active fragment or variant thereof, operatively linked to a gene encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof. Using this process it is possible to drive fructan accumulation in leaf blades, the plant organs that are primarily grazed by livestock, and which may not normally accumulate fructans. Thus, accumulation of fructans, especially those exhibiting a high degree of
25 polymerization ('high DP fructans'), provides more accessible nutrition for grazing animals. Fructans accumulate in the stems and leaf sheaths, with leaf fructans only forming during periods where CO₂ assimilation outperforms growth. Forage grasses may be nutritionally enhanced by expressing fructan genes in photosynthetic cells

where sucrose is synthesised, thus driving fructan accumulation preferentially in leaf blades and providing more energy to grazing livestock.

Fructans in forage grasses contribute significantly to the readily available energy in the feed for grazing ruminant animals. The fermentation processes in the rumen require
5 considerable readily available energy. The improvement of the readily available energy in the rumen can increase the efficiency of rumen digestion. An increased efficiency in rumen digestion leads to an improved conversion of the forage protein fed to the ruminant animal into milk or meat, and to a reduction in nitrogenous waste.

Applicants have also found that the methods of the present invention may be facilitated
10 by reprogramming photosynthetic cells for extended life, for example by delaying leaf senescence, to help increase plant biomass.

Applicants have found that a construct including a gene encoding a bacterial FT gene or functionally active fragment or variant thereof, may give superior results to a construct including a fusion gene encoding a translational fusion of two or more fructan
15 biosynthetic enzymes. Use of a bacterial FT gene, for example containing both 1-SST and 1-FFT activities, may be technically less difficult than fusing separate genes, and may result in a construct that is more readily introduced into plants and/or performs better than a construct including fused genes.

For example, by expressing a bacterial FT gene, for example containing both 1-SST
20 and 1-FFT activities, problems associated with differences in the expression patterns of these genes independently integrated into the plant genome may be alleviated, resulting in conversion of the sucrose molecules directly to fructans, those exhibiting a low degree of polymerisation ('low DP fructans') and a high degree of polymerization ('high DP fructans'). Furthermore, the FT protein may form a metabolic channel for the
25 efficient biosynthesis of fructans.

Expression of FT genes in photosynthetic cells leading to the accumulation of low and high DP fructans in photosynthetic cells may lead to a release from inhibition

mechanisms of photosynthesis, facilitating solar energy capture and increased CO₂ fixation.

Thus, applicants have found that reprogramming photosynthetic cells for extended life and enhanced fructan biosynthesis facilitates solar energy capture and increases plant biomass production including shoot and/or root growth.

Furthermore, since accumulation of low and high DP fructans has been associated with plant's tolerance to abiotic stress such as cold and drought; and since enhanced root growth and delayed leaf senescence has also been implicated in plant's tolerance of drought stress, reprogramming photosynthetic cells for extended life and enhanced fructan biosynthesis may facilitate yield stability and plants' tolerance of abiotic stresses.

Accordingly, in one aspect, the present invention provides a method of manipulating fructan biosynthesis in photosynthetic cells of a plant, said method including introducing into said plant an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial fructosyltransferase (FT) enzyme, or a functionally active fragment or variant thereof.

By 'manipulating fructan biosynthesis' is generally meant increasing fructan biosynthesis in a transformed plant relative to an untransformed control plant. However, for some applications it may be desirable to reduce or otherwise modify fructan biosynthesis in the transformed plant relative to the untransformed control plant. For example, it may be desirable to increase or decrease the activity of certain enzymes in the fructan biosynthetic pathway, in the transformed plant relative to the untransformed control plant.

By 'photosynthetic cells' is meant those cells of a plant in which photosynthesis takes place. Such cells generally contain the pigment chlorophyll and are otherwise known as green cells. Most photosynthetic cells are contained in the leaves of plants. Preferably, the genetic construct of the present invention is expressed in bundle sheath cells, more preferably in mesophyll and/or parenchymatous bundle sheath cells.

By 'an effective amount' is meant an amount sufficient to result in an identifiable phenotypic trait in said plant, or in a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

By 'genetic construct' is meant a recombinant nucleic acid molecule.

By a 'promoter' is meant a nucleic acid sequence sufficient to direct transcription of an operatively linked nucleic acid sequence.

By 'operatively linked' is meant that the nucleic acid(s) and a regulatory sequence, such as a promoter, are linked in such a way as to permit expression of said nucleic acid under appropriate conditions, for example when appropriate molecules such as transcriptional activator proteins are bound to the regulatory sequence. Preferably an operatively linked promoter is upstream of the associated nucleic acid.

By 'upstream' is meant in the 3'→5' direction along the nucleic acid.

By 'nucleic acid' is meant a chain of nucleotides capable of carrying genetic information. The term generally refers to genes or functionally active fragments or variants thereof and or other sequences in the genome of the organism that influence its phenotype. The term 'nucleic acid' includes DNA (such as cDNA or genomic DNA) and RNA (such as mRNA or microRNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases, synthetic nucleic acids and combinations thereof.

By a 'nucleic acid encoding a bacterial fructosyltransferase (FT) enzyme' or 'bacterial fructosyl transferase gene' is meant a nucleic acid encoding an enzyme normally present in a bacterium which catalyses the synthesis of oligo- and/or polyfructans by transferring fructosyl moieties from sucrose-containing saccharides to acceptor

molecules. The bacterial FT enzyme may include levansucrase activity and/or produce levan type fructan. The bacterial FT enzyme may include inulosucrase activity and/or produce inulin type fructan. A preferred bacterial FT includes both sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) enzymatic activities. A *SacB*, *Lsc* or *FTF* gene may be used. A *SacB* or *Lsc* gene is particularly preferred.

By 'functionally active fragment or variant' in relation to a promoter is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of directing transcription of an operatively linked nucleic acid. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence to which the fragment or variant corresponds, more preferably at least approximately 90% identity, even more preferably at least approximately 95% identity, most preferably at least approximately 98% identity. Preferably the fragment has a size of at least 20 nucleotides, more preferably at least 50 nucleotides, more preferably at least 100 nucleotides, more preferably at least 200 nucleotides, more preferably at least 300 nucleotides.

By 'functionally active' in relation to the nucleic acid encoding a bacterial FT enzyme is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of manipulating fructan biosynthesis in a plant by the method of the present invention, for example by being translated into an enzyme that is able to participate in the fructan biosynthetic pathway. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence to which the fragment or variant corresponds, more preferably at least approximately 90% identity, even more

preferably at least approximately 95% identity, most preferably at least approximately 98% identity. Such functionally active variants and fragments include, for example, those having conservative nucleic acid changes.

By 'conservative nucleic acid changes' is meant nucleic acid substitutions that result in
5 conservation of the amino acid in the encoded protein, due to the degeneracy of the genetic code. Such functionally active variants and fragments also include, for example, those having nucleic acid changes which result in conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence.

By 'conservative amino acid substitutions' is meant the substitution of an amino acid by
10 another one of the same class, the classes being as follows:

Nonpolar: Ala, Val, Leu, Ile, Pro, Met Phe, Trp

Uncharged polar: Gly, Ser, Thr, Cys, Tyr, Asn, Gln

Acidic: Asp, Glu

Basic: Lys, Arg, His

15 Other conservative amino acid substitutions may also be made as follows:

Aromatic: Phe, Tyr, His

Proton Donor: Asn, Gln, Lys, Arg, His, Trp

Proton Acceptor: Glu, Asp, Thr, Ser, Tyr, Asn, Gln

Particularly preferred fragments and variants include one or more conserved sucrose
20 binding/hydrolysis domains. Examples of such domains are shown in Figure 1 and include LDVWDSWP, QEWSGSA, LRDPH and DEIER.

Particularly preferred fragments and variants include a catalytic core. By a "catalytic core" is meant an internal region of the polypeptide excluding signal peptide and N- and C-terminal variable regions, which contains conserved sucrose binding and/or

hydrolysis domains. An example of a catalytic core is shown in Figure 1 and includes amino acid residues 65-468 of the SacB protein from *Bacillus subtilis*.

Particularly preferred fragments and variants include those lacking a signal peptide. By a "signal peptide" is meant an N-terminal signal sequence. An example of a signal
5 peptide is shown in Figure 1 and includes amino acids 1-27 of the SacB protein from *Bacillus subtilis*.

Particularly preferred fragments and variants have codon usage adapted for plants, including the start of translation for monocots and dicots.

Particularly preferred fragments and variants have cryptic splice sites and/or RNA
10 destabilizing sequence elements inactivated or removed.

Preferably, the nucleic acid encoding a bacterial FT is isolated from or corresponds to a gene or genes from a bacterial species selected from the group consisting of *Bacillus*, *Lactobacillus* and *Streptococcus*, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Lactobacillus johnsonii* and *Streptococcus mutans*. Even more preferably, the nucleic
15 acid encoding a bacterial FT enzyme is isolated from or corresponds to a *SacB* gene from *Bacillus subtilis* or *Bacillus amyloliquefaciens*, a *Lsc* gene from *Lactobacillus johnsonii* or a *FTF* gene from *Streptococcus mutans*.

In a particularly preferred embodiment the *SacB* gene includes a sequence selected from the group consisting of the sequence shown in Figure 2 and the nucleotide
20 sequences encoding the polypeptide sequence shown in Figure 3; and functionally active fragments and variants thereof.

In a particularly preferred embodiment the *Lsc* gene includes a sequence selected from the group consisting of the sequence shown in Figure 4 and the nucleic acid sequences encoding the polypeptide sequence shown in Figure 5; and functionally active
25 fragments and variants thereof.

Transgenic plants expressing bacterial levansucrases have been reported, in some instances, to display aberrant developmental phenotypes. While applicants do not wish

to be restricted by theory, this may arise from inadequate compartmentalization of the levansucrase enzymes and fructan polymers. Cytosolic expression of a bacterial *SacB* gene in transgenic tobacco and potato plants was shown to be particularly disruptive to plant development (Caimi et al., 1997). However, plants expressing a bacterial
5 fructosyltransferase fused to vacuole-targeting signals accumulate fructans with no discernible effect on plant development (Ebskamp et al., 1994, Ye et al. 2001).

To reduce the possibility of aberrant developmental phenotypes the bacterial FT gene may be modified to alter its targeting signal sequence to direct the bacterial FT proteins to compartments where high fructan levels exist.

- 10 More particularly, a chimeric sequence may be created, whereby the N-signal peptide of the bacterial FT gene may be removed and replaced by a sub-cellular targeting sequence, preferably a vacuolar targeting sequence.

In a preferred embodiment, the vacuolar targeting sequence may be from or correspond to a gene encoding a preprosporamin protein, such as SPOR531 of sweet potato.

- 15 In a particularly preferred embodiment, the vacuolar targeting sequence may include a sequence selected from the group consisting of the sequence shown in bold underline in Figure 6 and the nucleic acid sequences encoding the polypeptide shown in bold underline in Figure 7; and functionally active fragments and variants thereof.

- In a particularly preferred embodiment the nucleic acid encoding the bacterial FT
20 enzyme may be a *SPOR:SacB* chimeric sequence. Preferably the *SPOR:SacB* chimeric sequence includes a sequence selected from the group consisting of the sequence shown in Figure 8 and the nucleic acid sequences encoding the polypeptide sequence shown in Figure 9; and functionally active fragments and variants thereof.

- In a particularly preferred embodiment the nucleic acid encoding the bacterial FT
25 enzyme may be a *SPOR:Lsc* chimeric sequence. Preferably, the *SPOR:Lsc* chimeric sequence includes a sequence selected from the group consisting of the sequence shown in Figure 9 and the nucleic acid sequences encoding the polypeptide sequence shown in Figure 12; and functionally active fragments and variants thereof.

In a particularly preferred embodiment, the genetic construct includes a sequence selected from the group consisting of the sequences shown in Figures 8 and 11 and the nucleic acid sequences encoding the polypeptides shown in Figures 9 and 12; and functionally active fragments and variants thereof.

- 5 In another preferred embodiment, a chimeric sequence may be is created, whereby the N-signal peptide of the bacterial FT gene may be removed and replaced by a transmembrane domain of a fructosyltransferase enzyme such as 1-SST.

In a particularly preferred embodiment, the transmembrane domain includes a sequence selected from the group consisting of the sequence shown in bold italics in
10 Figure 15 and the nucleic acid sequences encoding the polypeptide shown in bold italics in Figure 16; and functionally active fragments and variants thereof.

In a particularly preferred embodiment, the genetic construct includes a sequence selected from the group consisting of the sequences shown in Figures 17 and 20 and the nucleic acid sequences encoding the polypeptides shown in Figures 18 and 21; and
15 functionally active fragments and variants thereof.

By a 'chimeric sequence' is meant a hybrid produced recombinantly by expressing a fusion gene including two or more linked nucleic acids which originally encoded separate proteins, or functionally active fragments or variants thereof.

By a 'fusion gene' is meant that two or more nucleic acids are linked in such a way as to
20 permit expression of the fusion protein, preferably as a translational fusion. This typically involves removing the stop codon from a nucleic acid sequence coding for a first protein, then appending the nucleic acid sequence of a second protein in frame. The fusion gene is then expressed by a cell as a single protein. The protein may be engineered to include the full sequence of both original proteins, or a functionally active
25 fragment or variant of either or both.

The genetic construct may also include a nucleic acid sequence encoding a linker between the two linked nucleic acids. A 'linker' is any chemical, synthetic, carbohydrate, lipid, polypeptide molecule (or combination thereof) positioned between and joined to

two adjacent active fragments in a fusion protein. A preferred linker of the invention is a flexible linker, such as a polypeptide chain consisting of one or more amino acid residues joined by amino acid bonds to the two active fragments. For example, a (Gly₄ Ser)₃ linker may be positioned between the two active fragments in the fusion protein.

- 5 The promoter used in the constructs and methods of the present invention may be a constitutive, tissue specific or inducible promoter. In a preferred embodiment, the promoter may be a light-regulated promoter, more preferably a photosynthetic promoter. By a 'light regulated promoter' is meant a promoter capable of mediating gene expression in response to light stimulus. By a 'photosynthetic promoter' is meant a
10 promoter capable of mediating expression of a gene encoding a protein involved in a photosynthetic pathway in plants.

Less fructans accumulate in mature leaf blades than in leaf sheaths and stems. In order to specifically increase the level of fructans in leaf blades, a strategic approach has been devised that co-ordinately expresses fructan biosynthesis genes in photosynthetic
15 cells. While applicants do not wish to be restricted by theory, the use of light-regulated or photosynthetic promoters may provide the following advantages:

- Photosynthetic promoters are active in a large group of cells including leaf blades, the upper and outer stem (>55% cells);
- They are active in sucrose producing cells (mesophyll and parenchymatous
20 bundle sheath cells);
- Their expression pattern temporally and spatially overlaps with sucrose accumulation;
- Fructosyltransferase activity will remove sucrose from the source thereby preventing feedback suppression on photosynthesis, and may facilitate increases
25 in CO₂ fixation;

Particularly preferred light-regulated promoters include a ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*) promoter and a chlorophyll a/b binding protein (*CAB*) promoter, and functionally active fragments and variants thereof.

The light-regulated promoter may be from any suitable plant species including monocotyledonous plants [such as maize, rice, wheat, barley, sorghum, sugarcane, energy cane, forage grasses (e.g. *Festuca*, *Lolium*, *Brachiaria*, *Paspalum*, *Pennisetum*), bioenergy grasses (e.g. switchgrass, *Miscanthus*)], dicotyledonous plants (such as
5 *Arabidopsis*, soybean, canola, cotton, alfalfa and tobacco) and gymnosperms.

For transformation of monocots, preferably the light-regulated promoter is isolated from or corresponds to a promoter from a monocot species, more particularly a *Triticum* or *Lolium* species, even more particularly *Triticum aestivum* or *Lolium perenne*.

For transformation of dicots, preferably the light-regulated promoter is isolated from or
10 corresponds to a promoter from a dicot species, more particularly an *Arabidopsis* species, even more particularly *Arabidopsis thaliana*.

In a particularly preferred embodiment, the *RbcS* promoter includes a sequence selected from the group consisting of the sequence shown in lower case italics in any one of Figures 23 to 26, and functionally active fragments and variants thereof.

15 In another particularly preferred embodiment, the *RbcS* promoter includes a sequence selected from the group consisting of the sequence shown in lower case italics in any one of Figures 29 to 36, and functionally active fragments and variants thereof.

The genetic constructs of the present invention may be introduced into the plants by any suitable technique. Techniques for incorporating the genetic constructs of the present
20 invention into plant cells (for example by transduction, transfection, transformation or gene targeting) are well known to those skilled in the art. Such techniques include *Agrobacterium*-mediated introduction, *Rhizobium*-mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues,
25 calli, immature and mature embryos, biolistic transformation, Whiskers transformation, and combinations thereof. The choice of technique will depend largely on the type of plant to be transformed, and may be readily determined by an appropriately skilled person.

Cells incorporating the genetic constructs of the present invention may be selected, as described below, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The
5 resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

The methods of the present invention may be applied to a variety of plants, including monocotyledons [such as grasses (e.g. forage and bioenergy grasses including perennial ryegrass, tall fescue, Italian ryegrass, red fescue, reed canarygrass, big
10 bluestem, cordgrass, napiergrass, wildrye, wild sugarcane, *Miscanthus*, switchgrass), corn or maize, rice, wheat, barley, sorghum, sugarcane, rye, oat) and energy crops (e.g. energy cane, energy sorghum)], dicotyledons [such as *Arabidopsis*, tobacco, soybean, canola, alfalfa, potato, cassava, clovers (e.g. white clover, red clover, subterranean clover), vegetable brassicas, lettuce, spinach] and gymnosperms.

15 In a further aspect of the present invention, there is provided a genetic construct capable of manipulating fructan biosynthesis in photosynthetic cells of a plant, said genetic construct including a light-regulated promoter, or functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof.

20 In a preferred embodiment, the genetic construct according to the present invention may be a vector.

By a 'vector' is meant a genetic construct used to transfer genetic material to a target cell.

The vector may be of any suitable type and may be viral or non-viral. The vector may be
25 an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, eg. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*; derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes;

bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable or integrative or viable in the plant cell.

In a preferred embodiment of this aspect of the invention, the genetic construct may
5 further include a terminator; said promoter, gene and terminator being operably linked.

The promoter, gene and terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that they are functional in the target plant cell.

A variety of terminators which may be employed in the genetic constructs of the present
10 invention are also well known to those skilled in the art. The terminator may be from the same gene as the promoter sequence or a different gene. Particularly suitable terminators are polyadenylation signals, such as the (CaMV)35S polyA and other terminators from the nopaline synthase (*nos*) and the octopine synthase (*ocs*) genes.

The genetic construct, in addition to the promoter, the gene and the terminator, may
15 include further elements necessary for expression of the nucleic acid, in different combinations, for example vector backbone, origin of replication (*ori*), multiple cloning sites, spacer sequences, enhancers, introns (such as the maize Ubiquitin *Ubi* intron), antibiotic resistance genes and other selectable marker genes [such as the neomycin phosphotransferase (*nptII*) gene, the hygromycin phosphotransferase (*hph*) gene, the
20 phosphinothricin acetyltransferase (*bar* or *pat*) gene], and reporter genes (such as beta-glucuronidase (GUS) gene (*gusA*)). The genetic construct may also contain a ribosome binding site for translation initiation. The genetic construct may also include appropriate sequences for amplifying expression.

In particular, the genetic construct may further include a nucleic acid sequence
25 encoding a linker between the two linked nucleic acids, as hereinbefore described.

Those skilled in the art will appreciate that the various components of the genetic construct are operably linked, so as to result in expression of said nucleic acid. Techniques for operably linking the components of the genetic construct of the present

invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

Preferably, the genetic construct is substantially purified or isolated. By 'substantially
5 purified' is meant that the genetic construct is free of the genes, which, in the naturally-
occurring genome of the organism from which the nucleic acid or promoter of the
invention is derived, flank the nucleic acid or promoter. The term therefore includes, for
example, a genetic construct which is incorporated into a vector; into an autonomously
replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or
10 which exists as a separate molecule (eg. a cDNA or a genomic or cDNA fragment
produced by PCR or restriction endonuclease digestion) independent of other
sequences. It also includes a genetic construct which is part of a hybrid gene encoding
additional polypeptide sequence. Preferably, the substantially purified genetic construct
is at least approximately 90% pure, more preferably at least approximately 95% pure,
15 even more preferably at least approximately 98% pure.

The term "isolated" means that the material is removed from its original environment
(eg. the natural environment if it is naturally occurring). For example, a naturally
occurring nucleic acid present in a living plant is not isolated, but the same nucleic acid
separated from some or all of the coexisting materials in the natural system, is isolated.
20 Such nucleic acids could be part of a vector and/or such nucleic acids could be part of a
composition, and still be isolated in that such a vector or composition is not part of its
natural environment.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for
selection of transformed host cells, the presence of the genetic construct in transformed
25 cells may be determined by other techniques well known in the art, such as PCR
(polymerase chain reaction), Southern blot hybridisation analysis, histochemical assays
(e.g. GUS assays), thin layer chromatography (TLC), northern and western blot
hybridisation analyses.

Applicant has also found that the methods of the present invention may result in enhanced biomass in the transformed plant relative to an untransformed control plant. This enhanced biomass may in turn be used as a selection tool for identifying transformed plants. This has the advantage that in some circumstances there may be
5 no need to include an antibiotic resistance or other marker to select for transformants, where subsequent removal of such markers (and for the creation of marker-free plants) may present difficulties.

By 'enhancing biomass' or 'enhanced biomass' is meant enhancement of, increase in, or increased stability of biomass yield, including shoot and/or root growth, in a
10 transformed plant relative to an untransformed control plant. For example, one or more growth characteristics selected from the group consisting of total leaf area, cumulative leaf area, leaf growth dynamics (ie. number of leaves over time), number of shoots, number of tillers, number of roots, root mass or weight, shoot mass or weight, root length, shoot length, stolon length, number of tubers, tuber weight, number of flowers,
15 number of fruits, number of seeds, seed weight, fruit weight, percentage of flowering plants and seed yield per flower or per area sown; may be enhanced, increased or more stable in a transformed plant relative to an untransformed control plant.

This technique is particularly applicable to plants that are substantially genetically uniform or genetically identical or exhibit small phenotype differences in biomass prior to
20 transformation.

Accordingly, in a further aspect of the present invention, there is provided a method of enhancing biomass in a plant, said method including introducing into said plant an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT-
25 enzyme, or a functionally active fragment or variant thereof. Preferably, the promoter is a light regulated promoter.

The methods, nucleic acids and genetic constructs of the present invention may be used in combination with other methods of genetic manipulation, or other transferred nucleic acids or genetic constructs, thereby stacking traits. Thus, transgenic plants,

plant cells, plant seeds or other plant parts with stacked genes (or stacked traits) may be produced. For example, the technology of the present invention may be combined with herbicide resistance technology (eg. glufosinate, glyphosate), insect resistance technology (eg. *Bacillus thuringiensis*) or delayed senescence technology. The nucleic acids or genetic constructs may be introduced into the plant by any suitable technique, as hereinbefore described, and may be introduced concurrently, sequentially or separately.

In a still further aspect of the present invention, there is provided a method of enhancing biomass in a plant, said method including introducing into said plant effective amount of

- 10 (a) a genetic construct capable of manipulating fructan biosynthesis in photosynthetic cells of the plant, said genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof; and
- 15 (b) a genetic construct capable of manipulating senescence in the plant.

The genetic constructs may be introduced into the plant by any suitable technique, as hereinbefore described, and may be introduced concurrently, sequentially or separately.

Preferably the genetic construct capable of manipulating fructan biosynthesis is as hereinbefore described.

- 20 Preferably the genetic construct capable of manipulating senescence in the plant is capable of manipulating senescence in photosynthetic cells of the plant.

Preferably the genetic construct capable of manipulating senescence includes a *myb* gene promoter or modified *myb* gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.

Suitable genetic constructs or vectors are described in International patent application PCT/AU01/01092 and United States patent application 11/789,526, the entire disclosures of which are incorporated herein by reference.

5 “Manipulating senescence” generally relates to delaying senescence in the transformed plant or cells or organs of the transformed plant, eg photosynthetic cells, relative to an untransformed control plant. However, for some applications it may be desirable to promote or otherwise modify senescence in the plant. Senescence may be promoted or otherwise modified for example, by utilizing an antisense gene.

10 The *myb* gene promoter may be of any suitable type. Preferably the *myb* gene promoter is a *myb32* gene promoter. Preferably the *myb* gene promoter is from *Arabidopsis*, more preferably *Arabidopsis thaliana*. Most preferably the *myb* gene promoter includes a nucleotide sequence selected from the group consisting of the sequence shown in Sequence ID No: 1 of PCT/AU01/01092 and functionally active fragments and variants thereof.

15 A suitable promoter is described in Li et al., Cloning of three MYB-like genes from *Arabidopsis* (PGR 99-138) Plant Physiology 121:313 (1999), the entire disclosure of which is incorporated herein by reference.

20 By a “modified *myb* gene promoter” is meant a promoter normally associated with a *myb* gene, which promoter is modified to delete or inactivate one or more root specific motifs and/or pollen specific motifs in said promoter.

Preferably the modified *myb* gene promoter is a modified *myb32* gene promoter. Preferably the modified *myb* gene promoter is modified from the *myb* gene promoter from *Arabidopsis*, or more preferably *Arabidopsis thaliana*.

25 A suitable promoter which may be modified according to the present invention is described in Li et al., Cloning of three MYB-like genes from *Arabidopsis* (PGR 99-138) Plant Physiology 121:313 (1999), the entire disclosure of which is incorporated herein by reference.

By a "root specific motif" is meant a sequence of 3-7 nucleotides, preferably 4-6 nucleotides, more preferably 5 nucleotides, which directs expression of any associated gene in the roots of a plant.

Preferably the root specific motif includes a consensus sequence ATATT or AATAT.

- 5 By a "pollen specific motif" is meant a sequence of 3-7 nucleotides, preferably 4-6 nucleotides, more preferably 4 or 5 nucleotides, which directs expression of an associated gene in the pollen of a plant.

Preferably the pollen specific motif includes a consensus sequence selected from the group consisting of TTTCT, AGAAA, TTCT and AGAA.

- 10 A root or pollen specific motif may be inactivated by adding, deleting, substituting or derivatizing one or more nucleotides within the motif, so that it no longer has the preferred consensus sequence.

Preferably the modified *myb* gene promoter includes a nucleotide sequence selected from the group consisting of the sequences shown in Sequence ID Nos: 2, 3 and 4 of US

- 15 11/789,526 and functionally active fragments and variants thereof.

- By a "gene encoding an enzyme involved in biosynthesis of a cytokinin" is meant a gene encoding an enzyme involved in the synthesis of cytokinins such as kinetin, zeatin and benzyl adenine, for example a gene encoding isopentenyl transferase (*ipt*), or *ipt*-like gene such as the *sho* gene (eg. from petunia). Preferably the gene is an isopentenyl transferase (*ipt*) gene or *sho* gene. In a preferred embodiment, the gene is from a species selected from the group consisting of *Agrobacterium*, more preferably *Agrobacterium tumefaciens*; *Lotus*, more preferably *Lotus japonicus*; and *Petunia*, more preferably *Petunia hybrida*.
- 20

- Most preferably the gene includes a nucleotide sequence selected from the group consisting of the sequences shown in Sequence ID Nos: 5, 7 and 9 of US 11/789,526, sequences encoding the polypeptides shown in Sequence ID Nos: 6, 8 and 10 of US 11/789,526, and functionally active fragments and variants thereof.
- 25

The present invention also provides a method of selecting for transformed plants, said method including introducing into said plants an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof and selecting plants with enhanced biomass. Preferably the promoter is a light regulated promoter.

In a further aspect of the present invention there is provided a transgenic plant cell, plant, plant seed or other plant part with modified fructan biosynthetic characteristics or enhanced biomass relative to an untransformed control plant; said plant cell, plant, plant seed or other plant part including a genetic construct or vector according to the present invention.

By "modified fructan biosynthetic characteristics" is meant that the transformed plant exhibits increased fructan biosynthesis and/or contains increased levels of soluble carbohydrate relative to an untransformed control plant.

In a preferred embodiment the a transgenic plant cell, plant, plant seed or other plant part with enhanced biomass has an increase in biomass of at least approximately 15%, more preferably at least approximately 25%, more preferably at least approximately 35%, more preferably at least approximately 50% relative to an untransformed control plant.

For example, biomass may be increased by between approximately 15% and 500%, more preferably between approximately 25% and 300%, more preferably between approximately 35% and 200%, more preferably between approximately 50% and 100% relative to an untransformed control plant.

In a preferred embodiment, the transgenic plant cell, plant, plant seed or other plant part with modified fructan biosynthetic characteristics has an increase in soluble carbohydrate of least approximately 15%, more preferably at least approximately 25%, more preferably at least approximately 35%, more preferably at least approximately 50% relative to an untransformed control plant.

For example, soluble carbohydrates may be increased by between approximately 15% and 500%, more preferably between approximately 25% and 300%, more preferably between approximately 35% and 200%, more preferably between approximately 50% and 100% relative to an untransformed control plant.

- 5 Preferably the transgenic plant cell, plant, plant seed or other plant part is produced by a method according to the present invention.

The present invention also provides a transgenic plant, plant seed or other plant part derived from a plant cell of the present invention and including a genetic construct or vector of the present invention.

- 10 The present invention also provides a transgenic plant, plant seed or other plant part derived from a plant of the present invention and including a genetic construct or vector of the present invention.

Preferably, the transgenic plant cell, plant, plant seed or other plant part is a *Lolium* species, more preferably *Lolium perenne* or *Lolium arundinaceum*.

- 15 Preferably, the transgenic plant cell, plant, plant seed or other plant part is a cereal grain, more preferably a *Triticum* species, more preferably wheat (*Triticum aestivum*).

For example, the present invention enables the production of transgenic perennial ryegrass plants with increased fructans in leaf blades, vigorous growth and greater tolerance to abiotic stress, for improved nutrition for grazing animals.

- 20 The present invention also enables the production of transgenic wheat plants with increased fructans, vigorous growth, and tolerance to abiotic stress, for increased mass of usable carbohydrates, eg. for bio-fuel production or animal feed.

- By 'plant cell' is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further
25 propagation is desired. Plant cell, as used herein includes, without limitation, algae,

cyanobacteria, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores.

By 'transgenic' is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into either the nuclear or plastidic genome.

Detailed description of the embodiments

The present invention will now be more fully described with reference to the accompanying examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the figures:

Figure 1: Schematic representation of SacB protein from *Bacillus subtilis*, member of GH68 family. The four different regions shown are: N-terminal signal sequence; N-terminal variable region; catalytic core; and C-terminal variable region. Amino acid residues, including the catalytic triad (**D86**, **D247** and **E342**) and sucrose binding (**W85**, **W163** and **R246**).

Figure 2. Nucleotide sequence of *SacB* gene from *Bacillus subtilis* (Levansucrase). Nucleotide sequence coding for the N-terminal signal peptide is in bold.

Figure 3. Amino acid sequence SacB protein from *Bacillus subtilis* (Levansucrase). The N-terminal signal peptide is in bold.

Figure 4. Nucleotide sequence of *Lsc* gene from *Lactobacillus johnsonii* NCC 533 (Inulosucrase). Nucleotide sequence coding for the N-terminal signal peptide is in bold.

Figure 5. Amino acid sequence of *Lsc* protein from *Lactobacillus johnsonii* NCC 533 (Inulosucrase). The N-terminal signal peptide is in bold.

Figure 6. Nucleotide sequence of SPOR531, Preprosporamin protein from *I. batatas*. Vacuolar targeting signal sequence is shown in bold underlined.

Figure 7. Amino acid sequence of SPOR531, Preprosporamin protein from *I. batatas*. Vacuolar targeting signal sequence is shown in bold underlined.

Figure 8. *SPOR:SacB* chimeric nucleotide sequence. The N-terminal signal sequence of SacB has been replaced by the vacuolar targeting signal of SPOR (indicated by bold underlined).

Figure 9. *SPOR:SacB* chimeric protein sequence. The N-terminal signal sequence of SacB has been replaced by the vacuolar targeting signal of SPOR (indicated by bold underlined).

Figure 10. Secondary Structure Prediction of SPOR-SacB fusion protein using Secondary Structure Prediction of Membrane Proteins software SOSUI <http://bp.nuap.nagoya-u.ac.jp/sosui/>

Figure 11. *SPOR:Lsc* chimeric nucleotide sequence. The N-terminal signal sequence of Lsc has been replaced by the vacuolar targeting signal of SPOR (indicated by bold underlined).

Figure 12. *SPOR:Lsc* chimeric protein sequence. The N-terminal signal sequence of Lsc has been replaced by the vacuolar targeting signal of SPOR (indicated by bold underlined).

Figure 13. Secondary Structure Prediction of SPOR-Lsc fusion protein using Secondary Structure Prediction of Membrane Proteins software SOSUI <http://bp.nuap.nagoya-u.ac.jp/sosui/>

Figure 14. Secondary Structure Prediction of Lp1-SST using Secondary Structure Prediction of Membrane Proteins software SOSUI <http://bp.nuap.nagoya-u.ac.jp/sosui/>

Figure 15. Lp1-SST nucleotide sequence from *L. perenne*. The Lp1-SST transmembrane domain coding sequence is shown in bold italics.

Figure 16. Lp1-SST protein sequence from *L. perenne*. The Lp1-SST transmembrane domain is shown in bold italics.

Figure 17. Lp1-SST-SacB chimeric nucleotide sequence. The N-terminal signal coding sequence of SacB has been replaced by the Lp1-SST transmembrane domain coding sequence (indicated by bold italics).

Figure 18. Lp1-SST-SacB chimeric protein sequence. The N-terminal signal sequence of SacB has been replaced by the Lp1-SST transmembrane domain (indicated by bold italics).

Figure 19. Secondary Structure Prediction of Lp1-SST-SacB fusion protein using
5 Secondary Structure Prediction of Membrane Proteins software SOSUI
<http://bp.nuap.nagoya-u.ac.jp/sosui/>

Figure 20. Lp1-SST-Lsc chimeric nucleotide sequence. The N-terminal signal coding sequence of Lsc has been replaced by the Lp1-SST transmembrane domain coding sequence (indicated by bold italics).

10 **Figure 21.** Lp1-SST-Lsc chimeric protein sequence. The N-terminal signal sequence of Lsc has been replaced by the Lp1-SST transmembrane domain (indicated by bold italics).

Figure 22. Secondary Structure Prediction of Lp1-SST-Lsc fusion protein using
Secondary Structure Prediction of Membrane Proteins software SOSUI
15 <http://bp.nuap.nagoya-u.ac.jp/sosui/>

Figure 23. Nucleotide sequences of the *AtRbcS::SPOR-SacB::NOS* expression cassette

Figure 24. Nucleotide sequences of the *AtRbcS::SPOR-Lsc::NOS* expression cassette

20 **Figure 25.** Nucleotide sequences of the *AtRbcS::Lp1-SST-SacB::NOS* expression cassette

Figure 26. Nucleotide sequences of the *AtRbcS::Lp1-SST-Lsc::NOS* expression cassette

Figure 27. Gateway pDestination vector

25 **Figure 28.** Gateway pDestination vectors for transformation of dicots:

A. *AtRbcS::SPOR-SacB::NOS*; B. *AtRbcS::SPOR-Lsc::NOS* C. *AtRbcS::Lp1-SST-SacB::NOS* and D. *AtRbcS::Lp1-SST-Lsc::NOS*.

Figure 29. Nucleotide sequences of the *TaRbcSp::SPOR-SacB::TaRbcst* expression cassette

Figure 30. Nucleotide sequences of the *TaRbcSp::SPOR-SacB::TaRbcst TaRbcst + AtMYB32::IPT::35S* expression cassette

Figure 31. Nucleotide sequences of the *TaRbcSp::SPOR-Lsc::TaRbcst* expression cassette

5 **Figure 32.** Nucleotide sequences of the *TaRbcSp::SPOR-Lsc::TaRbcst + AtMYB32::IPT::35S* expression cassette

Figure 33. Nucleotide sequences of the *TaRbcSp::Lp1-SST-SacB::TaRbcst* expression cassette

Figure 34. Nucleotide sequences of the *TaRbcSp::Lp1-SST-SacB::TaRbcst TaRbcst + AtMYB32::IPT::35S* expression cassette

Figure 35. Nucleotide sequences of the *TaRbcSp::Lp1-SST-Lsc::TaRbcst* expression cassette

Figure 36. Nucleotide sequences of the *TaRbcSp::SPOR-Lsc::TaRbcst + AtMYB32::IPT::35S* expression cassette

15 **Figure 37.** Gateway pDestination vector pBS:ubi::bar::NOS

Figure 38. Gateway pDestination vectors for transformation of monocots:

A. *TaRbcS::SPOR-SacB:: TaRbcS* and **B.** *TaRbcS::SPOR-SacB:: TaRbcS + AtMYB32::IPT::35S*

Figure 39. Gateway pDestination vectors for transformation of monocots:

20 **A.** *TaRbcS::SPOR-Lsc:: TaRbcS* and **B.** *TaRbcS::SPOR-Lsc:: TaRbcS + AtMYB32::IPT::35S*

Figure 40. Gateway pDestination vectors for transformation of monocots:

A. *TaRbcS::Lp1-SST-SacB:: TaRbcS* and **B.** *TaRbcS::Lp1-SST-SacB:: TaRbcS + AtMYB32::IPT::35S*

25 **Figure 41.** Gateway pDestination vectors for transformation of monocots:

A. *TaRbcS::Lp1-SST-Lsc:: TaRbcS* and **B.** *TaRbcS::Lp1-SST-Lsc:: TaRbcS + AtMYB32::IPT::35S*

Figure 42. Isolation of mesophyll-derived protoplasts of *Nicotiana tabacum*. A)-B) Dissection of 4-6 week-old *in vitro* leaf material; pre-enzymatic digestion; C) Digestion of 4-6 week-old *in vitro* leaf material; 16 hours incubation; D) Harvesting of protoplast suspension; E) Separation of protoplast-rich interphase; F)-G) Intact, chloroplast-rich protoplasts.

Figure 43. Isolation of mesophyll-derived protoplasts of *Nicotiana tabacum* for transient expression analysis. A)-B) Intact, chloroplast-rich protoplasts; C) Culturing of protoplasts in liquid enrichment medium; D) Viable protoplast; 48 hours post isolation.

Figure 44. Isolation of mesophyll-derived protoplasts of *Nicotiana tabacum* for stable transformations. A)-B) Intact, chloroplast-rich protoplasts; C) Protoplast-embedded sea plaque agarose plug, day 0; D) Viable protoplasts; 6 days post isolation and embedding; E)-F) Embedded and liberated protoplast-derived micro-calli; 4 weeks post isolation and embedding.

Figure 45. Regeneration of shoots from mesophyll protoplast-derived micro-calli of *Nicotiana tabacum*. A) Liberated micro-calli in liquid growth medium A, 6-7 weeks post isolation and embedding; B-C) Proliferation of calli on solidified growth medium; D)-E) Shoot induction and regeneration from mesophyll protoplast-derived calli; F) Root development from regenerated shoots; G)-H) Growth and development of plants under glasshouse containment.

Figure 46. Evaluation of untransformed protoplast viability; 48 hours post isolation.

Figure 47. Evaluation of PEG-transformed protoplast viability; 48 hours post isolation and transfection.

Figure 48. *Agrobacterium*-mediated transformation of tobacco leaf discs. A) Co-cultivation of transformed leaf discs, day 0; B) Stage 1 initiation of shoots; 3 days post co-cultivation.

Figure 49. Detection of *gfp* expression in transformed leaf discs of Tobacco. A) Untransformed leaf disc, white light; B) Untransformed leaf disc, gfp2 filter; C) Untransformed leaf disc, gfp3 filter; D)&G) Turbo gfp-transformed leaf discs, white light; E)&H) Turbo gfp-transformed leaf discs, gfp2 filter; F)&I) Turbo gfp-transformed leaf discs, gfp3 filter.

Figure 50. Electrophoresis of RT-PCR samples and controls.

Lane 1 and 13: 1kb+ DNA Ladder

Lane 2: Amplification of *sacB* transcript from 2 μ L cDNA generated by reverse transcription of mRNA from transfected protoplast 1 (sample 9A) with gene specific
5 primer

Lane 3: Amplification of *sacB* transcript from 1 μ L cDNA (sample 9A)

Lane 4: Control reaction performed without reverse transcriptase (sample 9A)

Lane 5: Control reaction performed without template (*sacB* primers)

Lane 6: Amplification of *sacB* transcript from 2 μ L cDNA generated by reverse
10 transcription of mRNA from transfected protoplast 1 (sample 12A) with gene specific primer

Lane 7: Amplification of *sacB* transcript from 1 μ L cDNA (sample 12A)

Lane 8: Control reaction performed without reverse transcriptase (sample 12A)

Lane 9: Amplification of *18S* transcript from 2 μ L cDNA generated by reverse
15 transcription of mRNA from untransfected protoplast with gene specific primer

Lane 10: Amplification of *18S* transcript from 1 μ L cDNA generated by reverse transcription of mRNA from untransfected protoplast with gene specific primer

Lane 11: Control reaction performed without reverse transcriptase (untransfected protoplast)

20 Lane 12: Control reaction performed without template (*18S* primers)

Examples

Example 1

Isolation of bacterial fructan biosynthesis genes

Figure 1 presents a schematic representation of SacB protein from *Bacillus subtilis*.

- 5 The four different regions shown are: N-terminal signal sequence; N-terminal variable region; catalytic core; and C-terminal variable region. Structurally, most of the bacterial inulosucrases and levansucrases share the N-terminal signal peptide, a catalytic triad. This sequence is removed during the sequence modification. The residues involved in sucrose binding are located inside the catalytic core sequences and remain untouched
- 10 during the modification.

The bacterial levansucrase (SacB) and inulosucrase (Lsc) nucleotide and protein sequences are provided in Figures 2 - 5, respectively. However, for transformation into plants the bacterial levansucrase and inulosucrase sequences are also modified in the following manner:

- 15
- Removal of the bacterial N-signal peptide;
 - Adaptation of codon usage, including the start of translation for monocots and dicots;
 - Removal of cryptic splice sites and RNA destabilizing sequence elements;
 - The coding sequence is further modified with putative sub-cellular targeting
- 20 sequences including vacuolar targeting sequences for monocots and dicots as well as including plant 1-SST-specific transmembrane domains.

Outlines of these changes are indicated in the following example.

Example 2

Modification of bacterial fructan biosynthesis genes

Targeting of bacterial FT genes to specific cellular compartments

To direct the bacterial FT genes away from the cytosol and to compartment where both
5 sucrose and fructan accumulate the coding sequences of *SacB* and *Lsc* are modified
with a putative vacuolar targeting sequence from the preprosporamin protein
(SPOR531) of sweet potato (*Ipomoea batatas*). The propeptide of a precursor to
sporamin is required for targeting of sporamin to the vacuole (Hattori et al., 1985). The
vacuolar targeting information of sporamin is encoded in an amino-terminal propeptide
10 and is indicated in Figures 5 and 6.

Sequence modification involves the removal of the N-signal peptide from both the *SacB*
and *Lsc* bacterial fuctan biosynthesis genes and the addition of SPOR531 vacuolar
targeting signal (Figures 7-8 and 10-11, respectively).

Prediction of subcellular localisation and topology of the modified proteins using the
15 Secondary Structure Prediction of Membrane Proteins software SOSUI
<http://bp.nuap.nagoya-u.ac.jp/sosui/> indicates a transmembrane localization triggered by
the vacuolar targeting signal (Figures 9 and 12).

Addition of transmembrane domains from Lp1-SST protein to bacterial FT genes

The SOSUI software was also used to predict the secondary structure of the *Lolium*
20 *perenne* 1-SST gene. This structure, indicating a transmembrane domain at the N
terminus is indicated in Figure 13. The transmembrane domain coding and protein
sequences are indicated in Figures 14 and 15, respectively.

Sequence modification involves the removal of the N-signal peptide from both the *SacB*
and *Lsc* bacterial fuctan biosynthesis genes and the addition of the Lp1-SST
25 transmembrane domain (Figures 16-17 and 19-20, respectively). The modified
sequences of *SacB* and *Lsc* were assessed using the Secondary Structure Prediction of
Membrane Proteins software SOSUI for subcellular localization and protein topology

and their predicted secondary structures are presented in Figures 18 and 21, respectively.

Example 3

Generating vectors for stable transformation in dicots

5 Synthesis of expression constructs

Expression constructs utilising photosynthetic promoters, the modified bacterial fructan biosynthesis genes indicated in Example 2 and the NOS terminator sequence for transformation into dicot plants is artificially synthesised.

10 The use of a photosynthetic promoter expresses the genes in tissues that accumulate fructans, while the modified sequences target the protein to specific plant cell compartments.

The Ribulose-1,5-bisphosphate carboxylase/oxygenase Small subunit (RbcS) is a well-characterised light-regulated gene in higher plants. A 1700 bp fragment of the *Arabidopsis thaliana* Ribulose-1,5-bisphosphate carboxylase/oxygenase Small subunit
15 (AtRbcS) promoter sequence has previously been cloned. Primers are designed to amplify a smaller fragment containing the TATA signal from the AtRbcS promoter for use in expression vectors.

The newly predicted sequences for the modified bacterial fructan biosynthetic genes are be artificially synthesised altering codon usage for expression in plants, as well as
20 removing cryptic splice sites and RNA destabilizing sequence elements, to optimise their performance in the plant cell.

Figures 23 - 26 represent the expression cassettes AtRbcS::SPOR-SacB::NOS, AtRbcS::SPOR-Lsc::NOS, AtRbcS::Lp1-SST-SacB::NOS and AtRbcS::Lp1-SST-Lsc::NOS, respectively, and have not yet had codon optimisation or removal of
25 destabilising elements.

Generation of constructs containing modified bacterial FT genes driven by an Arabidopsis photosynthetic promoter for transformation of dicots

Each synthesised expression cassette is placed in a Gateway enabled pDONOR vector for recombination into the final destination vector for transformation into plants.

- 5 A Gateway enabled destination vector, containing the 35Sp:hph:35St selectable marker cassette has been generated, pPZP200_35Sp_hph_35St_R4/R3 (Figure 27).

Gateway LR recombination reactions produce the following destination vectors for transformation into dicots:

AtRbcS::SPOR-SacB::NOS (Figure 28A);

- 10 AtRbcS::SPOR-Lsc::NOS (Figure 28B);

AtRbcS::Lp1-SST-SacB::NOS (Figure 28c) and

AtRbcS::Lp1-SST-Lsc::NOS (Figure 28d).

Example 4

Generating vectors for stable transformation in monocots

- 15 **Synthesis of expression constructs**

Expression constructs utilising the bread wheat photosynthetic promoter (*TaRbcsp*), the modified bacterial fructan biosynthesis genes, indicated in Example 2, and the *TaRbcS* terminator sequence for transformation into monocot plants are artificially synthesised. The use of a photosynthetic promoter expresses the genes in tissues that accumulate
20 fructans, while the modified sequences target the protein to specific plant cell compartments.

The bread wheat (*Triticum aestivum*), *TaRbcS* regulatory sequences (promoter and terminator) have previously been cloned (Zeng, et al., 1995; Sasanuma, 2001). A 695 bp promoter fragment from sequence previously published containing the TATA signal from

the *TaRbcS* gene (NCBI accession number AB042069) is amplified for use in expression vectors.

The newly predicted sequences for the modified bacterial fructan biosynthetic genes are artificially synthesised altering codon usage for expression in plants, as well as
5 removing cryptic splice sites and RNA destabilizing sequence elements, to optimise their performance in the plant cell.

Using the methods outlined above expression cassettes are synthesised to generate transgenic plants that contain both fructan biosynthetic genes and the LXR™ technology. LXR™ technology is based on an expression cassette containing one
10 candidate gene (IPT) for delayed leaf senescence under the control of the AtMYB32 gene promoter. The expression cassette AtMYB3p::IPT::35S is described in International patent application PCT/AU01/01092. The phenotype of transgenic LXR™ plants includes a decrease in leaf yellowing and chlorophyll loss associated with plant age leading to an increased photosynthetic ability resulting in improved tillering and
15 vegetative biomass.

Integration of the two technologies leads to an increased expression of fructans via an extension of activation of the photosynthetic promoters and may have significant impact on the efficacy of a variety of applications by increasing the range of productivity in plants.

20 Figures 29 - 36 represent the expression cassettes, TaRbcS::SPOR-SacB::TaRbcS, TaRbcS::SPOR-SacB::TaRbcS + AtMYB32::IPT::35S, TaRbcS::SPOR-Lsc::TaRbcS, TaRbcS::SPOR-Lsc::TaRbcS + AtMYB32::IPT::35S, TaRbcS::Lp1-SST-SacB::TaRbcS, TaRbcS::Lp1-SST-SacB::TaRbcS + AtMYB32::IPT::35S, TaRbcS::Lp1-SST-Lsc::TaRbcS and TaRbcS::Lp1-SST-Lsc::TaRbcS + AtMYB32::IPT::35S, respectively,
25 and have not yet had codon optimisation or removal of destabilising elements.

Generation of constructs containing modified bacterial FT genes driven by a *Triticum* photosynthetic promoter for transformation of monocots

Each synthesised expression cassette is placed in a Gateway enabled pDONOR vector for recombination into the final destination vector for transformation into plants.

- 5 A Gateway enabled destination vector, containing the Ubi::bar::NOS selectable marker cassette has been generated, pBS::Ubi::bar::NOS_R4/R3 (Figure 37). Gateway LR recombination reactions produce the following destination vectors for transformation in to monocots:

TaRbcS::SPOR-SacB:: TaRbcS (Figure 38A);

- 10 TaRbcS::SPOR-SacB:: TaRbcS + AtMYB32::IPT::35S (FigureB);

TaRbcS::SPOR-Lsc:: TaRbcS (Figure 39A);

TaRbcS::SPOR-Lsc:: TaRbcS + AtMYB32::IPT::35S (Figure 39B);

TaRbcS::Lp1-SST-SacB:: TaRbcS (Figure 40A);

TaRbcS::Lp1-SST-SacB:: TaRbcS + AtMYB32::IPT::35S (FigureB);

- 15 TaRbcS::Lp1-SST-Lsc:: TaRbcS (Figure 41A) and

TaRbcS:: Lp1-SST-Lsc:: TaRbcS + AtMYB32::IPT::35S (Figure 41B)

Example 5

Constructs for dicotyledons – *N. tabacum* protoplasts and *A. thaliana*

- 20 The following constructs were made in versions for direct delivery (transient expression in protoplasts) and versions in binary transformation vectors for stable delivery to *Arabidopsis* (*A. thaliana*) and tobacco (*N. tabacum*)

1. AtRbcS::1-SST-SacB*

2. AtRbcS::SPOR-SacB*

3. AtRbcS::1-SST-Lsc*

4. AtRbcS::SPOR-Lsc*

* The bacterial levansucrase and inulosucrase sequences are modified in the following manner:

- Removal of the bacterial N-signal peptide;
- 5 • Adaptation of codon usage, including the start of translation for monocots and dicots
- Removal of cryptic splice sites and RNA destabilizing sequence elements
- The coding sequence is further modified with putative sub-cellular targeting sequences including vacuolar targeting sequences for monocots and dicots as
10 well as including plant 1-SST- and FFT-specific trans-membrane domains.

Example 6

POLYETHYLENE GLYCOL-MEDIATED TRANSFORMATION OF MESOPHYLL-DERIVED PROTOPLASTS OF TOBACCO

This example describes delivery of the expression cassettes hereinbefore described to
15 tobacco protoplasts (see Figures 42-45).

I. ISOLATION OF MESOPHYLL-DERIVED PROTOPLASTS FOR DIRECT GENE TRANSFER

A. Digestion of In vitro Shoot Cultures to Yield Mesophyll-derived Protoplasts

Enzyme Solution

- 20 1.0 % (w/v) cellulase "Onozuka" R10 and 1.0 % (w/v) Macerozyme® R10 dissolved in K4 medium [medium K3 with 0.4 M sucrose instead of 0.3 M]. Spin down (Sorvall centrifuge, SS 34 rotor; at 7,000 rpm for 10 min.) in order to pellet contaminating starch

of the enzyme preparations. Adjust pH 5.6 with KOH and filtersterilize (0.2 µm pore size). Store at 4°C for no longer than 3-4 weeks.

Materials

- 400 ml culture vessels containing solidified MS medium with shoot cultures of
5 *Nicotiana tabacum*
- 90 x 20 mm sterile Petri dishes
- Forceps
- Scalpel
- Sterile scalpel blades; #11 or #22

10 **Solutions**

- Enzyme solution; 1 % Cellulase and 1 % Macerozyme dissolved in K4 medium
- Sterile water

Procedure

1. Into sterile 90 x 20 mm Petri dishes, decant a volume of enzyme solution sufficient to
15 generously cover plate base; 15 ml should suffice.
2. Transfer 2-4 healthy, fully expanded leaves of a 4-6 week-old shoot culture to an empty 90 X 20 mm Petri dish.
3. With the abaxial-side up, carefully remove the mid-rib of one leaf, ensuring a sharp, sterile blade is used to minimise tearing of surrounding leaf tissue.
20 Repeat for remaining 3 leaves.

Handle small quantities of leaf material (maximum 4) at any one time to minimise desiccating effect of laminar flow.

4. Gently stack leaf halves and, with a sharp, sterile blade, slice into 1-2 mm strips.
5. Carefully transfer leaf segments into a Petri dish containing enzyme solution (abaxial-side down). Seal dish with Parafilm® and incubate overnight for 16 – 18 hours at 25°C in the dark *without* shaking.

B. Isolation of Mesophyll-derived Protoplasts

Materials

- 10
 - Sterile 5 ml pipettes
 - Sterile 10 ml pipettes
 - Pipette boy
 - Sterilised protoplast filtration unit: 100 µm stainless steel mesh sieve resting on a 100 ml glass beaker
- 15
 - Sterile 14 ml plastic round-bottomed centrifuge tubes
 - Clements Orbital 500 bench centrifuge
 - Waterbath

Media

- 90 x 20 mm sterile Petri dish/es containing digesting leaves of *Nicotiana tabacum*
- 20
 - Solidified 1:1 mix of K3:H medium containing 0.6% Sea Plaque™ agarose

Solutions

- Autoclaved W5 Solution
- Autoclaved K3 Solution

Procedure

- 5 6. Gently agitate the overnight digest to release protoplasts into the enzyme solution.

Agitation should be gentle, yet thorough, and performed in a side-to-side (horizontal) motion.

7. Angle plate slightly to aid transfer of digesting suspension (enzyme solution and plant debris). Using a 10 ml sterile pipette, transfer digesting suspension
10 to a sterilised protoplast filtration unit to separate protoplast suspension from plant debris.

8. Tap filtration unit gently to release excess liquid caught in sieve.

9. Mix the protoplast suspension gently and distribute into 14 ml sterile plastic round-bottomed centrifuge tubes, filling to approximately 8 ml (maximum 9
15 ml).

10. Re-distribute suspension to obtain a uniform distribution of volumes (max. 9 ml).

11. Carefully overlay each suspension with 1.5 ml W5 solution.

- 20 To aid dispensing W5 solution, place suspension-filled tube on an angle and allow pipette tip to touch wall surface near tube opening before slowly lowering to just above the suspension surface. Slowly dispense W5 solution, adding drop-by-drop, ensuring to keep pipette tip as close to the

suspension surface as possible. Minimal agitation of protoplast suspension and, thus, mixing with W5 solution will result if correctly performed.

12. Carefully replace lids and centrifuge tubes for 5 minutes at 70g (Clements
5 Orbital 500 bench centrifuge, swing-out rotor, 400 rpm). Protoplasts will float at the interphase.
13. Keeping protoplast-filled tube upright, carefully lower a sterile 5 ml pipette to a point just above the layer of protoplasts and collect the protoplasts at the interphase, taking as little as possible of the lower phase.
- 10 W5 solution will be collected simultaneously.
14. Collect and transfer protoplasts to one new 14 ml centrifuge tube. Upon completing protoplast collection, gently mix protoplast suspension by gently pipetting up and down.
15. Determine protoplast yield by removing a 100 μ l aliquot of the protoplast
15 suspension and transferring to a tube containing 900 μ l W5 solution. Count the protoplasts in a haemocytometer and determine the number of protoplasts per ml.
16. Calculate the total volume required to obtain approximately 1×10^6 (maximum
1.5 $\times 10^5$) protoplasts per ml. Distribute protoplast suspension in new 14 ml
20 round-bottomed centrifuge tubes, ensuring equal volumes are obtained.
17. Using a 10 ml pipette, fill each protoplast-containing tube with W5 solution up to a total volume of 10 ml. To minimise disruption to the protoplasts, spray W5 solution along the tube wall when filling.

18. Replace lids and resuspend the protoplasts by gently inverting the capped tube once.
19. Pellet the protoplasts [spin 70g (Clements Orbital 500 bench centrifuge, 400 rpm) for 5 min.] before removing all W5 solution, leaving pure protoplast suspension.
20. Resuspend protoplast suspensions by gently shaking.
21. Fill each protoplast-containing tube to a total volume of 5 ml with W5 solution and incubate at room temperature for a minimum of 1 hour and a maximum of 4.
22. During 1-4 hour incubation time, organise the following components in preparation for direct gene transfer into isolated protoplasts:
- Remove 40% PEG solution from -20 °C storage and store at room temperature. 30 minutes prior to proceeding with the direct gene transfer, incubate PEG solution in a beaker of hot water.
 - Melt solidified K3:H medium in microwave. Once completely melted, place in a 40 °C water-bath until ready to use.

II. DIRECT GENE TRANSFER TO PROTOPLASTS USING POLYETHYLENE GLYCOL

Transforming DNA

Plasmid DNA is sterilized by precipitation and washing in 100% (v/v) ethanol and dried in a laminar flow hood [precipitation of plasmid DNA in 70% ethanol is also possible, but DNA pellet will take longer to dry]. DNA pellet is resuspended in 30 µl sterile double-distilled water at a final concentration of 0.7 µg/µl for transient transformations. The physical structure of the DNA should be supercoiled for transient and linearized - outside of the gene of interest - for stable transformations. Addition of carrier DNA (e.g.

fish-sperm DNA) to the transforming plasmid DNA usually gives better stable transformation frequencies. For stable transformations 10 µg of linearized plasmid DNA and 40 µg of sheared fish-sperm DNA are co-precipitated as indicated above, dried and dissolved in 30 µl of sterile double-distilled water.

5 Transformation Buffer

15 mM MgCl₂, 0.1% (w/v) morpholinoethanesulphonic acid (MES) and 0.5 M mannitol. After dissolving in distilled water, adjust pH 5.8 with KOH and autoclave. Store at 4°C.

PEG Solution

40% (w/v) PEG 4000 in 0.4 M mannitol and 0.1 M Ca(NO₃)₂. Dissolve PEG in 0.4 M
10 mannitol and 0.1 M Ca(NO₃)₂ (the final concentration of these two components will be lower due to the volume of PEG). Adjust pH 8-9 and autoclave (the pH will take several hours, e.g. overnight, to stabilize in this solution and will drop to pH 6-7 after autoclaving).

Materials

- 15 • Sterile 1 ml pipettes
- Sterile 5 ml pipettes
- Sterile 10 ml pipettes
- Pipette boy
- Sterile 14 ml plastic round-bottomed centrifuge tubes
- 20 • Clements Orbital 500 bench centrifuge
- Waterbath

- 50 x 10 mm Petri dishes

Media

- 14 ml round-bottomed centrifuge tubes containing isolated protoplast suspension, pelleted
- 5 • Solidified 1:1 mix of K3:H medium containing 0.6% Sea Plaque™ agarose

Solutions

- Autoclaved W5 Solution
 - Autoclaved K3 Solution
 - Autoclaved H Solution
- 10 • 40% PEG Solution
- Transformation Buffer
 - 10 µg Transforming DNA dissolved in 30 µl sterile double-distilled water.

Procedure

1. Pellet the protoplasts [spin 70g (Clements Orbital 500 bench centrifuge, 400 rpm) for
15 5 min.] before removing all W5 solution, leaving pure protoplast suspension.
2. Using a 1 ml pipette, add (*drop-wise*) 300 µl (approximately 7 drops) of transformation buffer to each 14 ml round-bottomed centrifuge tube containing isolated protoplasts.
3. Carefully resuspend pellet by gently tapping base of tube.

4. To each protoplast suspension, add 10 µg (30 µl) of transforming DNA before adding 300 µl (approximately 7 drops when using a 1 ml pipette for dispensing) of pre-warmed PEG solution. Mix protoplast suspension by gently tapping tube base.

5 Time interval between resuspending protoplasts in transformation buffer and the addition of transforming DNA and PEG should be kept at a minimum.

5. Incubate transformation mix for 15 minutes at room temperature with no agitation.

6. Using a 10 ml pipette, gradually add 10 ml W5 solution to each tube in intervals of:

- 1 ml (approximately 12 drops) drop-wise to each tube. Gently invert all tubes to mix.
- 10 • 1 ml (approximately 12 drops) drop-wise to each tube. Gently invert all tubes to mix.
- 1 ml (approximately 12 drops) drop-wise to each tube. Gently invert all tubes to mix.
- 2 ml as a gentle stream to each tube. Gently invert all tubes to mix.
- 15 • 2 ml as a gentle stream to each tube. Gently invert all tubes to mix.
- 3 ml as a gentle stream to each tube. Gently invert all tubes to mix.

To aid dispensing, in a 10 ml pipette collect the total volume required at each interval to fill each tube with the required volume of W5 solution, prior to dispensing. Repeat at each interval.

- 20 7. Pellet the protoplasts [spin 70g (Clements Orbital 500 bench centrifuge, 400 rpm) for 10 min.] before removing all W5 solution, leaving pure protoplast suspension. Tap all tube bases once before proceeding.

FOR TRANSIENT TRANSFORMATIONS

8. Resuspend protoplast pellet in equal volumes of K3 medium and H medium up to a total volume of 5 ml (2.5 ml of each solution).
9. Slowly transfer the liquid K3:H + protoplast-suspension-mix to the centre of a 50 x 10 mm Petri dish.
10. Seal all dishes with Parafilm® and culture protoplasts for 24 – 72 hours under dim light at 24°C, before proceeding with transient expression analysis.

FOR STABLE TRANSFORMATIONS

11. Continue with “Part III. Culture of Mesophyll-derived Protoplasts and Regeneration of Plants”, below.

III. CULTURE OF MESOPHYLL-DERIVED PROTOPLASTS AND REGENERATION OF PLANTS

For Steps 1 & 2, each protoplast-containing tube must be handled one tube at a time.

Materials

- Sterile 1 ml pipettes
- Sterile 5 ml pipettes
- Sterile 10 ml pipettes
- Pipette boy
- Clements Orbital 500 bench centrifuge
- Waterbath

- 50 x 10 mm Petri dishes
- Autoclaved stainless steel spatula

Media

- 14 ml round-bottomed centrifuge tubes containing isolated protoplast suspension,
5 pelleted
- Solidified 1:1 mix of K3:H medium containing 0.6% Sea Plaque™ agarose; 40 °C
- Autoclaved K3 Solution
- 250 ml culture vessel containing 20 ml A medium
- 12-well Costar® plates containing solidified MS Morpho medium
- 10 • 250 ml culture vessels containing solidified MS Morpho medium
- 250 ml culture vessels containing solidified MS medium

Procedure

1. Add 0.5 ml K3 medium close to the protoplast pellet to resuspend the protoplasts.
2. Slowly transfer the K3 + protoplast-suspension-mix to the centre of a 50 x 10 mm
15 Petri dish.
3. Repeat Steps 1 and 2 for all protoplast-containing tubes before proceeding.
4. Add 5 ml pre-warmed 1:1 mix of K3:H medium containing 0.6% Sea Plaque™ agarose one plate at a time. In a gentle swirling motion, shake plate *once only* to evenly distribute protoplast suspension in medium. Repeat for all plates.

5. Leave plates to stand, untouched, until medium has solidified (10-30 minutes depending on ambient temperature).
6. Seal all dishes with Parafilm® and culture protoplasts for 24 h in complete darkness at 24°C, followed by 6 days under continuous dim light (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes), where first and multiple cell divisions occur.
7. Using a sterile spatula, divide the protoplast-containing sea plaque agarose plugs into quadrants and place into 250 ml plastic culture vessels containing 20 ml of A medium supplemented with appropriate antibiotic (1 quadrant per 250 ml vessel). Incubate on a rotary shaker at 80 rpm and 1.25 cm throw at 24°C in continuous dim light.
8. Replace liquid A medium + appropriate antibiotic every 2 weeks, monitoring growth of protoplast-derived colonies.
9. When protoplast-derived colonies are approximately 2-3 mm in diameter (5-6 weeks incubation in liquid A medium), transfer colonies into individual wells of a 24-well Costar® plate containing solidified MS Morpho medium.
10. Incubate plate/s for 1-2 weeks at 24°C under continuous dim light (5 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes), where calli proliferate and reach a size of 8-10 mm in diameter.
11. When protoplast-derived calli are approximately 1-2 cm in diameter, transfer calli to individual 250 ml culture vessels containing solidified MS Morpho medium. Incubate vessels at 24°C under 16 hour light/8 hour dark conditions (20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes). Within 1-2 weeks, multiple shoots should be visible.
12. Transfer shoots of 3-4 cm lengths to 250 ml culture vessels containing solidified MS medium to encourage root formation. Incubate vessels at 24°C under 16 hour light/8 hour dark conditions (20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes). Within 3 weeks, signs of root formation should be visible.

13. Plantlets with an established root system should be maintained as *in vitro* plant cultures as sources for mesophyll protoplasts of Tobacco.

Example 7

EVALUATION OF TOBACCO PROTOPLAST VIABILITY USING EVANS BLUE

5 STAIN

See Figures 46 and 47.

BACKGROUND INFORMATION

- Evans blue stain (EVB; 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo)bis[4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid] tetrasodium salt).
- 10 • Non-fluorescent dye.
- Method of action: Living cells retain the ability to exclude Evans blue at the plasma membrane and remain their natural colour. Cells damaged by salt or osmotic stress are unable to exclude Evans blue, are stained deep blue, and are readily distinguished upon microscopic examination.
- 15 • Method of preparation: 400 mg/l stock solution (solvent: 0.65 M Mannitol)
- Method of staining: Evans blue stock solution was added to an equal volume of protoplast suspension, gently mixed and incubated at room temperature for 10 minutes prior to microscopic visualisation.
- Method of detection: Leica MZFL III Light Dissecting Microscope.

20 Example 8

GENE EXPRESSION ANALYSIS IN *NICOTIANA TABACUM* PROTOPLASTS

Aim of the experiment: The expression of the cloned genes AtRbcS::1-SST-SacB and AtRbcS::SPOR-SacB in transfected tobacco protoplasts was tested using RT-PCR

Materials and methods: 3 plates of protoplasts named

- i) Transfected protoplasts (sample 9A): AtRbcS::1-SST-SacB
- 5 ii) Transfected protoplasts (sample 12A): AtRbcS::SPOR-SacB
- iii) Untransfected protoplasts

Steps involved in the expression analysis:

- 1. Primer design and optimisation of PCR
- 2. Total RNA isolation
- 10 3. RT reaction
- 4. qRT-PCR assay

Primer design and PCR product identity: Primer pairs were designed to amplify the gene of interest using beacon design software (Premier Biosoft International) and the
15 gene sequences available in gene bank. The gene specific primers were chosen so that the resulting PCR product size ranged from 200 to 250 bp. The PCR products were identified by melt curve analysis and size based on gel electrophoresis.

Total RNA isolation: Total RNA was isolated by SV Total RNA isolation system by PROMEGA from 1×10^6 protoplasts per treatment.
20 <http://www.promega.com/tbs/tm048/tm048.pdf>

Yield of total RNA:

Sample	Quantity
9A	3.4ng/ μ l

12A	4.2ng/ μ l
Control (untransfected protoplasts)	10.2 ng/ μ l

Reverse Transcriptase Reaction

RT reaction was performed by using QIAGEN RT kit. Four RT reactions were performed (9, 12, control and WT tobacco RNA) by using primer mix (QIAGEN).

- 5 <http://www1.qiagen.com/products/pcr/QuantiTectPcrSystems/QuantiTectRevTranscriptonKit.aspx#Tabs=t2>

A replicate of each of the above samples was subjected to RT reaction using gene-specific primers. Specifically, samples 9A and 12A were transcribed using the reverse *sacB* primer, and the control (untransfected) sample with the reverse *18S* primer.

10 PCR

Reactions were set up as follows:

	2x GoTaq® Green MasterMix (Promega)	10 μ L
	cDNA template	2.0 μ L
	Forward primer (10 μ M)	0.5 μ L
15	Reverse primer (10 μ M)	0.5 μ L
	Nuclease free water	7.0 μ L

Reactions were cycled:

Step 1:	95 °C	2 min
Step 2:	95 °C	30 sec

50

Step 3: 55 °C 30 sec

Step 4: 72 °C 60 sec

25 cycles from Step 2 (*18S*) or 35 cycles from Step 2 (*sacB*)

Step 5: 4 °C hold

- 5 Reaction products were visualised under UV light after electrophoresis through 1% (w/v) agarose in 1x TBE buffer, staining with SYBR (50 µL/L).

Detection of the *sacB* transcript was shown in both transfected protoplast cDNA samples (9 and 12), while the method used was validated by amplification of *18S* from the untransfected protoplast cDNA sample (Figure 50).

- 10 Expression of chimeric *sacB* genes under control of light regulated promoters was observed in transfected protoplasts. No products were observed for no-RT controls and no Template controls. *sacB* gene expression could be detected in protoplasts transfected with vectors used in samples 9A and 12A.

Example 9

15 **AGROBACTERIUM TUMEFACIENS-MEDIATED LEAF DISC TRANSFORMATION OF TOBACCO**

This example describes stable transformation of tobacco leaf discs using *Agrobacterium* carrying binary vectors engineered with the expression cassettes hereinbefore described (see Figures 48 and 49).

20 **INTRODUCTION**

- Utilising *Agrobacterium tumefaciens*-mediated leaf disc transformation is an efficient method of producing transgenic plants. *A. tumefaciens* is a natural dicot pathogen that contains the genetic machinery to infect the plant and incorporate the bacterial DNA into the plant genome. As a result of this capability, *A. tumefaciens* can be adopted as a
- 25 cloning vehicle to incorporate DNA of specific interest into tobacco, for example.

The method can be used to generate a model system in tobacco, to assess the function of cDNA heterologous clones for the gene of interest.

MATERIALS AND CHEMICALS

Equipment and instruments

- 5 Laminar flow hood with horizontal flow (series HWS180, CLYDE-APAC, a division of Evans Deakin Pty. Ltd., Woodville North, South Australia 5012, Australia), rotary shaker (Infors type RC-406, Infors AG, CH-4103 Bottmingen, Switzerland), bench centrifuge with swing-out rotor (Clements Orbital 500), forceps (bend, cat no. 2108/160, Crown Scientific, Rowville, Victoria 3178, Australia), scalpel handles (No 3, cat no. SHN3,
- 10 Crown Scientific, Rowville Victoria 3178, Australia) with sterile surgical scalpel blades (size 11, cat no. 1838, Laboratory Supply Pty. Ltd., Milperra DC, New South Wales 1891, Australia) were used.

Stock Solutions

- The macro-elements, micro-elements and vitamins required for all culture media must
- 15 be prepared as concentrated stocks (macro-elements stock: 10-fold concentrated; micro-elements and vitamins stocks: 100-fold concentrated) to aid in their addition. All stocks, except that containing the micro-elements are prepared at room temperature. Preparation of the micro-elements stock requires the heating of components prior to mixing. $\text{Na}_2\text{-EDTA}$ and $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$ must each be dissolved in 400 mL distilled water
- 20 (for a total volume of 1000 mL) prior to mixing. Mix dissolved solutions and heat at ca. 60°C until solution turns yellow in colour. Allow solution to cool before adding remaining components. Make solution up to 1000 mL with distilled water. Store all stocks at 4°C. Dissolve hormones [2,4-D (2,4-dichlorophenoxyacetic acid) and kinetin] in 1 M KOH and dilute with distilled water to prepare 100 mg/liter concentrated stocks.

Culture Media

The composition of the media used at the final concentrations of their individual ingredients is given in Appendix 1: MS micro, MS macro, B5 vitamins, Lauria Bertani media, Wash media, PC media, SEL media, RM media and SEM media.

5 Chemicals

2,4-D: 2,4-dichlorophenoxyacetic acid, Activated charcoal, Timentin (can be replaced by Cefotaxime at same concentrations), BAP (6-benzylaminopurine), Zeatin, AgNO_3 , Rifampicin, Agar (Difco, Bacto-Agar, cat. no: 0140-01) is used as the gelling agent. Other chemicals (PEG 4000, Tween 80, KOH, NH_4OH , NaCl, KCl, $\text{Ca}(\text{NO}_3)_2$, MgCl_2 and CaCl_2) were purchased from BDH; MES (2-[N-morpholino] ethanesulfuric acid) from Sigma (Cat. No. N-8250), kanamycin sulphate were from Sigma; hygromycin B was purchased from Calbiochem; $\text{Ca}(\text{OCI})_2$ (-65%) and phosphinotricin were from Roth and Riedel de Haen, respectively. Sucrose was purchased from Fluka (cat. no: 84100). Parafilm® "M" (American National Can™, Greenwich, CT 06836, USA) was used as sealing tape. Sterile disposable bottle-top filters (0.2 μm vacucap 90; cat. no. 4622, Gelman Sciences® Pty. Ltd., Cheltenham, Victoria 3192, Australia) and disposable filter units (0.2 μm ; cat. no. 16534, Sartorius AG, 37070 Gottingen, Germany) were used for filter-sterilisation. Sterile disposable pipettes: 1 mL_ (TRP®; cat. no. 94001) 5 mL_ (TRP®; cat. no. 94005) and 10 mL_ (TRP®; cat. no. 94010); all from Life Technologies Pty. Ltd., Mulgrave, 3170 Australia, sterile plastic centrifugal tubes with screw cap (14 mL, TRP®; cat. no.91016, Life Technologies Pty. Ltd., Mulgrave, 3170 Australia); sterile plastic petri dishes (90 x 14 mm; cat. no. 82.9923.484, and 60 x 14 mm, cat. no. 83.1801.011, Sarstedt® Australia Pty. Ltd., Technology Park, South Australia 5095, Australia and 90 x 20 mm; cat. no. 664160, Greiner Labortechnik GmbH, 72636 Frickenhausen, Germany); and autoclavable culture vessels (250 mL, cat. no. 75.9922.519, Sarstedt Australia Pty. Ltd, Technology Park, South Australia 5095, Australia) were used.

Plant Material

- A) Sterile shoot cultures of *N. tabacum* cv. Petit Havana SR1 can be utilised. They are established from corresponding seeds surface-sterilised in hypochlorite solution [1.4% (w/v) $\text{Ca}(\text{OCl})_2$, 0.05% (v/v) Tween 80] for 15 min., and after 3-4 rinses in sterile distilled water, plated for germination on half-strength MS medium (Appendix 1) solidified with 0.8% (w/v) agar. Shoots with 2-3 leaves are cut and grown in 250 mL culture vessels on 0.8% (w/v) agar-solidified MS medium at 25°C in 16h/d light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes). Rooted shoots are subcultured at 6 weeks intervals as stem cuttings, several times before use.
- 10 B) Glasshouse grown *N. tabacum* can also be utilised, but requires leaf surface sterilisation as described below [I. B)]. Plant seeds in sterile soil ensuring they are not planted too deeply and that they remain moist. Grow under 16h/d light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$, Osram L36 W/21 Lumilux white tubes) conditions 25°C, fertilising with Osmocote slow release fertiliser.
- 15 C) The strain of *Agrobacterium tumefaciens* utilised for leaf disc transformations is AGL1.

I. Preparation of *Agrobacterium tumefaciens* for Transforming Tobacco Leaf Discs with vector pBinhph200

Commence pre-culture of transformed *Agrobacterium tumefaciens* strain AGL1 two days prior to tobacco transformation date ensuring sterile conditions are maintained, (see Appendix 2 for the ID card for pBinhph200)

1. Scratch the surface of -70°C frozen glycerol bacterial stock with an inoculation loop and inoculate 2 mL of LB (containing 20 mg/mL rifampicin, plus 10 mg/L spectinomycin) in a sterile tube. Incubate for 24 hours at 28°C at 150rpm.
- 25 2. Inoculate 4 mL of LB plus antibiotics (in a 12 mL sterile tube) with 0.25 mL of the 24 hour pre-culture. Incubate for 6-7 hours at 28°C.

3. Inoculate 25mL LB with no antibiotics (in a 150 mL sterile flask) with 0.025ml of 6-7 hour pre-culture and incubate overnight at 28°C and 150 rpm.
4. Add 25ml of LB to the 25ml overnight pre-culture and continue to grow at 28°C for a further 90 minutes at 150 rpm.
5. Transfer 50ml pre-culture to centrifuge tubes and spin for 12 minutes at 2,000 rpm at room temperature in a Clements bench centrifuge.
6. Remove supernatant and gently resuspend the pellet in 20 mL of WM. Measure OD₆₀₀. Add further WM to bacterial suspension to provide a final OD₆₀₀ of 0.45. This preparation is for use in step III)1.

10 II. Preparation of Leaf Discs

A) Preparation of Leaf Discs using Tobacco Shoot Cultures

1. In a laminar flow, harvest 4-6 leaves from tobacco plants grown on MS media. Place the leaves in 1.5 x 9 cm petri dishes containing WM. Using a scalpel, remove the mid-rib and cut the leaf tissue into squares ~1cm². The tissue or leaf discs are now ready for transformation with *Agrobacterium tumefaciens* (AGL1). The discs should be transformed within an hour.

B) Preparation of Leaf Discs using Non-sterile Tobacco Tissue

1. Harvest 4 young leaves (~8cm long) from a glasshouse grown tobacco plant.
2. Place leaves into a sterile beaker containing 70% ethanol, cover with aluminium foil and swirl gently on an orbital shaker (Bio-Line Orbital Shaker, Edwards Instrument Company) for 1 minute.
3. Remove 70% ethanol and replace with 1% Ca(OCl)₂. Swirl tissue for 8 minutes. Wash leaves in sterile water at least 3 times.

4. In a laminar flow, remove the mid-rib and cut the remaining leaf tissue into squares $\sim 1\text{cm}^2$. Place the prepared discs in a 1.5 x 9 cm petri dish containing WM. The discs are now ready for transformation with *Agrobacterium tumefaciens* (AGL1).

III. Incubation and Co-cultivation of Leaf Discs with *Agrobacterium*

5 *tumefaciens*

1. Replace WM (from last step in leaf disc preparation) with *Agrobacterium* culture and incubate for 1-2 minutes.
2. Remove bacterial suspension and rinse explants briefly with WM. Blot explants on sterile napkins before plating onto PC media. Place in the growth room
10 (16h/d light ($20\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, Osram L36 W/21 Lumilux white tubes) conditions 25°C) for 3 day co-cultivation.

IV. Pre-regeneration of Leaf Discs Following *Agrobacterium*-mediated Transformation

1. Transfer explants (5/plate) to SEL media and return to growth room for a further 7
15 days.

V. Regeneration

1. Transfer explants (5/plate) to RM and return to growth room. Shoot formation should occur within 3-6 weeks. Transfer explants to fresh RM after 4 weeks.
2. If calli becomes too large, and particularly if not all shoots are in contact with the
20 media, divide calli using a scalpel. Expose as many of the shoots to selection as possible.

VI. Shoot Elongation and Root Development

1. After eight weeks on selection, or when the untransformed control explants on selection are dead, transfer green shoots (5/plate) to SEM media (include IBA (1 mg/L)) in 9 x 2 cm petri dishes. Roots should appear in 4-5 weeks.
25

VII. *Invitro* Plantlet Development

1. When roots appear, transfer rooted plantlets to SEM media in tissue culture vessels.

References

- 5 Stewart, C.N. Jr., Adang, M.J., All, J.N., Rayner, P.L., Ramachandran, S. and Parrott, W.A. (1996) Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis cryI*Ac gene. *Plant Physiol* 112: 115-120.

APPENDICES***Appendix 1: Stock Solutions and Media***

MS Media	1 Litre
MS powder	4.74 g
3% sucrose	30 g
dH ₂ O	Up to 1 L
Adjust pH to 5.8-6.0 with 1M NaOH	
Autoclave	

MS Macro (10 x)

	1 Litre	2 Litre
NH ₄ NO ₃	16.5 g	33.0 g
KNO ₃	19.0 g	38.0 g
CaCl ₂ x 2 H ₂ O	4.4 g	8.8 g
MgSO ₄ x 7 H ₂ O	3.7 g	7.4 g
KH ₂ PO ₄	1.7 g	3.4 g

MS Micro (100 x)

	1 Litre	2 Litre
KI	0.083 g	0.166 g
H ₃ BO ₃	0.62 g	1.24 g
MnSO ₄ x H ₂ O	1.69 g	3.38 g
ZnSO ₄ x 7 H ₂ O	0.86 g	1.72 g
Na ₂ MoO ₄ x 2 H ₂ O	0.025 g	0.05 g
CuSO ₄ x5H ₂ O	0.0025 g	0.005 g

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$\text{CoCl}_2 \times 6 \text{ H}_2\text{O}$	0.0025 g	0.005 g
$\text{Na}_2\text{-EDTA}$	3.73 g	7.46 g
$\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$	2.78 g	5.56 g

Dissolve $\text{Na}_2\text{-EDTA}$ and $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$ in 400 ml ddH₂O, respectively, mix and heat (do not boil). Let cool down and add the other Micro salts in the remaining volume.

MS Vitamins (100 x)

	1 Litre	2 Litre
Inositol	10g	20 g
Nicotinic acid	0.05 g	0.1 g
Pyridoxine HCl	0.05 g	0.1 g
Thiamine	0.01 g	0.02 g
Glycine	0.2 g	0.4 g

5 B5 Vitamins (100 x)

	1 Litre	2 Litre
Inositol	10g	20 g
Nicotinic acid	0.1 g	0.2 g
Pyridoxine HCl	0.1 g	0.2 g
Thiamine	1.0 g	2.0 g

RM Media: regeneration media

	stock	/1 litre
MS Macro	10x	100 ml
MS Micro	100 x	10 ml
B5 Vitamins	100 x	10 ml
MES		500 mg

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Sucrose 30 g

Adjust pH 5.80 with KOH (1M)

Agar 8.0 g

Autoclave

Once cool add: **stock** **/1 litre**

BAP 2 mg/ml 2 ml

Zeatin 1 mg/ml 2 ml

AgNO₃ 5 mg/ml 1 ml

Timentin 250 mg/ml 1 ml

Hygromycin 25 mg/ml 0.4 ml

To prepare BAP and Zeatin weigh powder into a small vessel and start dissolving with 0.5 -1 ml of 1 M KOH. Transfer the solution to ddH₂O and fill up to the final volume.

SEL media:selection media

	stock	/litre
MS Macro	10 x	100 ml
MS Micro	100 x	10 ml
B5 Vitamins	100 x	10 ml
MES		500 mg
Sucrose		30 g

Adjust pH 5.80 with KOH (1M)

Agar 8.0 g

Autoclave

Once cool add: **stock** **/litre**

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2,4-D	1 mg/ml	1 ml
Timentin	250 mg/ml	1 ml
Hygromycin	25 mg/ml -	0.4 ml

To prepare 2,4-D weigh the powder into a small vessel and start dissolving with 0.5 - 1 ml of 1 M KOH. Transfer the solution to ddH₂O and fill up to the final volume.

SEM Media

	stock	/litre
MS Macro	10 x	50ml
MS Micro	100 x	5 ml
B5 Vitamins	100 x	5 ml
MES		500 mg
Sucrose		10g

Adjust pH 5.8 with KOH(1 M)

Agar	8.0 g
Activated Charcoal	0.5 g

Autoclave

Once cool add:	stock	/litre
Timentin	250mg/ml	1 ml

5 WM medium: wash media

	stock	/litre
MS Macro	10 x	100ml

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MS Micro	100X	10ml
B5 Vitamins	100 x	10 ml
MES		500 mg
Sucrose		30 g

Adjust pH 5.8 with KOH (1M)

Autoclave

Example 10

STABLE TRANSFORMATION OF ARABIDOPSIS USING AGROBACTERIUM
5 TUMEFACIENS CARRYING BINARY VECTORS ENGINEERED WITH THE SAME
EXPRESSION CASSETTES VIA THE 'FLORAL DIP' METHOD.

Preparation of Electrocompetent *Agrobacterium tumefaciens* Cells

Experimental Procedure

1. Streak out *Agrobacterium tumefaciens* (AGL1 strain) from a frozen -80°C glycerol
10 stock onto MGL agar containing 20 mg/L rifampicin and 100 mg/L ampicillin, and
incubate at 27°C for two days.
2. Measure 5 ml MGL into a 50 ml Falcon tube and add rifampicin to a final
concentration of 20 mg/L and ampicillin 100 mg/L. Inoculate with a single colony of
Agrobacterium tumefaciens AGL1.
- 15 3. Incubate at 27°C for 24 h on an orbital shaker at 150 rpm in a tilted rack (ca. 30
degrees).
4. In late afternoon inoculate 100 ml MGL containing 20 mg/L rifampicin and 100 mg/L
ampicillin (in a 500 ml flask) with the 500 μl of an overnight culture.
5. Incubate at 27°C overnight on an orbital shaker at 150 rpm until an OD_{600} reading
20 between 0.4 - 0.6 (max. 0.6) is obtained. See comments below if overgrown.

6. Transfer cells to an autoclaved JA10 centrifuge tube and chill on ice for 10 min.
7. Centrifuge for 10 min, 9000 rpm, at 4°C using the JA10 rotor.
8. Carefully discard supernatant (pellet is not very stable) by pouring into the 500 ml flask used for culture.
- 5 9. Add 20 ml ice cold 10% glycerol to the pellet in the JA10 centrifuge tube and resuspend pellet by vortexing.
10. Pour the suspension into a JA20 centrifuge tube and spin for 10 min, 10000 rpm, at 4°C using the JA20 rotor.
11. Discard supernatant by pouring into the 500 ml flask.
- 10 12. Add 15 ml ice cold 10% glycerol to the pellet and resuspend by pipetting. Centrifuge for 10 min, 10000 rpm, at 4°C in the JA20 rotor.
13. Repeat steps 11 and 12 twice.
14. Resuspend the pellet in 1 ml ice cold 10% glycerol (pipette or vortex) and transfer to a sterile microfuge tube.
- 15 15. Spin for 3 min, 13000 rpm, at 4°C in microfuge.
16. Finally resuspend pellet in 1 ml 10% ice cold glycerol.
17. Aliquot 50 µl batches into labelled 1.7 ml microtubes, snap freeze in liquid nitrogen.
18. Remove tubes from liquid nitrogen and store competent cells at –80°C.

Comments

- 20 Wear latex gloves while handling *A. tumefaciens* bacterial cultures. Collect all bacterial waste in 500 ml flask and autoclave. If the *A. tumefaciens* 100 ml culture overgrows,

dilute 1/3 – 1/4 with fresh MGL medium containing 20 mg/L rifampicin, and incubate for further 1 – 2 h.

Transformation of *Agrobacterium tumefaciens* via Electroporation

Experimental Procedure

- 5 1. Pre-chill Gene pulser cuvette holder on ice.
2. Remove aliquots of competent *Agrobacterium* (AGL1) cells from –80°C and thaw on ice.
3. Turn on main switch at the back of the Gene pulser. Adjust the voltage to 2.5 kV (use the 'raise' button until '2.5' registers on the display), capacitance 25 µFD and
10 resistance 600 Ω.
4. Add 0.1 µg of DNA in a volume not smaller than 50 µl of thawed cells.
5. Mix by pipetting and transfer the cell/DNA mix to a pre-chilled 0.2 cm gap Gene Pulser cuvette.
6. Carefully tap or shake the cells to the bottom of the cuvette so that the cells touch
15 both electrodes.
7. Dry the outside of the cuvette with a tissue and place it into the cuvette holder. A notch on the cuvette ensures correct orientation. Slide the cuvette holder into the chamber until the cuvette is seated between the contacts at the base of the chamber.
- 20 8. Pulse the cells by depressing both red buttons until a beep sounds. The machine will display CHG whilst charging and will beep as it discharges. Place cells back on ice for 1 min to assist recovery.
9. Add 1 ml LB medium to the cells in the cuvette with a glass transfer pipette.

10. Mix the suspension up and down then transfer to a sterile 15 ml tube.
11. Incubate at 27°C on an orbital shaker at 150 rpm for 1 to 2 hours using a tilted rack (ca. 30 degree).
12. Add 9 ml of LB to the cell suspension mix thoroughly and plate out 100 µl of this culture onto an LB plate containing 20 mg/L rifampicin and the appropriate antibiotic (e.g. 100 mg/L spectinomycin for pPZP series of vectors). Transfer 100 µl of this suspension into a 1.7 ml microtube containing 900 µl of LB broth, mix thoroughly and plate out 100 µl onto another LB plate containing the appropriate antibiotics. Remove 100 µl from the above suspension and place into a fresh 1.7 ml microtube and add 900 µl of LB broth. Mix thoroughly and plate out 100 µl onto another LB plate containing the appropriate antibiotics.
13. Seal plates with Parafilm and incubate at 27°C for 2- 3 days until single large colonies become visible.

Storage of Transformed *Agrobacterium*

15 Experimental Procedure

1. In a 50 ml sterile tube inoculate a single colony into 5 ml LB broth containing 20 mg/L rifampicin and appropriate selection antibiotic (i.e. 100mg/L spectinomycin for pZP series and 50mg/L kanamycin for pBin series). This is best done early morning so that you can closely monitor the degree of growth the following day.
2. Incubate tubes in the dark at 27 °C for 24 - 36 hours shaking at 250 rpm. Regularly observe culture growth after first 24 hours of incubation. Remove from incubation once cells are actively growing (highly visible). Rapid growth will occur soon after the first signs of turbidity. Growing time depends on individual strains and transformants.
3. Each culture should be checked to verify that AGL1 contains the desired binary vector. This is done using the protocol set out in section 5.2.

4. Aliquot 500 µl of culture into a cuvette. Measure OD₆₀₀ reading, blanking with 500 µl of LB broth, containing the appropriate antibiotics, between each reading.
5. Allow cultures to grow until the OD₆₀₀ reading ranges between 0.8 to 1.0.
6. In a sterile 15 ml tube add 5.0 ml of culture and 5.0 ml of conservation stock. Mix thoroughly before proceeding to step 7.
7. Aliquot 500 µl into fully labelled sterile cyrotubes. Invert all tubes before snap freezing in liquid nitrogen. Store at -80°C until required. Discard any stock if shown to be PCR negative.

PCR Analysis of *Agrobacterium*

1. Add 1 µl of *Agrobacterium* culture to 9 µl of sterile MQ H₂O in a sterile PCR tube.
2. Incubate cells at 98°C for 5 mins. Transfer tubes to ice.
3. Add 10 µl of the prepared 2 x PCR master mix. 10 µl of 2 x PCR master mix contains the following:

10 x Dynazyme Buffer	2 µl
10 mM dNTPs	1.0 µl
10 µM forward primer (10µM)	1.0 µl
10 µM reverse primer (10µM)	1.0 µl
Dynazyme II polymerase	1 µl
MQ water	3 µl
4. Include a positive control (50 ng of the original plasmid DNA) and a negative control (no template DNA). Carry out a total of 35 cycles using standard PCR conditions

1. 95°C for 3 mins
2. 94°C for 30 secs
3. 55°C for 30 secs
- 4.. 72 °C for 1 mins
- 5 5. 72 °C 10 mins

Repeat steps 2 to 4 a total of 35 times.

5. Analyse the PCR product on a 1% agarose gel.

Comments

- Wear gloves while handling *Agrobacterium*. Collect and autoclave all bacterial and DNA waste. Gene pulser cuvettes are reusable: Soak lids in 70% EtOH and autoclave cuvettes in a closed container with water to remove *Agrobacterium*. Cuvettes can then be stored in 70% EtOH and be reused after drying.

In planta Transformation of *Arabidopsis thaliana*

Experimental procedure

15 Preparation of plant material

1. Fill seedling punnets with seed raising mixture to form a mound. Cover with two layers of anti-bird netting and secure with rubber bands at each end. Saturate the soil by sitting punnets in a tray of water. Sow sufficient seed to obtain ~40 plants per punnet.
- 20 2. Vernalise the seed by placing the punnets at 4°C for 2-3 days. Transfer punnets to a growth chamber at 22°C under fluorescent light (constant illumination, 55 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and feed with Miracle-Gro or Aquasol once per week.

3. Remove primary bolts when they appear and allow secondary bolts to grow until around 2-10 cm tall (this should take around 4–6 days, the plants should have numerous unopened floral buds and few siliques). Using forceps carefully remove any siliques or open flowers. Water plants well the day before infiltration so that the stomata will be open. Prior to infiltration saturate the soil with water to minimise absorption of bacterial solution into the soil.
4. Enter details into LWS to generate barcodes. Label punnets with LWS barcode details.

Preparation of *Agrobacterium tumefaciens*

1. In the morning inoculate 200 ml LB media containing the appropriate selection antibiotic (ie 100 mg/ml of spectinomycin for pPZP vector) with a single 500 µl starter culture of *Agrobacterium* conservation stock (section 5.1). Incubate for 24 hours at 27°C in an orbital shaker at 250 rpm. A 200 ml culture is sufficient to infiltrate about 2 punnets of plants.
2. Centrifuge overnight cultures in 500 ml centrifuge bottles at 5500g at room temperature for 15 mins to pellet cells. Discard the supernatant removing as much liquid as possible. Resuspend the pellet in infiltration media (see appendix 1) to an OD₆₀₀ reading of approximately 0.7 to 0.9

Agroinfiltration

1. Place half of the *Agrobacterium* solution into a 250 ml vessel.
2. Invert the punnet immersing the entire plant including rosette leaves in the bacterial solution and shake gently to dislodge air bubbles. Co-cultivate the plants for 2 mins.
3. Remove the punnet and briefly drain, however, the thin layer of film surrounding the plants should be retained. Cover the plants with plastic film to maintain humidity and return to the growth room away from direct light. Autoclave waste solution and dispose of in a chemical waste drum for correct disposal.

4. Repeat steps one to three for all punnets of *A. thaliana* to be transformed.
5. Enter details into LWS to generate barcodes. Label individual punnets with LWS barcode details.
6. The next day, uncover the pots and place back into direct light. Water the plants
5 until plants have fully developed siliques.

Seed collection

1. Once plants have dried out, remove the silique bearing stems and place them into a paper bag and leave to dry for one week at 37°C. Label bags with LWS barcode. Crush the dried siliques in the paper bag. This will shatter the siliques and release
10 the seed.
2. Place a 200 micron sieve onto a fresh piece of A4 paper and tip the seed and crushed siliques into it. Tap the sieve gently allowing the seed to fall onto the paper underneath. Discard the plant material that remains in the sieve. Repeat this process until the majority of the plant material has been removed (note plant material can be
15 a source of contamination in subsequent steps). Place seeds into a 1.7 ml microfuge tube and label with LWS barcode details. Place the tube into a small manila envelope and label with LWS barcode. Note that this barcode will relate back to the original transformation event.
3. Store seeds at -20°C for 24 hours before transferring the seeds to 4°C for storage.

20 Selection of Positive T₁ Transgenic Arabidopsis thaliana plants

Surface sterilisation of seeds

1. Working in a laminar flow hood, place seed to be sterilised (40 mg = ~2000 seeds per 150x15 mm plate) into a 2.0 ml microtube.
2. Add 1000 µl 70% ethanol and leave for two mins.

3. Remove the ethanol and add 1000 µl of seed sterilisation solution (4% chlorine: water: 5% SDS at a ratio of 8:15:1 respectively) and mix thoroughly by vortexing.
4. Place the tubes on the Ratek 'ferris wheel' to ensure mixing of the seeds and solution, leave for ten mins.
5. In the laminar flow, remove the sterilisation solution and replace with sterile water. Vortex the tube(s) and spin for 30 seconds in a bench top centrifuge to sediment the seeds. Remove the water and replace with another 1 ml of sterile water. The seed washing steps should be repeated until no visible bubbles are apparent (at least 4 times). After the final wash, leave approximately 200 µl of water covering the seeds.

10 Selection of T₁ transformants

1. Prepare 150 x 15 mm plates with selection germination medium (SGM) containing the appropriate selection antibiotic (eg. Hygromycin at 8mg/L for PZP200 series). Include Timentin (250 mg/L) to inhibit growth of *Agrobacterium*. Approximately 125 ml of SGM is required for each plate.
2. Working in a laminar flow hood, run a sterile scalpel across the surface of the SGM agar plate in a parallel fashion (see Figure 4). This will help to spread the seeds.
3. Using a sterile 1ml tips, with its end removed, pipette the sterilised seeds onto a plate. Distribute the seeds with a sterile disposable spreader.
4. Cold treat the seeds at 4°C for two days, and then grow under continuous fluorescent light (55 µmol m⁻²s⁻¹) at 22°C.
5. When putative transformants are at the 6-8 leaf stage they can be transferred to soil. With a pair of forceps carefully remove plants from the tissue culture media ensuring the roots remain intact. Transplant into moist *in-vitro* mix soil using the ARASYSTEM (see Figure 5 in appendix 2) Cover with a plastic tube. Create new LWS barcode and label tubes. Cover top of tubes with plastic wrap for a few days to assist recovery.

Verification of integration of transgene: alkali-treated leaf tissue as a source of genomic DNA

Three days after putative transformants have been transferred to soil, individual plants can be molecularly characterised for presence of the transgene using the following
5 protocol.

1. Prepare a 1x PCR buffer mix for every alkali-treated leaf tissue to be tested (see below for details).
2. In a 1.7 ml microtube, add 200 μ l of 0.25 M NaOH to a small young leaf (removed from a T₁ plant).
- 10 3. Immerse the tube in boiling water for 2 min. Note, to prevent lid popping during boiling, secure the lid with a microtube lid lock or pierce the lid with a fine needle.
4. After boiling, remove the tube from the water and add 200 μ l of 0.25 M HCl and 100 μ l of 0.25% (v/v) igepal [0.5 M Tris HCl pH 8.0]. Immerse the tube in boiling water for a further 4 mins.
- 15 5. Remove a small portion of the alkali-treated leaf ($\sim 2 \text{ mm}^2$) and place in the pre-prepared PCR mix:

	10 x PCR reaction buffer (including Mg(SO ₄).7H ₂ O)	5.0 ml
	10 mM dNTP's	1.0 μ l
	10 μ M forward primer	1.0 μ l
20	10 μ M reverse primer	1.0 μ l
	PWO DNA polymerase	1.0 μ l
	MQ H₂O	41.5 μl

6. Carry out 35 cycles using standard PCR conditions;

1. 95°C for 3 mins

2. 94°C for 30 secs

3. 55°C for 30 secs

5 4. 72 °C for 1 mins

5. 72 °C 10 mins

Repeat steps 2 to 4 a total of 35 times.

7. Analyse the PCR product on a 1% agarose gel.

10 Note if an insert is not amplified using alkali-treated leaf tissue the first time, re-boil the tissue for a further 2 mins and repeat the PCR amplification of the transgene. If this second PCR fails, extract a small quantity of genomic DNA from leaf tissue using Qiagen plant genomic DNA extraction kit. Update LWS.

Seed collection

15 1. Once plants have dried out, remove the silique bearing stems and place them a paper bag and leave to dry for one week at room temperature. Label bags with LWS barcode. Crush the dried siliques in the paper bag. This will shatter the siliques and release the seed.

20 2. Place a 200 micron sieve onto a fresh piece of A4 paper and tip the seed and crushed siliques into it. Tap the sieve gently allowing the seed to fall onto the paper underneath. Discard the plant material that remains in the sieve. Repeat this process until the majority of the plant material has been removed (note plant material can be a source of contamination in subsequent steps). Place seeds into a 1.7ml microfuge tube and label with LWS barcode details. Place the tube into a small manila

envelope and label with LWS barcode. Note that this barcode will relate back to the original transformation event.

3. Store the T₂ seed at -20°C for 24 hours (helps reduce chances of fungal contamination during selection for positive transgenic T₂ plants) before storing the seeds at 4°C.

Comments

All containers that come into contact with *Agrobacterium*, including the ARASYSTEM trays, holders, etc should be thoroughly cleaned using commercial bleach and 70% ethanol.

- 10 Depending on the experiment, T₁ plants can be used for phenotypic characterisation, i.e. reporter gene analysis, and it may not be necessary to continue these lines beyond the T₁ stage.

Generation of Homozygous T₃ seeds

Introduction

- 15 The protocols detailed below describe the methods employed to select for homozygous plants carrying a single copy of a transgene. Integration of the transgene into *Arabidopsis thaliana* using the infiltration method occurs in the gynoecium prior to fertilisation of the ovary. Therefore any seeds produced by infiltration that carry the transgene are considered to be T₁. T₁ seeds germinate to produce T₁ plants, which in turn produce T₂ seeds. The aim is to obtain at least five independent transgenic plants per construct that have a single insert and are expressing the transgene. Each LWS barcode generated for T₁ seeds represents a distinct transformation event. As each T₁ plant is harvested for T₂ seeds they are given a new LWS barcode number. This LWS barcode will relate back to the original transformation event.

Selection of T₂ transformants to generate T₃ seeds

1. Working in a laminar flow hood surface sterilise approximately 100 T₂ seeds (see section 7.1)
2. Plate out approximately 25 seeds per plate (4 in total) onto selection SGM media containing 250mg/L timetin and 8mg/L hygromycin (selection agent for pPZP200-35s-hph-35st).
3. Cold treat the seeds at 4°C for two days then transfer to growth room with constant illumination (55 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) at 22°C.
4. After two weeks, segregation analysis of plants resistant or sensitive to hygromycin is performed.
5. When the putative transformants are at the 6 to 8-leaf stage, transfer at least 10 individual plants into soil using the Arasystem. Generate new LWS barcode (relates back to original transformation) and label each plant individually with the barcode. Cover tubes with plastic film for a few days to aid recovery.
6. To confirm that each individual plant has the transgene integrated, use the alkali treated leaf tissue method (section 7.3). Update LWS.
7. Once plants have dried out, remove the silique bearing stems and place them into a paper bag and leave to dry for one week at 37°C. Label bags with LWS barcode. Crush the dried siliques in the paper bag. This will shatter the siliques and release the seeds.
8. Place a 200 micron sieve onto a fresh piece of A4 paper and tip the seed and crushed siliques into it. Tap the sieve gently allowing the seed to fall onto the paper underneath. Discard the plant material that remains in the sieve. Repeat this process until the majority of the plant material has been removed (note plant material can be a source of contamination in subsequent steps). Place seeds into a 1.7 ml microfuge tube and label with LWS barcode details. Place the tube into a small manila

envelope and label with LWS barcode. Note that this barcode will relate back to the original transformation event.

9. Store the seed at -20°C for 24 hours (helps reduce chances of fungal contamination during selection for positive transgenic T₃ plants) before transferring the seeds to
5 4°C for storage.

Segregation Analysis

1. Score the total number of T₂ plants from each line that is either resistant or sensitive to hygromycin.
2. If the T-DNA is inserted at one locus, the ratio of resistant to sensitive plants should
10 be 3:1. If the T-DNA locus is inserted at two loci, the ratio of resistant to sensitive plants should be 15:1. If the T-DNA is inserted at more than two loci, the ratio of resistant to sensitive plants should be > 15:1.
3. Use the Chi-square (χ^2) statistical test to determine how well the segregation data fits a particular hypothesis.
- 15 4. Continue growing transgenic lines that Chi-square analysis indicated contained a single copy of the transgene.
5. Update LWS

Verification of Integration of Transgene: Alkali-treated Leaf Tissue as a Source of Genomic DNA

- 20 1. Harvest one small leaf for each T₂ plant.
2. Follow alkali-treated leaf protocol (section 7.3) to determine presence of a transgene.
 3. Update LWS.

Selection for homozygous T₃ lines

1. Continue growing T₂ transgenic lines that indicate that they contain a single insertion of the transgene.
2. Collect T₂ seeds (section 8.2), and update LWS and generate new barcodes.
- 5 3. Germinate ~ 40 T₃ seeds on SGM
4. After 2 weeks score the total number of T₃ plants from each line that is either resistant or sensitive to hygromycin.
5. Homozygous T₃ lines will be indicated by the absence of sensitive plants.
6. When the putative transformants are at the 6 to 8-leaf stage, transfer at least 20
10 individual plants into soil using the Arasystem. Generate new LWS barcode and label each plant individually with the barcode. Cover tubes with plastic film for a few days to aid recovery.
7. To further validate that a line is homozygous for a single insertion use the alkali treated leaf tissue method (section 7.3) to confirm that all plants contain a transgene.
15 Update LWS.
8. Harvest sufficient material from putative homozygous lines to perform a Southern Hybridisation to confirm transgene integrated number. Update LWS.
9. Harvest seeds from T₃ homozygous lines following the protocol set out in section 8.2.

References

- Caimi PG, McCole LM, Klein TM, Hershey HP. 1997. Cytosolic expression of the *Bacillus amyloliquefaciens* SacB protein inhibits tissue development in transgenic tobacco and potato. *New Phytologist* 136, 19-28
- 5
- Caimi PG, McCole LM, Klein TM, Kerr PS. 1996. Fructan accumulation and sucrose metabolism in transgenic maize endosperm expressing a *Bacillus amyloliquefaciens* *sacB* gene. *Plant Physiology* 110, 355-363
- Cairns AJ. Fructan biosynthesis in transgenic plants. 2003. *J Expt Biol* 54: 549-67
- 10 Clough, S. J. and Bent, A. F., 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* 16: 735-743.
- Ebskamp MJM, van der Meer IM, Spronk BA, Weisbeek PJ, Smeekeens SCM. 1994. Accumulation of fructose polymers in transgenic tobacco. *Bio/Technology* 12, 272-275
- Hattori T, Nakagawa T, Maeshima M, Nakamura K, Asahi T (1985) Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. *Plant Mol Biol* 5: 313-320
- 15
- Klimyuk, V. I., Carroll, B. J. Thomas, C. M. and Jones, J. D. (1993) *The Plant Journal* 3(3):493-494
- Sasanuma, T. (2001). Characterization of the *rbcS* multigene family in wheat: subfamily classification, determination of chromosomal location and evolutionary analysis. *Mol Genetics Genomics* 265(1): 161-171.
- 20
- Ye XD, Wu XL, Zhao H, Frehner M, Noesberger J, Potrykus I, Spangenberg G. 2001. Altered fructan accumulation in transgenic *Lolium multiflorum* plants expressing a *Bacillus subtilis* *sacB* gene. *Plant Cell Reports* 20, 205-212

Zeng, W. K., et al. (1995). PCR Amplification and Sequencing of a Wheat rbcS Gene Promoter. *Acta Bot Sinica* 37, 496-500.

CLAIMS:

1. A method of manipulating fructan biosynthesis in photosynthetic cells of a plant, said method including introducing into said plant an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial fructosyltransferase (FT) enzyme, or a functionally active fragment or variant thereof.
2. A method according to claim 1 wherein said promoter is selected from the group consisting of constitutive, tissue specific and inducible promoters.
3. A method according to claim 2 wherein said promoter is a light regulated promoter.
4. A method according to claim 1 wherein said bacterial FT enzyme includes both sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) enzymatic activities.
5. A method according to claim 4 wherein said nucleic acid encoding a bacterial FT enzyme is selected from the group consisting of *SacB*, *Lsc* and *FTF* genes.
6. A method of enhancing productivity of a biochemical pathway in a plant, said method including introducing into said plant an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a FT enzyme, or a functionally active fragment or variant thereof.
7. A method of enhancing biomass in a plant, said method including introducing into said plant an effective amount of a genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to nucleic acids encoding one or more fructan biosynthetic enzymes, or functionally active fragments or variants thereof.
8. A method of selecting for transformed plants, said method including introducing into said plants an effective amount of a genetic construct including a promoter, or a

functionally active fragment or variant thereof, operatively linked to nucleic acids encoding one or more fructan biosynthetic enzymes, or functionally active fragments or variants thereof, and selecting plants with enhanced biomass.

9. A genetic construct capable of manipulating fructan biosynthesis in photosynthetic cells of a plant, said genetic construct including a promoter, or functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof.
10. A genetic construct according to claim 9 wherein said promoter is selected from the group consisting of constitutive, tissue specific and inducible promoters.
11. A genetic construct according to claim 10 wherein said promoter is a light regulated promoter.
12. A genetic construct according to claim 9 wherein said bacterial FT enzyme includes both sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) enzymatic activities.
13. A genetic construct according to claim 12 wherein said nucleic acid encoding a bacterial FT enzyme is selected from the group consisting of *SacB*, *Lsc* and *FTF* genes.
14. A method of enhancing biomass in a plant, said method including introducing into said plant effective amounts of
- (a) a genetic construct capable of manipulating fructan biosynthesis in photosynthetic cells of the plant, said genetic construct including a promoter, or a functionally active fragment or variant thereof, operatively linked to a nucleic acid encoding a bacterial FT enzyme, or a functionally active fragment or variant thereof; and
- (b) a genetic construct capable of manipulating senescence in the plant.

15. A method according to claim 14 wherein the promoter is a light regulated promoter.
16. A method according to claim 14 wherein the genetic construct capable of manipulating senescence includes a *myb* gene promoter or modified *myb* gene promoter, or a functionally active fragment or variant thereof, operatively linked to a
5 gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.
17. A transgenic plant cell, plant, plant seed or other plant part with modified fructan biosynthetic characteristics or enhanced biomass relative to an untransformed control plant; said plant cell, plant, plant seed or other plant part including a genetic
10 construct according to claim 9.
18. A transgenic plant cell, plant, plant seed or other plant part according to claim 17 having increased biomass of at least approximately 50% relative to an untransformed control plant.
19. A transgenic plant cell, plant, plant seed or other plant part according to claim 17
15 having increased soluble carbohydrate of least approximately 50% relative to an untransformed control plant.

FIGURE 1

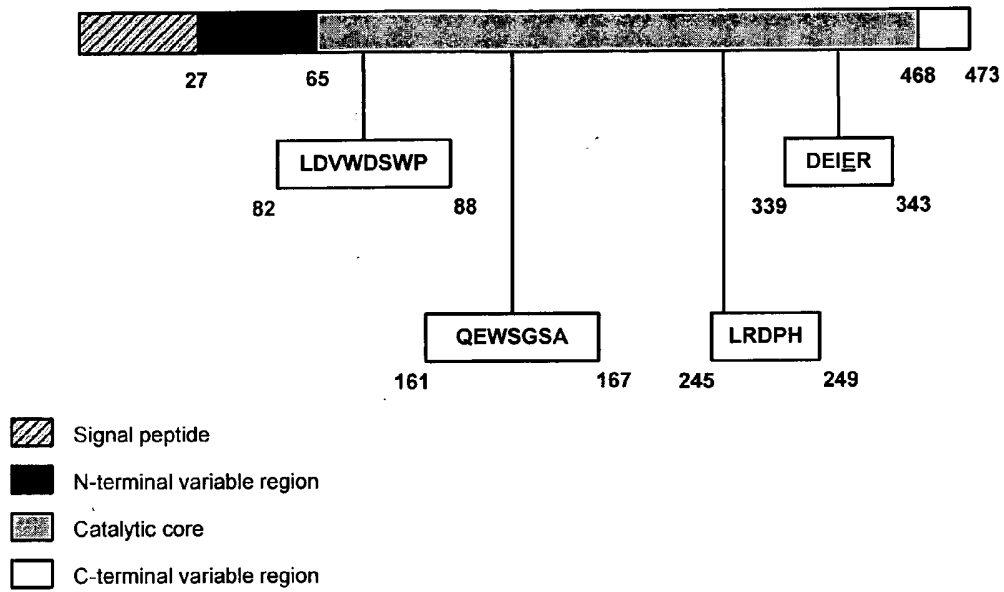


FIGURE 2

**ATGAACATCAAAAAGTTTGCAAAACAAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGGGCGCAACTCA
AGCGTTTGCGAAAGAAACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCTCATATTACACGCCATGATATGC
TGCAAATCCCTGAACAGCAAAAAAATGAAAAATATCAAGTTCCTGAATTTCGATTCGTCCACAATTAAAAATATCTCT
TCTGCAAAAGGCCTGGACGTTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGCTA
CCACATCGTCTTTGCATTAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTACATGTTCTATCAAAAAGTCG
GCGAACTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCT
ATCCTAAAGACCAAAACACAAGAATGGTCAGTTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACAC
TGATTTCTCCGTAAACATTACGGCAAACAAACACTGCAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTT
TGAACATCAACGGTGTAGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTC
ATCGATGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAA
ATACTTAGTATTTGAAGCAAACACTGGAACGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACT
ATGGCAAAAGCACATCATTCTTCCGTCAAGAAAGTCAAAAACCTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTA
GCAAACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATC
TAACACAGTAACAGATGAAATTTGAACGCGCGAACGTCTTTAAATGAACGGCAAATGGTACCTGTTCACTGACTCCC
GCGGATCAAAAATGACGATTGACGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACT
GGCCCATACAAGCCGCTGAACAAAACCTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTA
CTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACG
CAGACAAACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAAACATCTGTTGTCAAAGACAGC
ATCCTTGAACAAGGACAATTAACAGTTAACAAATAA**

FIGURE 3

MNIKKFAKQATVLTFTTALLAGGATQAFKETNQKPYKETYGISHITRHDMLQIPEQQKNEKYQVPEFDSSTIKNIS
SAKGLDVWDSWPLQNADGTVANYHGYHIVFALAGDPKNADDTSIYMFYQKVGETSIDSWKNAGRVFKDSDKFDANDS
ILKDQTQEWSGSATFTSDGKIRLFYTD FSGKHYGKQTLTTAQVNV SASDSSLNINGVEDYKSIFDGDGKTYQNVQQF
IDEGNYSSGDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSTSFFRQESQKLLQSDKKRTAEL
ANGALGMIELNDDYTLKKVMKPLIASNTVTDEIERANVFKMNGKWYLF TDSRGSKMTIDGITSNDIYMLGYVSNSLT
GPYKPLNKTGLVLKMDLDPNDVTFTYSHFAVPQAKGNNVVITSYMTNRGFYADKQSTFAPSFLNLIK GKKTSVVKDS
ILEQGQLTVNK

FIGURE 4

ATGTTGGAAAATAAAAATCATAAAAAGATATCTTTAAGCGGAAAATCTTTGTTAATGGGAACCTTGTC AACAGCAGC
AATTGTATTAAGTGCATCAACTGCAAAATGCTGCGACTATTAATGCAGACAATGTTAATGAAAATCAAACGTAGAG
TAACTGCTAGTTCAGTAAACAATGAAAATAATAAGCAAGTAAC TGAAAAAGATAGTGCAGATAAAAGTACTAGTGAT
GTGGCTGAAGATGCTAACACCAAGAAAATCAAACGAAAATACAGAACTACAGAAAAGAATACTCAAACAGTTGTTAC
TAATGCGCCAGTAAAGTGATGTGAAAAATACAAACACAGTTACCGCTGAAACACCTGTTGATAAAGTAGTAAATAATA
GTGATCAAAGACAAC TAATGCTGCAACTACTGATACTAAAAAGATGATGTAAAACAAGTTGAAAAGAAAAGACTCA
GTAGATAAAACAAATGCTGAGGAAAATAAAGATAGTTT CAGTAAAGCCAGCTGAAAATGCTACTAAGGCTGAATTA
GGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTTGATACTAGCAAGTTAACTAATGATCAAATTAATGAATTA
ATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGGTACTCAGTTAACTTACAACGACTTTAAAAAAATTGCTAAAC
TTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCAATGCAAGTAAAATTA AAAATATGCCTGCTGCTAAAC
ACTTGATGCTCAAAGTGGAAGTAGAAGATTTGGAATTTGGGATTCATGGCCTGTTCAAGATGCAAAAACCTGGTT
ACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTATGATGGGAGTTCAAACGTCAATGATAACCACATTTAT
CTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATTGGAAGAATGCCGGTCTATTTTCGGTCTAGGTACTCC
AGTTATTCAACAATGGTCTGGATCAGCAACTTTAAATAAAGATGGCTCAATTCAACTTTACTACACTAAGGTTGATA
CTAGTGATAATAATACTAACCACCAAAAACCTCGCTAGTGCAACTGTTTACTTAAATCTTGAAAAGATCAAGATAAG
ATTTCTATTGCTCATGTTGACAACGACCATATTGCTTTGAAGGTGATGGTTACCACTACCAAACTTATGACCAATG
GAAAGAACTAACAAGGGTGCTGACAATATCGCAATGCGTGATGCACACGTGATTGATGATGATAATGGTAATCGTT
ACCTTGTTGTTTGAAGCAAGTACTGGAACCGAAAATTATCAAGGTGATGATCAAATTTATCAATGGTTAAATTACGGC
GGTACTAACAAGGATAATTTAGGTGATTTCTTCCAAATTTTATCTAACTCCGATATTAAAGATAGAGCTAAATGGTC
AAACGCTGCAATTTGGTATCATTAATTAATGATGATGTTAAGAATCCAAGTGTGCAAAGGTCTACAGCCCACTTA
TTAGTGACCAATGGTAAGTGATGAAATTGAACGCCCTGATGTTGTTAAATTAGGTAATAAGTATTACTTATTTGCT
GCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGGATGGCAACTAACAAAGCAGTTGGTGATAACGTAGCTAT
GATTGGTTATGTTTCTGATAACTTAACTCATGGTTATGTTCCATTGAATGAATCTGGCGTTGTTTTAACTGCATCTG
TACCGGCTAACTGGCGTACTGCAACTTATTCATACTATGCAGTTCCAGTAGAAGGAAGAGATGATCAACTTTTAATT
ACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGGGTATGCATGCAACTTGGGCACCAAGTTTCTTGTTACA
AATTAATCCAGATAACACTACTACTGTTTTAGCTAAAAAGACTAACCAGGGGATTGGATTTGGGATGATAGTAGTG
AAAATCCAGATATGATGGGTGTACTTGAAAAGATGCTCCAAATAGTGCTGCCCTTCTGGAGAATGGGGAAAACCA
GTTGATTGGGATTTAATTGGTGATACAACCTGAAGCCACACCAACCTGTAACCTCTATTCCAAATGTACCAACTAC
TCCTGAAACCCCAACCACACCAGATAAGCCAGAGGTACCAACTACCCCTGAAGTTCCAACCACTCCAGAACTCCAA
CTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAAC TAGTCAGTCTAACTTCCAAAGGCTGGAGATAAAAATAGCTTT
GCAGCAGTTGTTTTAGGTGCTGTAAGTTCAATATTAGGTGCTGTTGGTTTAACAGGTGTTTCAAACGTAAACGTAA
TAATTAA

FIGURE 5

MLENKNHKKISLSGKSLLMGTLSTAAIVLSASTANAATINADNVNENQTV EVTASSVNNENNKQVTEKDSADKSTSD
VAEDANTKKS NENTETTEKNTQT VVTNAPVSDVKNTNTVTAETPVDKVVNNSDQKTNAATTDTKDDVKQVEKKDS
VDKTNAEENKDS SVKPAENATKAELKGQVKDIVEESGVDTSKLTNDQINELNKINFSKEAKSGTQLTYNDFKKIAKT
LIEQDARYAIPFFNASKIKNMPAAKTLDAQSGKVEDLEIWDSWPVQDAKTGYVSNWNGYQLVIGMMGVPNVNDNHIY
LLYNKYGDNDFNHWK NAGPIFGLGTPVIQQWGSATLNKDGS IQLYYTKVDTSDNNTNHQKLASATVYLNLEKDQDK
ISIAHVDNDHIVFEGDGYHYQTYDQWKETNKGADNIAMRDAHVIDDDNGNRYLVFEASTGTENYQGDDQIYQWLNYG
GTNKDNLGDDFFQILSNSDIKDRAKWSNAAIGIIKLND DVKNPSVAKVYSPLISAPMVSDEIERPDVVKLG NKYYLFA
ATRLNRGSND DAWMATNKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAVPVEGRDDQLLI
TSYITNRGEVAGKGMHATWAPS FLLQINPDNTTTVLAKMTNQGDWIWDDSENPDMMGVLEKDAPNSAALPGEWGKP
VDWDLIGGYNLKPHQPVTPIPNVPTTPETPTTPDKPEVPTTPEVPTTPETPTPEAPKNPVKKTSQSKLPKAGDKNSF
AAVVLGAVSSILGAVGLTGVS KRKRNN

FIGURE 6

ATGAAAGCCTTCACACTCGCTCTCTTTCTTAGCTCTTTTCCTCTATCTCCTGCCCAATCCAGCCCATTCCAGGTTCAA
TCCCATCCGCCTCCCCACCACACACGAACCCGCCTCCTCTGAAACTCCAGTACTCGACATCAACGGCGACGAGGTCC
GCGCCGGCGGGAACACTACTACATGGTCTCCGCCATATGGGGAGCCGGCGGGGGAGGGCTAAGACTCGCCCACCTTGGAC
ACGATGTCCAAATGCGCCAGCGACGTATCGTATCCCCAACGACTTAGACAACGGCGACCCCATCACCATCAGCC
GGCGACGGCCGACCCGGAATCCACCGTGGTCATGGCGTCGACCTACCAGACTTTCGGTTCAATATCGCCAACAACA
AACTGTGCGTGAAGAACGTGAACTGGGGAATCCAGCAGCAGACGCGGTCCGGGCAGTATTTCTGAAAGACGGCGAG
TTTGTCTCCGACAATAGCAACCAGTTCAAGATTGAGGTGGTGGATGCCAACCTTAACTTCTACAAACTCACTTACTG
TCAGTTCGGCTCCGACAAATGCTACAACCTGCGGCAGATTCCACGACCCCATGTTGAGGACCACGCGCTTGGCTCTCT
CCAATTCTCCCTTCGTTTTTGTTCATCAAACCTACCGATGTG

FIGURE 7

MKAF~~TLALFLALFLYLLPNPAHS~~RFPNPIRLPTTHEPASSETPVLDINGDEV
RAGGNYYMVSAIWGAGGGGLRLAHLDTMSKASDVIVSPNDLDNGDPITITPATADPESTVVMAS
TYQTFRFNIANNKLCVKNVNWGIQHDSASGQYFLKDGEFVSDNSNQFKIEVVDANLNFYKLT
YCQFGSDKCYNCGRFHDPMRLRTTRLALSNSPFVFIKPTDV

FIGURE 8

ATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTTCCTCTATCTCCTGCCCAATCCAGCCCATTCCACGAACCA
AAAGCCATATAAGGAAACATACGGCATTTCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAA
ATGAAAAATATCAAGTTCCCTGAATTCGATTTCGTCCACAATTAATAATATCTCTTCTGCAAAAGGCCTGGACGTTTGG
GACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCGG
AGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAACTTCTATTGACAGCTGGA
AAAACGCTGGCCGCTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCAACACAAGAA
TGGTCAGGTTTACGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTACGG
CAACAAACACTGACAACGACCAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATT
ATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTTCATCGATGAAGGCAACTACAGCTCA
GGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACAC
TGGAACCTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAGCATACTATGGCAAAAGCACATCATTTCTCC
GTCAAGAAAGTCAAAAACCTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATT
GAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATGGA
ACGCGCGAACGTCTTTAAATGAACGGCAAATGGTACCTGTTCAGTACTCCCGCGGATCAAAAATGACGATTGACG
GCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACAAA
ACTGGCCTTGTTGTTAAATGGATCTTGATCCTAACGATGTAACTTTACTTACTCACACTTCGCTGTACCTCAAGC
GAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGC
CAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACA
GTTAACAATAA

FIGURE 9

MKAFTLALFLALFLYLLPNPAHSTNQKPYKETYGISHITRHDMLQIPEQQKNEKYQVPEFDSSTIKNISSAKGLDVW
DSWPLQNADGTVANYHGYHIVFALAGDPKNADDTSIYMFYQKVGETSIDSWKNAGRVPKDSKFDANDSILKDQTQE
WSGSATFTSDGKIRLFYTDGSGKHYGKQTLTTAQVNVASDSSLNINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSS
GDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSTSFFRQESQKLLQSDKKRTAELANGALGMI
ELNDDYTLKKVMKPLIASNTVTDEIERANVFKMNGKWYLF TDSRGSKMTIDGITSNDIYMLGYVSNLTGPYKPLNK
TGLVLKMDLDPNDVTFTYSHFAVPQAKGNNVVITSYMTNRGFYADKQSTFAPSFLLNKGGKTSVVKDSILEQGQLT
VNK

FIGURE 10

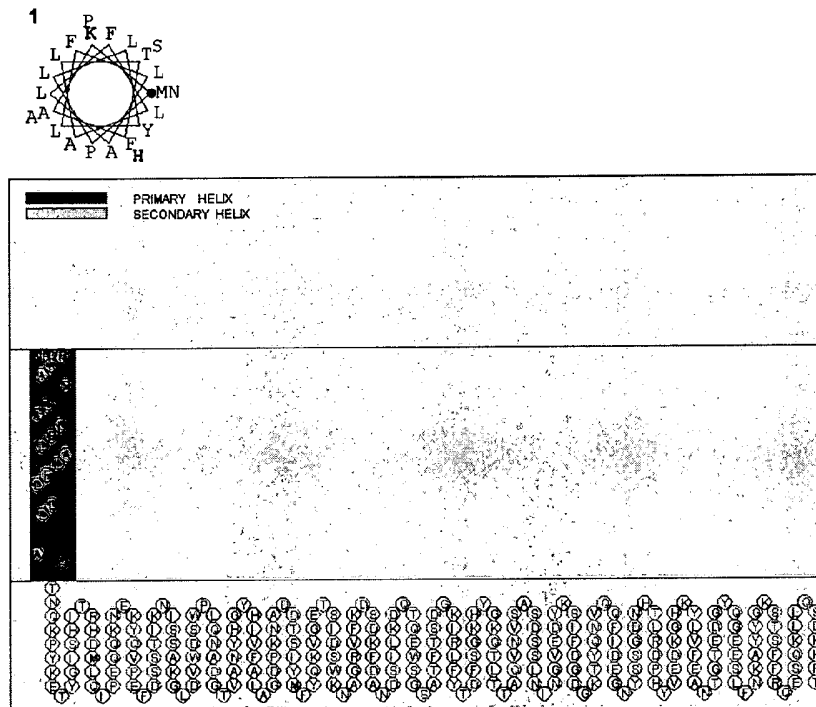


FIGURE 11

ATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTCTCTATCTCCTGCCCAATCCAGCCCATTCCACTATTAA
TGCAGACAATGTTAATGAAAATCAAACGTAGAGTAAGTACTGCTAGTTCAGTAAACAATGAAAATAATAAGCAAGTAA
CTGAAAAAGATAGTGCAGATAAAAGTACTAGTGTGCTGGAAGATGCTAACACCAAGAAAATCAAACGAAAATACA
GAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTTAC
CGCTGAAACACCTGTTGATAAAGTAGTAAATAATAGTGATCAAAAGACAACTAATGCTGCAACTACTGATACTAAAA
AAGATGATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAATAAAGATAGTTCAGTA
AAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAAGGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTGATAC
TAGCAAGTTAACTAATGATCAAATTAATGAATTAATAAAATTAATTCTCCAAAGAAGCAAAAAGTGGTACTCAGT
TAACTTACAACGACTTTAAAAAAATTGCTAAACCTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCAAT
GCAAGTAAAATTAATAATATGCCTGCTGCTAAAACACTTGATGCTCAAAGTGGAAGTAGAAGATTTGGAAATTTG
GGATTCATGGCCTGTTCAAGATGCAAAAACCTGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTTGGTATGA
TGGGAGTTCCAAACGTCAATGATAACCACATTTATCTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATTGG
AAGAATGCCGGTCTATTTTCGGTCTAGGTACTCCAGTTATTCAACAATGGTCTGGATCAGCAACTTTAAATAAAGA
TGGCTCAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCACCAAAAACCTCGCTAGTGCAA
CTGTTTACTTAAATCTTGAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTGAA
GGTGATGGTTACCACTACCAAACCTTATGACCAATGGAAAGAACTAACAAGGGTGCTGACAATATCGCAATGCCGTGA
TGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTTGAAGCAAGTACTGGAACCGAAAATTATCAAG
GTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTTCTTCCAAATTTTA
TCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAAATTAATGATGATGTAA
GAATCCAAGTGTGCAAAGGTCTACAGCCCACTTATTAGTGACCAATGGTAAGTGATGAAATTGAACGCCCTGATG
TTGTTAAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGGATG
GCAACTAACAAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATACTTAACTCATGGTTATGTTCC
ATTGAATGAATCTGGCGTTGTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCTACTATGTCAG
TTCCAGTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGGGT
ATGCATGCAACTTGGGCACCAAGTTTCTTGTTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAAATGAC
TAACCAAGGGGATTGGATTTGGGATGATAGTAGTGAATAATCCAGATATGATGGGTGTACTTGAAAAGATGCTCCAA
ATAGTGCTGCCCTTCTGGAGAATGGGGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACTTGAAGCCACAC
CAACCTGTAACCTCTATTCCAAATGTACCAACTACTCCTGAAACCCCAACCACACCAGATAAGCCAGAGGTACCAAC
TACCCCTGAAGTTCCAACCACTCCAGAACTCCAACCTCAGAAGCTCCAAAGAATCCAGTTAAGAAAACAGTCAGT
CTAAACTTCAAAGGCTGGAGATAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAAGTTCAATATTAGGTGCT
GTTGGTTTAACAGGTGTTTCAAACGTAAACGTAATAATTAA

FIGURE 12

MKAFTLALFLALFLYLLPNPAHSTINADNVNENQTVEVTASSVNNENNKQVTEKDSADKSTSDVAEDANTKKSNE
ENTTEKNTQTVVTNAPVSDVKNTNTVTAETPVDKVVNNSDQKTTNAATTDTKDDVKQVEKKDSVDKTNAEENKDSSV
KPAENATKAELKGQVKDIVEESGVDTSKLTNDQINELNKINFSKEAKSGTQLTYNDFKKIAKTLEQDARYAIPFFN
ASKIKNMPAAKTLDAQSGKVEDLEIWDSWPVQDAKTGYVSNWNGYQLVIGMMGVPNVNDNHIIYLLYNKYGDNDFNHW
KNAGPIFGLGTPVIQQWGSATLNKDGSIQLYYTKVDTSDNNTNHQKLASATVYLNLEKDQDKISIAHVDNDHIVFE
GDGYHYQTYDQWKETNKGADNIAMRDAHVIDDDNGNRYLVFEASTGTENYQGDDQIYQWLNYYGGTNKDNLGDDFFQIL
SNSDIKDRAKWSNAAIGIIKLNDVKNPSVAKVYSPLISAPMVSDEIERPDVVKLGNKYYLFAATRLNRGSNDDAWM
ATNKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAVPVEGRDDQLLITSYITNRGEVAGKG
MHATWAPSFLQINPDNTTTLAKMTNQGDWIWDDSENPDMMGVLEKDAPNSAALPGEWGKPVWDWDLIGGYNLKPH
QPVTPIPNVPTTPETPTTPDKPEVPTTPEVPTTPETPTPEAPKNPVKKTSSQSKLPKAGDKNSFAAVVLGAVSSILGA
VGLTGVSKRRNN

FIGURE 13

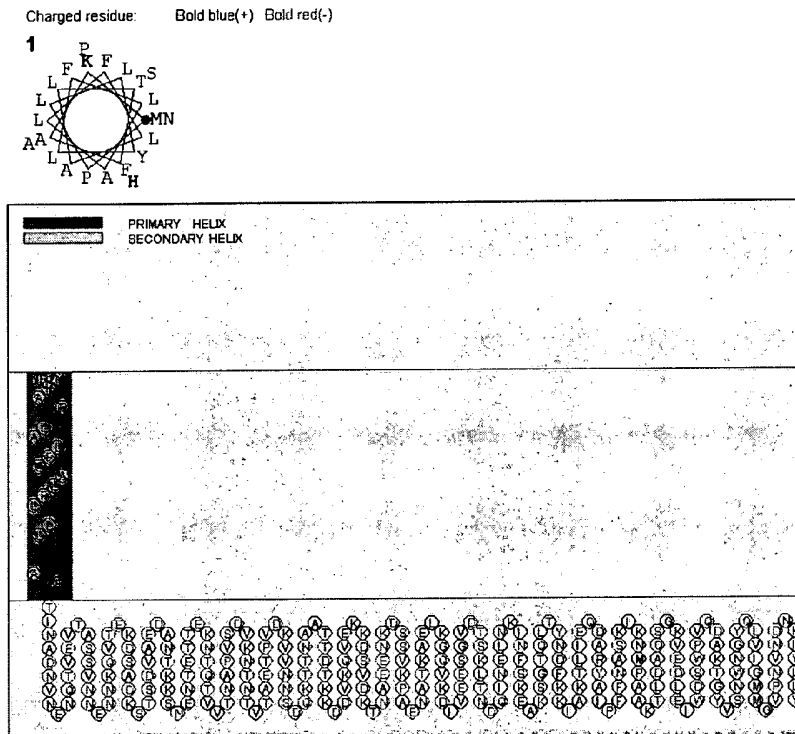


FIGURE 14

1-SST

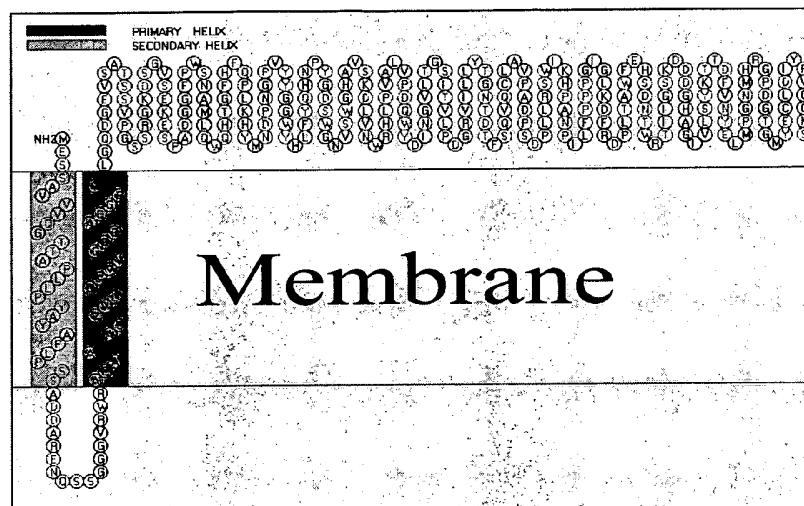


FIGURE 15

ATGCAACCAGGGAACGTCGTCCACCCCTTCCACATCCTGTATCAAATTAAGGAACGGGCGCTGAGCCTATGCCGAGA
CATATATAATGCGGCGACTCGGACATGGAGGGCCTCAGGCATAGCCAGCTAGTTATCTCATTTCTCTCCTTAGCAA
TAATACTTAGCACCATGGCCCCCGCGGTGGAATTCATGGAGTCCCCAAGCGCCGTCGTCCCCGGCACCACGGCGCCG
CTGCTTCTTATGCGTACGCGCCGCTGCCGTCGTCCGCCGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCG
CGCGTGCGCCCGCGTGCTGGCCGCATCGGCGTTGGCGGTGGTTCGTCTGGTCGGGCTCCTCGCGGGCGGCAGGGTGG
ATCGGGTCCCCGGCCGGCGGAGACGTGGCGTCGGCCACGGTGCCGGCCGTGCCGATGGAGTTCCCGAGGAGCCGGGGC
AAGGACTTCGGCGTGTGCGAGAAGTCTCCGGTGCCTACTCCACCGACGGCGGGTTCCCGTGAGCAACGCCATGCT
GCAGTGGCAGCGCACCGGGTTCCATTTCCAGCCGGAGCAGCACTACATGAACGATCCCAACGGCCCCGTGTACTACG
GCGGATGGTACCACCTCTTCTACCAGCACAAACCCCAAGGGCGACAGCTGGGGCAACATCGCGTGGGGCCACGCCGTC
TCCAAGGACATGGTCAACTGGCGCCACCTCCCTCTCGCCATGGTTCCCGACCAGTGGTACGACAGCAACGGCGTCTCT
CACCGGCTCCATCACCGTGCTCCCCGACGGCCAGGTTCATCTGCTCTACACCGGCAACACCGACACCCTAGCCAGG
TCCAGTGCTCGCCACGCCCGCCGACCCGTCGACCCGTCCTCCGCGAGTGGGTCAAGCACCCCGCCAACCCATC
CTCTACCTCCCCCGGCATCGGCCTCAAGGACTTCCGCGACCCCTCACCGCTGGTTTCGACCACTCCGACCACAC
CTGGCGCACCGTCATCGGCTCCAAGGACGACGACGGCCACGCCGCGCATCATCTCAGCTACAAGACCAAGGACTTCG
TCAACTACGAGCTCATGCCGGGAACATGCACCGCGGGCCCGACGGCACCGGAATGTACGAGTGCATCGACCTCTAC
CCCGTCGGCGGCAACTCGTCCGAGATGCTCGGCGGCGACGACTCGCCCGCGTGCTCTTCGTGCTCAAGGAGAGCAG
CGACGACGAGCGCCACGACTACTACGCGCTCGGAAGGTTTCGACGCCGTCGCCAACGTTTGGACGCCCATCGACCGGG
AGCTGGACCTTGGGATCGGGCTCAGATACGACTGGGGAAAGTACTACGCCTCCAAGTCCTTCTACGACCAGAAGAAG
AACCGCCGCATCGTATGGGCATACATCGGCGAGACCGACTCCGAGCAGGCCGACATCACCAAGGGATGGGCCAATCT
CATGACGATTTCAAGAACGGTGGAGCTTGACAGGAAGACCCGCACAAACCTCATCCAATGGCCAGTGGAGGAGGTG
ACACCCTCCGCAGGAACCTCCACGGACCTCGGTGCGATCACCGTCAACGCCGGCTCCGTCATTGCTCTCCCTCCCTCCAC
CAGGGCGCTCAACTCGACATCGAGGCCTCCTTCCAACCTCAACTCTTCCGACGTGGATGCTATCAACGAGGCCGACGT
CGGCTACAACCTGCAGCACCAGTGGTGCCGCCGTACGGGGGGCGCTCGGCCCTTTGGCCTCCTCGTCCTTGCCAACG
GCCGCACCGAACAGACGGCTGTGTACTTCTACGTGTCCAAGGGCGTCGACGGTGCCCTCCAGACCCACTTCTGCCAC
GACGAGTCACGGTCAACGCGGGCAAAGGATGTCGTGAATAGGATGATTGGCAGCATCGTGCCGGTGCTTGACGGTGA
GACCTTTTCGGTGAGGGTGCTAGTGGACCACTCCATCGTGCAGAGCTTCGCGATGGGCGGGAGGATCACGGCGACGT
CGCGGGCGTACCCGACGGAGGCCATCTACGCGGCCGCGGGGGTCTACCTCTTCAACAACGCCACGGGCGCCACCGTC
ACCGCCGAGAGGCTCGTCTGTCACGAGATGGCCTCAGCTGACAACCATATCTTCACGAACGACGACTTG

FIGURE 16

MESPSAVVPGTTAPLLPYAYAPLPSSADDARQNRSGGRWRACAAVLAASALAVVVVVGLLAGGRVDRVPAGGDVASA
TVPAVPMEFPRSRGKDFGVSEKSSGAYSTDGGFPWSNAMLQWQRTGFHFQPEQHYMNDPNGPVYYGGWYHLFYQHNP
KGDSWGNIAWAHAVSKDMVNRHLPLAMVPDQWYDSNGVLTGSITVLPDGQVILLYTGNTDTLAQVQCLATPADPSD
PLLREWVKHPANPILYPPPGIGLKDFRDPLTAWFDHSDHTWRTVIGSKDDDGHAGIILSYKTKDFVNYELMPGNMHR
GPDGTGMYECIDLYPVGGNSSEMLGGDDSPGVLFVLKESSDDERHDYYALGRFDAVANVWTPIDRELDLGIGLRYDW
GKYYASKSFYDQKKNRRIVWAYIGETDSEQADITKGWANLMTIPRTVELDRKTRTNLIQWPVEEVDTLRRNSTDLGR
ITVNAGSVIRLPLHQGAQLDIEASFQLNSSDVDAINEADVGYNCSTSGAAVRGALGPFGLLVLANGRTEQTAVYFYV
SKGVDGALQTHFCHDESSTRAKDVVNRMIGSIVPVL DGETFSVRVLVDHSIVQSFAMGGRITATSRAYPTEAIYAA
AGVYLFNNATGATVTAERLVVHEMASADNHIFTNDDL

FIGURE 17

ATGGAGTCCCCAAGCGCCGTCGTCCCCCGGCACACGGCGCCGCTGCTTCCTTATGCGTACGCGCCGCTGCCGTCG
TCCGCCGACGACGCCCGTCAAAACGGAGTGGCGGGAGGTGGCGCGCGTGCGCCGCGTGCTGGCCGCATCGGCG
TTGGCGGTGGTCGTCGTGGTCGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGGCCGGGAACGAACAA
AAGCCATATAAGGAAACATACGGCATTTCCTCATATTACAGCCATGATATGCTGCAAATCCCTGAACAGCAAAAA
AATGAAAAATATCAAGTTCCTGAATTCGATTTCGTCACAAATTAATAATATCTCTTCTGCAAAGGCTGGACGTT
TGGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGCTACCACATCGTCTTTGCATTA
GCCGGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGAC
AGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAGACCAA
ACACAAGAATGGTCAGGTTTCAGCCACATTTACATCTGACGGAATAATCCGTTTATTCTACACTGATTCTCCGGT
AAACATTACGGCAAACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAAC
GGTGTAGAGGATTATAAATCAATCTTTGACGGTGACGGAATAACGTATCAAAATGTACAGCAGTTTCATCGATGAA
GGCAACTACAGCTCAGGCGACAACCATAACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTA
GTATTTGAAGCAAACACTGGAAGTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGC
AAAAGCACATCATCTTCCGTCAAGAAAGTCAAAAACCTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCA
AACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCT
AACACAGTAACAGATGAAATTGAACGCGCAACGTCTTTAAATGAACGGCAAATGGTACCTGTTCACTGACTCC
CGCGGATCAAAAATGACGATTGACGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTA
ACTGGCCCATACAAGCCGCTGAACAAAACCTGGCCTTGTTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTT
ACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGA
TTCTACGCAGACAAACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTC
AAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAACAAATAA

FIGURE 18

MESPSAVVPGTTAPLLPYAYAPLPSSADDARQNRSGGRWRACAAVLAASALAVVVVVGLLAGGRVDRVPAGGTNQ
KPYKETYGISHITRHDMLQIPEQQKNEKYQVPEFDSSTIKNISSAKGLDVWDSWPLQNADGTVANYHGYHIVFAL
AGDPKNADDTSIYMFYQKVGETSIDSWKNAGR VFKDSKFDANDSILKDQTQEWSGSATFTSDGKIRLFYTDFSG
KHYGKQTLTTAQVNVASDSSLNINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSSGDNHTLRDPHYVEDKGHKYL
VFEANTGTEDGYQGEESLFNKAYYGKSTSFFRQESQKLLQSDKKRTAELANGALGMIELNDDYTLKKVMKPLIAS
NTVTDEIERANVFKMNGKWYLF TDSRGSKMTIDGITSNDIYMLGYVSNLTGPYKPLNKTGLVLKMDLDPNDVTF
TYSHFAVPQAKGNV VITSYMTNRGFYADKQSTFAPSFLLNKGGKTSVVKDSILEQGQLTVNK

FIGURE 20

ATGGAGTCCCCAAGCGCCGTCGTCCCCGGCACCACGGCGCCGCTGCTTCCTTATGCGTACGCGCCGCTGCCGTCG
TCCGCCGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCGTGCGCCGCGTGCTGGCCGCATCGGCG
TTGGCGGTGGTTCGTTCGTGGTTCGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGCCGGCGGAACTATTAAT
GCAGACAATGTTAATGAAAATCAAACGTAGTAAGTAACTGCTAGTTTCAGTAAACAATGAAAATAATAAGCAAGTA
ACTGAAAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACACCAAGAAATCAAACGAAAAT
ACAGAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAATACAAACACA
GTTACCGCTGAAACACCTGTTGATAAAAGTAGTAAATAATAGTGATCAAAAGACAACATAATGCTGCAACTACTGAT
ACTAAAAAGATGATGTAAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAAACAAATGCTGAGGAAAATAAAGAT
AGTTCAGTAAAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAAGGGCCAAGTTAAAGATATCGTTGAAGAATCT
GGTGTGATACTAGCAAGTTAACTAATGATCAAAATTAATGAATTAATAAAATTAATTTCTCCAAAGAAGCAAAA
AGTGGTACTCAGTTAACTTACAACGACTTTAAAAAAATTGCTAAACCTTTAATTGAACAAGATGCTCGTTATGCT
ATTCATTCTTCAATGCAAGTAAATTAATAATATGCCTGCTGCTAAACACTTGATGCTCAAAGTGAAAAGTA
GAAGATTTGGAAATTTGGGATTCATGGCCTGTTCAAGATGCAAAACTGGTTACGTATCTAAGTGAATGGCTAC
CAATTAGTGATTGGTATGATGGGAGTTCCAAACGTCAATGATAACCACATTTATCTTCTTTACAACAAGTATGGT
GATAATGACTTTAATCATTGGAAGAATGCCGGTCTATTTTCGGTCTAGGTACTCCAGTTATTCAACAATGGTCT
GGATCAGCAACTTTAAATAAAGATGGCTCAATTCAACTTTACTACACTAAGTTGATACTAGTGATAATAATACT
AACCACCAAAAACCTCGCTAGTGCAACTGTTTACTTAAATCTTGAAAAAGATCAAGATAAGATTTCTATTGCTCAT
GTTGACAACGACCATATTGTCTTTGAAGGTGATGGTTACCACTACCAAACCTTATGACCAATGGAAGAACTAAC
AAGGGTGCTGACAAATATCGCAATGCGTGATGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTT
GAAGCAAGTACTGGAACCGAAAATTATCAAGGTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAAC
AAGGATAATTTAGGTGATTTCTTCCAAATTTTATCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCT
GCAATTGGTATCATTAAATTAATGATGATGTTAAGAAATCCAAGTGTGCAAAGGTCTACAGCCCACTTATTAGT
GCACCAATGGTAAGTGATGAAATGAACGCCCTGATGTTGTTAAATTAGGTAATAAGTATTACTTATTTGCTGCT
ACTAGATTAAACCGTGGTAGTAACGATGATGCTTGATGGCAACTAACAAAGCAGTTGGTGATAACGTAGCTATG
ATTGGTTATGTTTCTGATAACTTAACTCATGGTTATGTTCCATTGAATGAATCTGGCGTTGTTTTAACTGCATCT
GTACCGGCTAACTGGCGTACTGCAACTTATTCTACTATGCAGTTCCAGTAGAAGGAAGAGATGATCAACTTTTA
ATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGGGTATGCATGCAACTTGGGCACCAAGTTTCTTG
TTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAAATGACTAACCAAGGGGATGGATTTGGGATGAT
AGTAGTGAAAATCCAGATATGATGGGTGACTTGAAAAAGATGCTCCAAATAGTGCTGCCCTTCTGGAGAATGG
GGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACCTGAAGCCACACCAACCTGTAACCTCTATTCCAAAT
GTACCAACTACTCTGAAACCCCAACCAACAGATAAGCCAGAGGTACCAACTACCCCTGAAGTTCCAACCACT
CCGAAAACCTCAACTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAACCTAGTCAGTCTAAACTTCCAAAGGCTGGA
GATAAAAATAGCTTTGCAGCAGTTGTTTGGTGGCTGTAAGTTCAATATTAGGTGCTGTTGGTTTAAACAGGTGTT
TCAAAACGTAAACGTAATAATTAA

FIGURE 21

MESPSAVVPGTTAPLLPYAYAPLPSSADDARQNRSGGRWRACAAVLAASALAVVVVVGLLAGGRVDRVPAGGTINAD
NVNENQTV EVTASSVNNENNKQVTEKDSADKSTSDVAEDANTKKS NENTETTEKNTQTVVTNAPVSDVKNTNTVTAE
TPVDKVVNSDQKTTNAATTDTKKDDVKQVEKKDSVDKTNAEENKDS SVKPAENATKAE LKGQVKDIVEESGVDTSK
LTNDQINELNKINFSKEAKSGTQLTYNDFKKIAKTLIEQDARYAIPFFNASKIKNMPAAKTLDAQSGKVEDLEIWDS
WPVQDAKTGYVSNWNGYQLVIGMMGVPNVNDNHIYLLYNKYGDNDFNHWKNAGPIFGLGTPVIQQWSGSATLNKDGS
IQLYYTKVDTS DNNTNHQKLASATVYLNLEKDQDKISIAHVDNDHIVFEGDGYHYQTYDQWKETNKGADNIAMRDAH
VIDDDNGNRYLVFEASTGTENYQGDDQIYQWLN YGGTNKDNLG DFFQILSNSDIKDRAKWSNAAIGI IKLND DVKNP
SVAKVYSPLISAPMV SDEIERPDVVKLG NKYYLFAATRLNRGSND DAWMATNKAVGDNVAMIGYVSDNLTHGYVPLN
ESGVVLTASVPANWRTATYSYYAVPVEGRDDQLLITSYITNRGEVAGKGMHATWAPS FLLQINPDNTTTVLAKMTNQ
GDWIWDDSSENPDMMGVLEKDAPNSAALPGEWGKPDWDLIGGYNLKPHQPVTPIPNVPTTPETPTTPDKPEVPTTP
EVPTTPETPTPEAPKNPVK KTSQSKLPKAGDKNSFAAVVLGAVSSILGAVGLTGVS KRKRNN

FIGURE 22

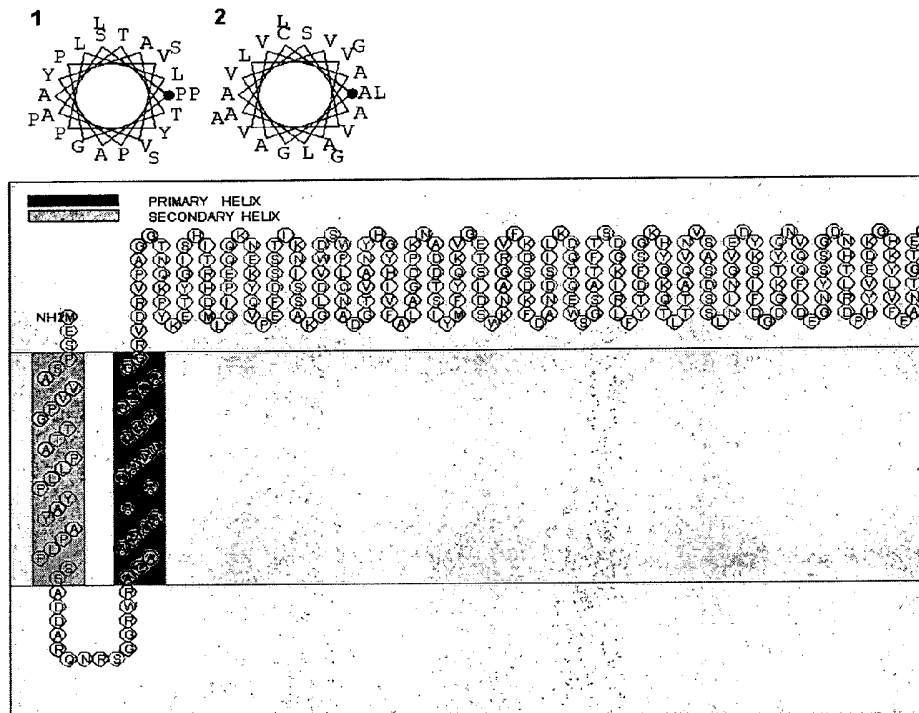


FIGURE 23

cgtggtcgagattgtgtattattcttttagttattacaagacttttagctaaaatttgaaagaatttactttaagaaa
atcttaacatctgagataatctcagcaatagattatatttttcattactctagcagtatttttgcagatcaatcgca
acatatatggttgttagaaaaatgcactatatatatatatattttttcaattaaaagtgcagatatataata
tatatatatatatatatgtgtgtgtgtatatggtcaaagaaattctttatacaaatatacacgaacacatatattt
gacaaaatcaaagtattacactaaacaatgagttggtgcatggccaaaacaaatatgtagattaaaaattccagcct
ccaaaaaaaatccaagtgtgttaaagcattatatatatatagtagatcccaaattttgtacaattccacactgat
cgaatttttaaagttgaatatctgacgtaggatttttttaatgtcttacctgaccatttactaataacattcatacg
ttttcatttgaaatatctctataattatattgaatttggcacataataagaaacctaatgtgtgatttattttact
agtaaattttctggtgatgggctttctactagaaagctctcggaaaatcttgaccaaatccatattccatgacttcg
attgttaaccctatttagttttcacaaacatactatcaatatcattgcaacggaaaaggtacaagtaaacattcaat
ccgatagggaagtgtatgtaggaggttgggaagacaggcccagaaagagatttatctgacttgttttgtgtatagttt
tcaatgttcataaaggaagatggagacttgagaagtttttttggactttgttttagctttgttggcggtttttttt
tttgatcaataaactttgttgggcttatgatttgaatattttcgtggactcttttagtttttagacgtgctaactt
tgttgggcttatgacttggttgaacatatgttaacagatgacttgatgtgactaatctttacacattaaacatag
ttctgttttttgaaagttcttattttcatttttatttgaatgttatatatattttctatatatttataattctagtaaaa
ggcaaattttgcttttaaatgaaaaaatatatattccacagtttcacctaattcttatgcatttagcagtacaaatt
caaaaattttccattttttattcatgaatcataccattatatattaactaaatccaaggtaaaaaaagggtatgaaag
ctctatagtaagtaaaatataaattccccataaggaaaggccaagtccaccaggcaagtaaaatgagcaagcacca
ctccaccatcacacaattttcactcatagataacgataagattcatggaattatcttccacgtggcattattccagcg
gttcaagccgataaggggtctcaacacctctccttaggcctttgtggcggttaccagtaaaattaacctcacacata
tccacactcaaaatccaacggtgtagatcctagtcacttgaaatctcatgtatcctagacctccgatcactccaaa
gcttgttctcattgttgttatcattatatatagatgaccaaagcactagaccaaacctcagtcacacaaagagtaaa
gaagaaca**ATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTTCCTCTATCTCCTGCCAATCCAGCCCATTCC**
ACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCTATTTACACGCCATGATATGCTGCAATCCCTGAACA
GCAAAAAATGAAAAATATCAAGTTCCTGAATTCGATTTCGTCCACAATTAATAATATCTCTTCTGCAAAAGGCCTGG
ACGTTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGCTACCACATCGTCTTTGCA
TTAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTACATGTTCTATCAAAAAGTCGGCGAACTTCTATTGA
CAGCTGGA AAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAATGATTCTATCCTAAAAGACCAAA
CACAAGAATGGTCAGGTTACGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTCTCCGGTAAA
CATTACGGCAACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGT
AGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACT
ACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAA
GCAAACTGGAACCTGAAGATGGCTACCAAGCGAAGAATCTTTATTTAACAAGCATACTATGGCAAAAGCACATC
ATTCTTCCGTCAAGAAAGTCAAAAATCTTGCAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCG
GTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGAT

FIGURE 23 (CONT'D)

GAAATTGAACGCGCGAACGTCTTTAAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGAC
GATTGACGGCATTACGTCTAACGATATTTACATGCTTGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGC
TGAACAAAACCTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTA
CCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAAC
GTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGAC
AATTAACAGTTAACAATAAGAtcggttcaaacatttggcaataaagtttcttaagaatgaatcctgttgccggtctt
gcgatgattatcatataatcttctgttgaattacgttaagcatgaaataattaacatgtaatgcatgacgtaatttat
gagatgggtttttatgattagagtcccgcaattatacatttaatacgcgatagaaaacaaaatatagcgcgcaact
aggataaattatcgcgcgcggtgtcatcaatgttactagatc

FIGURE 24

cgtggcgcgagattgtgtattattccttagttattacaagacttttagctaaaatttgaaagaatttactttaaaaa
 atcttaacatctgagataatttcagcaatagattatatttttcattactctagcagtagttttgagatcaatcgca
 acatatatggttgttagaaaaatgcactatatatatatatattttttcaattaaaagtgcagatataataa
 tatatatatatatatatgtgtgtgtgtatattggtcaaagaaattcttatatacaaatatacacgaacacatatatt
 gacaaaatcaaagtattacactaaacaatgagttggtgcatggccaaaacaaatatgtagattaaaaattccagcct
 ccaaaaaaaaaatccaagtgtgtgaaagcattatatatatatagtagatcccaaattttgtacaattccacactgat
 cgaatttttaaaagtgaatatctgacgtaggatttttttaattgtcttacctgaccatttactaataacattcatacg
 ttttcatttgaaatatcctctataattatattgaatttggcacataataagaaacctaatgtgtgatttattttact
 agtaaatttctggtgatgggctttctactagaaagctctcggaatcttgaccaaatccatattccatgacttcg
 attgttaaccctatttagttttcacaaacatactatcaatatcattgcaacggaaaaggtacaagtaaaacattcaat
 ccgataggggaagtgtgtaggaggttgggaagacaggcccagaaagagatttatctgacttggtttgtgtatagttt
 tcaatgttcataaaggaagatggagacttgagaagttttttttgactttgttttagctttgttggcggtttttttt
 tttgatcaataactttgttgggcttatgatttgaatatatttcgtggactcttttagttatttagacgtgctaactt
 tgttgggcttatgacttggttgaacatattgtaacagatgacttgatgtgactaatctttacacattaaacatag
 ttctgttttttgaaagttcttattttcatttttatttgaatgttatatatattttctataatttataattctagtaaaa
 ggcaaattttgcttttaaatgaaaaaatatatattccacagtttcacctaattctatgcatttagcagtacaaatt
 caaaaattttccattttttattcatgaatcataccattatatattaactaaatccaaggtaaaaaaaaggatgaaag
 ctctatagtaagtaaaatataaaattcccataaggaagggccaagtccaccaggcaagtaaaatgagcaagcacca
 ctccaccatcacacaatttctactcatagataacgataagattcatggaattatcttccacgtggcattattccagcg
 gttcaagccgataagggctcacaacacctctccttaggcctttgtggcggttaccaagtaaaattaacctcacacata
 tccacactcaaaatccaacggtgtagatcctagtccacttgaatctcatgtatcctagacctccgatcactccaaa
 gcttgttctcattgttgttatcattatatatagatgaccaaagcactagaccaaacctcagtcacacaaagagtaaa
 gaagaaca**ATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTTCTCTATCTCCTGCCAATCCAGCCCATTCC**
 ACTATTAATGCAGACAATGTTAATGAAAATCAAAGTGTAGAAGTAACTGCTAGTTCAGTAAACAATGAAAAATAATA
 GCAAGTAACTGAAAAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACACCAAGAAATCAAACG
 AAAATACAGAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAAC
 ACAGTTACCGCTGAAACACCTGTTGATAAAGTAGTAAATAATAGTGATCAAAAGACAATAATGCTGCAACTACTGA
 TACTAAAAAAGATGATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAAATAAGATA
 GTTCAGTAAAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAAGGCCAAGTTAAAGATATCGTTGAAGAATCTGGT
 GTTGATACTAGCAAGTTAACTAATGATCAAATTAATGAATTAATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGG
 TACTCAGTTAACTTACAACGACTTTAAAAAATTGCTAAAACTTTAATTGAACAAGATGCTCGTTATGCTATTCCAT
 TCTTCAATGCAAGTAAATTAATAATATGCCTGCTGCTAAAACACTTGATGCTCAAAGTGAAAAAGTAGAAGATTTG
 GAAATTTGGGATTCATGGCCTGTTCAAGATGCAAAAAGTGGTTACGTATCTAAGTGAATGGCTACCAATTAGTGAT
 TGGTATGATGGGAGTTCCAAACGTCAATGATAACCAATTTATCTTTTACAACAAGTATGGTGATAATGACTTTA
 ATCATTGGAAGAATGCCGGTCTATTTTCGGTCTAGGTACTCCAGTTATTCAACAATGGTCTGGATCAGCAACTTTA
 AATAAAGATGGCTCAATTCAACTTTACTACAC' 'TGATAATAATACTAACCACCAAAAAGTTCGCG

FIGURE 24 (CONT'D)

TAGTGCAACTGTTTACTTAAATCTTGAAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTG
TCTTTGAAGGTGATGGTTACCACTACCAAACCTTATGACCAATGGAAAGAACTAACAAGGGTGCTGACAATATCGCA
ATGCGTGATGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTTGAAGCAAGTACTGGAACCGAAAA
TTATCAAGGTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTTCTTCC
AAATTTTATCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTTAAATTAAATGAT
GATGTTAAGAATCCAAGTGTGCAAAGGTCTACAGCCCACTTATTAGTGACCAATGGTAAGTGATGAAATTGAACG
CCCTGATGTTGTTAAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATG
CTTGATGGCAACTAACAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATAACTTAACTCATGGT
TATGTTCCATTGAATGAATCTGGCGTTGTTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCATA
CTATGCAGTTCCAGTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTG
GAAAGGGTATGCATGCAACTTGGGCACCAAGTTTCTTGTTACAAATTAATCCAGATAACACTACTACTGTTTTAGCT
AAAATGACTAACCAAGGGGATTGGATTGGGATGATAGTAGTGAAAATCCAGATATGATGGGTGTACTTGAAAAAGA
TGCTCCAAATAGTGCTGCCCTTCCTGGAGAATGGGGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACTTGA
AGCCACACCAACCTGTAACCTCCTATTCCAAATGTACCAACTACTCCTGAAACCCCAACCACACCAGATAAGCCAGAG
GTACCAACTACCCCTGAAGTTCCAACCACTCCAGAACTCCAACTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAAC
TAGTCAGTCTAACTTCCAAAGGCTGGAGATAAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAAGTTCAATAT
TAGGTGCTGTTGGTTTAAACAGGTGTTTCAAAACGTAAACGTAATAATTAagatcggttcaaacatttggcaataaagt
ttcttaagaatgaatcctggtgcccgtcttgcgatgattatcatataatttctggtgaattacgttaagcatgaaat
aattaacatgtaatgcatgacgtaatttatgagatgggtttttatgattagagtcccgcaattatacattttaatacg
cgatagaaaacaaaatatagcgcgcaaactaggataaattatcgcgcgcggtgtcatcaatgttactagatc

FIGURE 25

cgtggtcgagattgtgtattattcttttagttattacaagacttttagctaaaatttgaaagaatttactttaagaaa
 atcttaacatctgagataatttcagcaatagattatatttttcattactctagcagtatttttgcagatcaatcgca
 acatatatggttgttagaaaaatgcactatatatatatatattttttcaattaaaagtgcagatataata
 tatatatatatatatatgtgtgtgtgtatatggtcaaagaaattctttatacaaatatacacgaacacatatattt
 gacaaaatcaaagtattacactaaacaatgagttggtgcatggccaaaacaaatatgtagattaaaaattccagcct
 ccaaaaaaaaaatccaagtgtgttaaagcattatatatatatagtagatcccaaattttgtacaattccacactgat
 cgaatttttaaaagtgaatatctgacgtaggatttttttaatgtcttacctgaccatttactaataacattcatacg
 ttttcatttgaaatatcctctataattatattgaatttggcacataataagaaacctaattggtgatttattttact
 agtaaatttctggtgatgggctttctactagaaagctctcggaatcttggaacaaatccatattccatgacttcg
 attgttaaccctatttagttttcaciaacatactatcaatatcattgcaacggaaaaggtacaagtaaaacattcaat
 ccgatagggaaagtgtgtaggaggttgggaagacaggcccagaaagagatttatctgacttggtttgtgtatagttt
 tcaatgttcataaaggaagatggagacttgagaagttttttttgactttgttttagctttgttggcgctttttttt
 tttgatcaataactttgttgggcttatgatttgaatatatttcgtggactcttttagtttatttagacgtgctaactt
 tgttgggcttatgacttggttgaacataattgtaacagatgacttgatgtgacgactaatctttacacattaaacatag
 ttctgttttttgaaagttcttattttcatttttatttgaatgttatatatattttctatatatttataattctagtaaaa
 ggcaaattttgcttttaaatgaaaaaatatatattccacagttttcacctaattcttatgcatttagcagtacaaatt
 caaaaatttcccatttttattcatgaatcataccattatatattaactaaatccaaggtaaaaaaaaggatgaaag
 ctctatagtaagtaaaatataaattccccataaggaaagggccaagtccaccaggcaagtaaaatgagcaagcacca
 ctccaccatcacacaatttctcatagataacgataagattcatggaattatcttccacgtggcattattccagcg
 gttcaagccgataaagggtctcaacacctctccttaggcctttgtggcggttaccaagtaaaatcaacctcacacata
 tccacactcaaaaatccaacggtgtagatcctagtcacttgaaatctcatgtatccttagacctccgatcactccaaa
 gcttgttctcattgttgttatcattatatatagatgaccaaagcacttagaccaaacctcagtcacacaaagagtaaa
 gaagaaca**ATGGAGTCCCCAAGCGCCGTCGTCCCCGGCACCACGGCGCCGCTGCTTCCTTATGCGTACGCGCCGCTG**
CCGTGCTCCGCCGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCGTGCGCCGCCGTGCTGGCCGCATC
GGCGTTGGCGGTGGTTCGTGCTGGTTCGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGGCCGGCGGAACGAACC
 AAAAGCCATATAAGGAAACATACGGCATTTCCTATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAA
 AATGAAAAATATCAAGTTCCTGAATTCGATTTCGTCCACAATTAAAAATATCTCTTCTGCAAAAGGCCTGGACGTTTG
 GGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCG
 GAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGACAGCTGG
 AAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAATGATTCTATCCTAAAAGACCAACACAAGA
 ATGGTTCAGGTTACGCCACATTTACATCTGACGGAATAATCCGTTTATCTACACTGATTTCTCCGGTAAACATTACG
 GCAACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGAT
 TATAAATCAATCTTTGACGGTGACGGAATAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCTC
 AGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACA
 CTGGAAGTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACATCATTTCTTC
 CGTCAAGAAAGTCAAAAACCTTCTGCAAAGCGA'

'TGAGTTAGCAAACGGCGCTCTCGGTATGAT

FIGURE 25 (CONT'D)

TGAGCTAAACGATGATTACACACTGAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATTG
AACGCGCGAACGTCTTTAAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGAC
GGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACAA
AACTGGCCTTGTTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAG
CGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCG
CCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAAC
AGTTAACAAATAAgatcggttcaaacatthggcaataaagtttcttaagaatgaatcctggtgcccgtcttgcgatga
ttatcatataatthctggtgaattacggttaagcatgaaataattaacatgtaatgcatgacgtaatttatgagatgg
gtttttatgattagagtcgcaattatacatttaatacgcgatagaaaacaaatatagcgcgcaaactaggataa
attatcgcgcgcggtgtcatcaatggttactagatc

FIGURE 26

cgtggtcgagattgtgtattattcttttagttattacaagacttttagctaaaatttgaaagaatttactttaagaaa
atcttaacatctgagataatttcagcaatagattatatttttcattactctagcagtatttttgcagatcaatcgca
acatatatggttgttagaaaaatgcactatatatatatatattattttttcaattaaaagtgcagatataata
tatatatatatatatatatgtgtgtgtgtatatggtcaaagaaattcttatacaaatatacacgaacacatatattt
gacaaaatcaaagtattacactaaacaatgagttggtgcatggccaaaacaaatatgtagattaaaaattccagcct
ccaaaaaaaatccaagtgtttaaagcattatatatatatagtagatcccaaattttgtacaattccacactgat
cgaatttttaaaagtgaatatctgacgtaggatttttttaatgtcttacctgaccatttactaataacattcatacg
ttttcatttgaaatatctctataattatattgaatttggcacataataagaaacctaatgtgtgatttattttact
agtaaatttctggtgatgggctttctactagaaagctctcggaatcttggaacaaatccatattccatgacttcg
attgttaaccctatttagttttcacaaacatactatcaatatcattgcaacggaaaaggtacaagtaaaacattcaat
ccgatagggaagtgtatgtaggaggttggaagacaggcccagaaagagatttatctgacttggtttgtgtatagttt
tcaatgttcataaaggaagatggagacttgagaagtttttttggactttgttttagctttgttggcggtttttttt
tttgatcaataactttgttgggcttatgatttgaatatatttcgtggactcttttagtttatttagacgtgctaactt
tgttgggcttatgacttggttgaacatatgtgaacagatgacttgatgtgactaatctttacacattaaacatag
ttctgttttttgaaagttcttatttttcattttttattgaatgttatatatattttctatatatttataattctagtaaaa
ggcaaattttgcttttaaatgaaaaaatatatattccacagtttcacctaattcttatgcatttagcagtacaaatt
caaaaattttccattttttattcatgaatcataccattatatattaactaaatccaaggtaaaaaaaaggtatgaaag
ctctatagtaagtaaaatataaattccccataaggaagggccaaagtccaccaggcaagtaaaatgagcaagcacca
ctccaccatcacacaatttctcatagataacgataagattcatggaattatcttccacgtggcattattccagcg
gttcaagccgataagggtctcaacacctctccttaggcctttgtggcggttaccaagtaaaattaacctcacacata
tccacactcaaaaatccaacggtgtagatcctagtcacttgaaatctcatgtatccttagacctccgatcactccaaa
gcttgttctcattgttgttatcattatatatagatgaccaaagcactagaccaaacctcagtcacacaaagagtaaa
gaagaaca**ATGGAGTCCCCAAGCGCCGTCTCCCGGCACACGGCGCCGCTGCTTCCTTATGCGTACGCGCCGCTG**
CCGTCTGTCGCGGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCTGCGCCGCGTCTGGCCGCATC
GGCGTTGGCGGTGGTCTGCTGGTCTGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGGCCGGCGGAACTATTA
ATGCAGACAATGTTAATGAAAATCAAACCTGTAGAAGTAACTGCTAGTTCAGTAAACAATGAAAATAATAAGCAAGTA
ACTGAAAAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACACCAAGAAATCAAACGAAAATAC
AGAAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTTA
CCGCTGAAACACCTGTTGATAAAGTAGTAAATAATAGTGATCAAAAGACAATAATGCTGCAACTACTGATACTAAA
AAAGATGATGTAAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAAATAAGATAGTTCAGT
AAAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAGGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTGATA
CTAGCAAGTTAACTAATGATCAAATTAATGAATTAATAAAATTAATTTCTCAAAGAAGCAAAAAGTGGTACTCAG
TTAACTTACAACGACTTTAAAAAAATTGCTAAAACTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCAA
TGCAAGTAAAATTAAAAATATGCCTGCTGCTAAAACACTTGATGCTCAAAGTGGAAGTAGAAGATTTGGAAATTT
GGGATTCATGGCTGTTCAGATGCAAAAACCTGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTATG
ATGGGAGTTCAAACGTCAATGATAACCAT' .CAAGTATGGTGATAATGACTTTAATCATTG

FIGURE 26 (CONT'D)

GAAGAATGCCGGTCCTATTTTCGGTCTAGGTACTCCAGTTATTCAACAATGGTCTGGATCAGCAACTTTAAATAAAG
ATGGCTCAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCACCAAAAACTCGCTAGTGCA
ACTGTTTACTTTAAATCTTGAAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTGA
AGGTGATGGTTACCACTACCAAACCTTATGACCAATGGAAAGAACTAACAAGGGTGCTGACAATATCGCAATGCGTG
ATGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTTGAAGCAAGTACTGGAACCGAAAATTATCAA
GGTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTTCTTCCAAATTTT
ATCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAAATTAAATGATGATGTTA
AGAATCCAAGTGTGCAAAGGTCTACAGCCCCTTATTAGTGCACCAATGGTAAGTGATGAAATTGAACGCCCTGAT
GTTGTTAAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGGAT
GGCAACTAACAAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATAACTTAACTCATGGTTATGTTT
CATTGAATGAATCTGGCGTTGTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCATACTATGCA
GTCCAGTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAGGG
TATGCATGCAACTTGGGCACCAAGTTTCTTGTTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAAATGA
CTAACCAAGGGGATTGGAATTTGGGATGATAGTAGTGAAAATCCAGATATGATGGGTGTACTTGAAAAAGATGCTCCA
AATAGTGCTGCCCTTCCTGGAGAATGGGGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACTTGAAGCCACA
CCAACCTGTAACCTCCTATTCCAAATGTACCAACTACTCCTGAAACCCCAACCACACCAGATAAGCCAGAGGTACCAA
CTACCCCTGAAGTTCCAACCACTCCAGAACTCCAACCTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAAGTACTAG
TCTAAACTTCCAAAGGCTGGAGATAAAAATAGCTTTCAGCAGTGTGTTTATAGGTGCTGTAAGTTCAATATTAGGTGC
TGTTGGTTTAAACAGGTGTTTCAAACGTAACGTAATAATTAAgatcggttcaaacatttggcaataaagtttcttaa
gaatgaatcctgttgccggtcttgcgatgattatcatataatttctgttgaattacgttaagcatgaaataattaac
atgtaatgcatgacgtaatttatgagatgggtttttatgattagagtcccgcgaattatacatttaatacgcgataga
aaacaaaatatagcgcgcaaaactaggataaattatcgcgcgcggtgtcatcaatgttactagatc

FIGURE 27

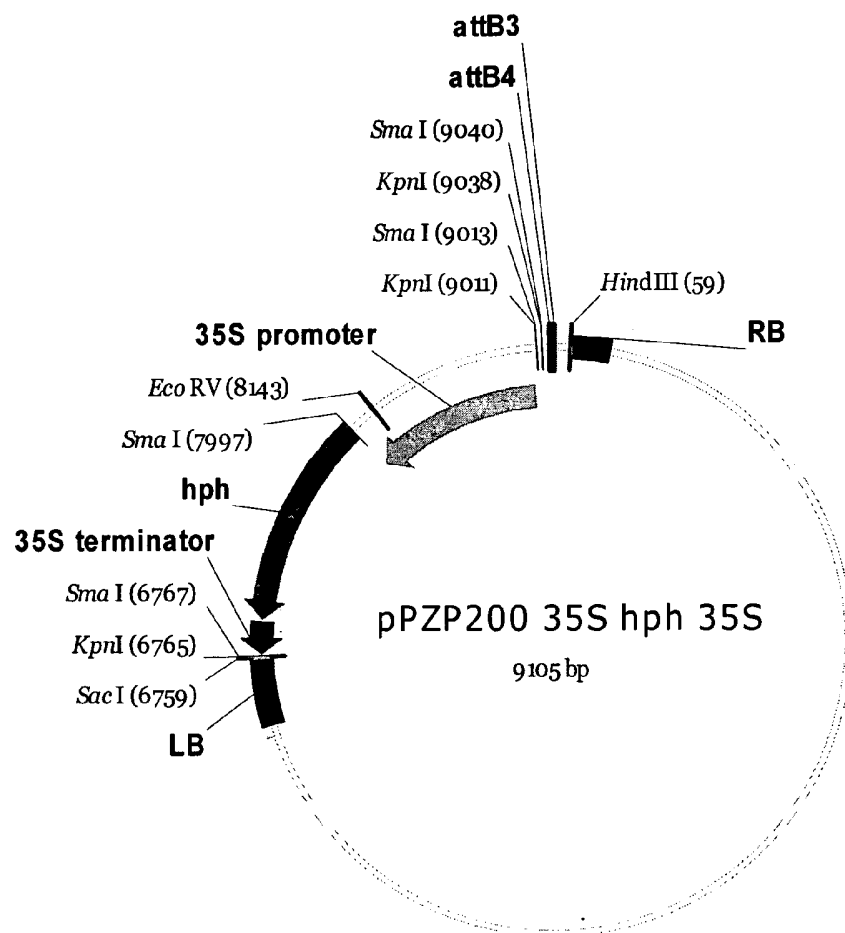


FIGURE 28

A

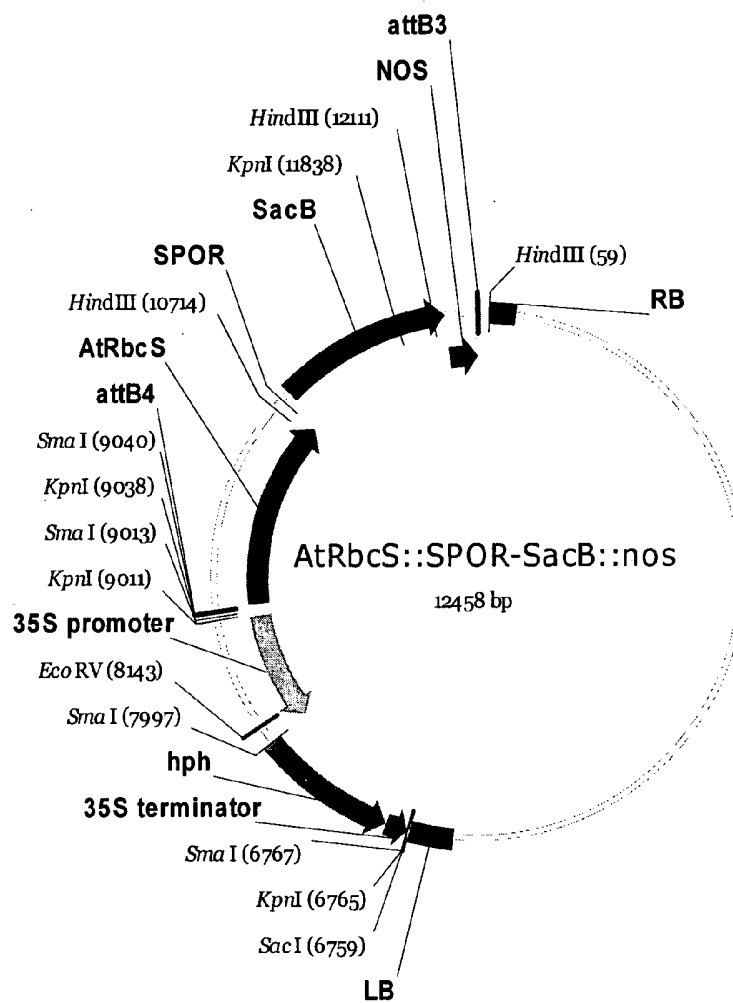


FIGURE 28 (CONT'D)

B

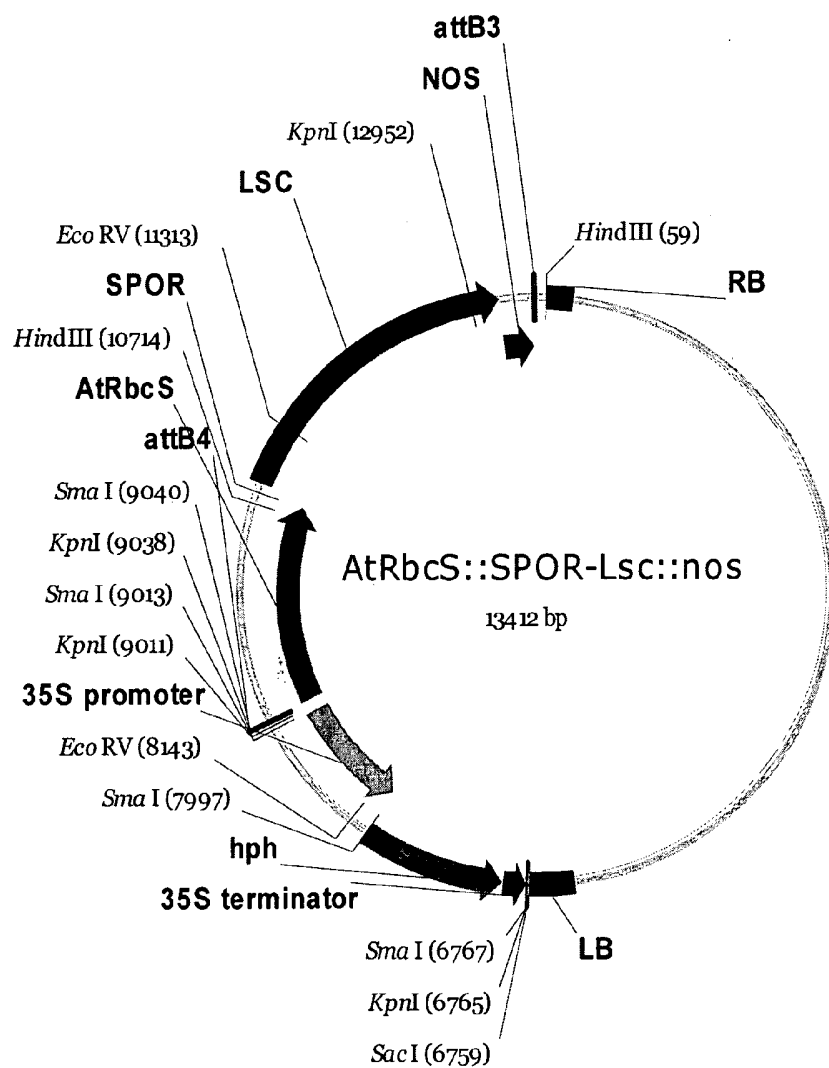


FIGURE 28 (CONT'D)

C

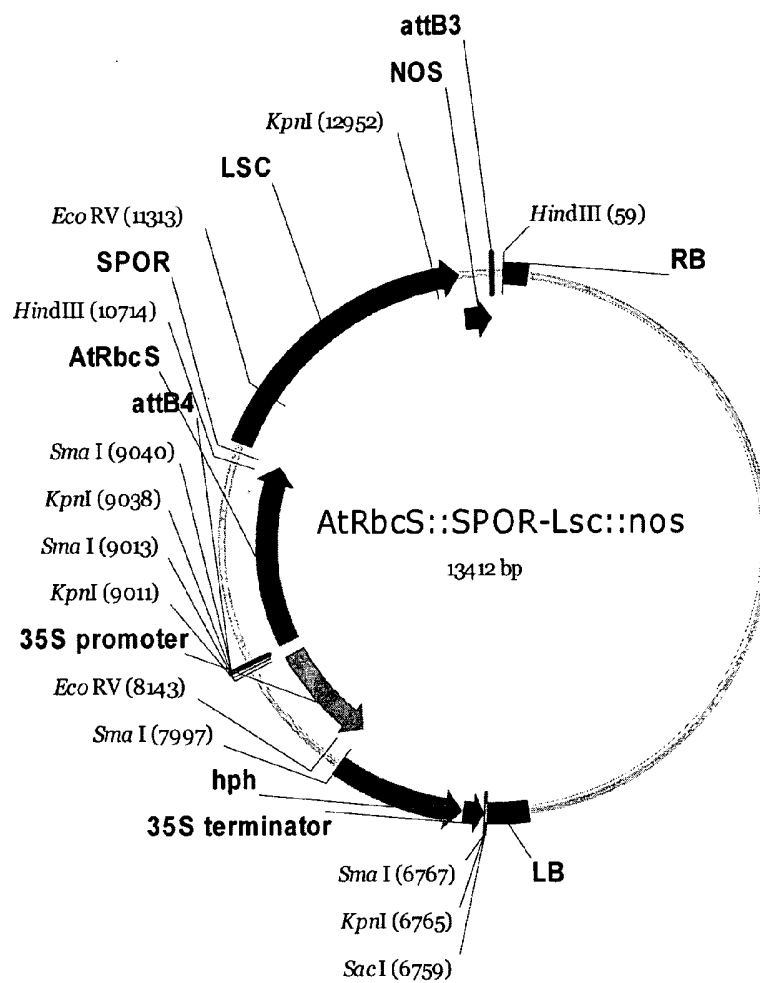


FIGURE 28 (CONT'D)

D

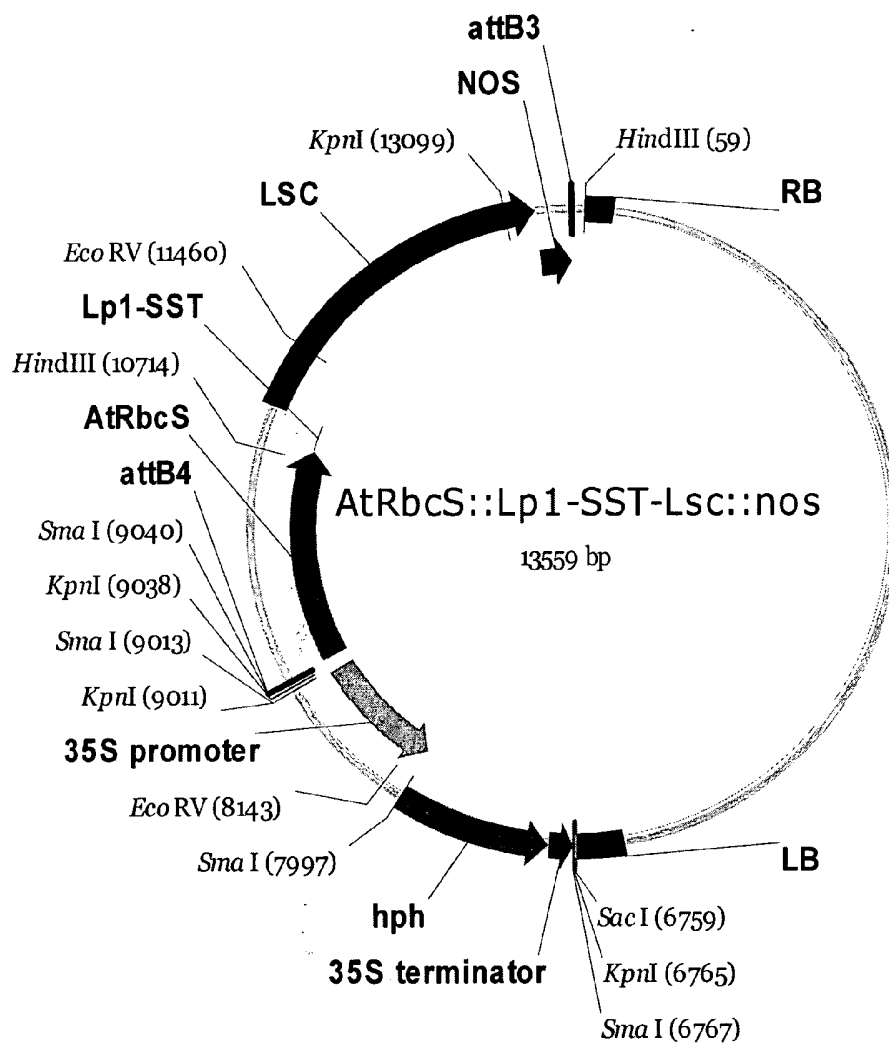


FIGURE 29

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgttttagatcactacttctttacagagaatttccttccctccaagggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatatcttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccaccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagttagccagcaaattttatgataaaatgaaatattttgccca
gccaaactcagtcgcatcctcggacaatttgttatcaaggaactcacccaaaaacaagcaaagctagaaaaaggttgt
gtggcagccacctaatgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaacgaggcaaccagcgaacgtgagccatcccaaccacatctgtaccaagaaacgggg
ctatatataccgtggtgacccggcaatggggtcctcaactgtagccggcatcctcctctcctccgataatacaata
ccATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTCTCTCTATCTCCTGCCCAATCCAGCCCATTCCACGAAC
CAAAAGCCATATAAGGAAACATACGGCATTTCCTCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAA
AAATGAAAAATATCAAGTTCCTGAATTCGATTCGTCCACAATTAAAAATATCTCTTCTGCAAAGGCCTGGACGTTT
GGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGCTACCACATCGTCTTTGCATTAGCC
GGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGACAGCTG
GAAAAACGCTGGCCGCTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCAACACAAG
AATGGTCAGGTTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTAC
GGCAAACAACACTGACAACGACAAAGTTAAGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGA
TTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCT
CAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAAC
ACTGGAACGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAAACAAAGCATACTATGGCAAAGCACATCATTCTT
CCGTCAAGAAAGTCAAAAACCTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGA
TTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCCTGATTGCATCTAACACAGTAACAGATGAAATT
GAACGCGCAACGTCTTTAAATGAACGGCAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGA
CGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACA
AACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAA
GCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGC
GCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAA
CAGTTAACAAATAAactatgagttgaaacaatggcctatctcatatgaagatcttttgtgaatttcacttttgtcca
cgacctctgttgacgactctgctttccgaccggagcataccttttgttctatatgatttgtgtatgtatgtaggaa
cctatgttctcgagcatgcatacataattcctcataggtctatatacaccggctatccatatgcaaacctgtgtaa
tatttgttatatacaacacgaggaccattgtcttctgtgttattaattcttttttcccgcaaaaaggaaaagtttct
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tgcaattgcaaatttcagtttctttacttatatcactacaaaagtcttattgtttcttttccacgtcattaccatct
gtccattgttttttctagtagaataggatgaagcatggacacagattaactgagctcgagctcatatgagctcgg
gtgaacaataaaatctgaaaatacttagaaagaattcaaaattttctgttttttgggcaaaatttgacaaatgta
taaagcttgcaagtttcatcatagaacgacattcgtggatgtcatggcaaaaacaaattcagcactctgaaaat
aacttttttgaagtatcg

FIGURE 30

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgtttagatcactacttctttacagagaatttccttccctccaagggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccacccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagttagccagcaaattttatgataaaatgaaatattttgccca
gccaaactcagtcgcatcctcggacaatttggtatcaaggaactcacccaaaaacaagctagaaaaaggttgt
gtggcagccacctaataatgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaacgaggaaccagcgaacgtgagccatcccaaccacatctgtaccaaagaaacgggg
ctatatataccgtggtgacccggcaatggggctcctcaactgtagccggcatcctcctcctcctcgataatacaata
ccATGAAAGCCTTCACACTCGCTCTCTTTCTTAGCTCTTTTCTCTATCTCTCTGCCCAATCCAGCCCATTCCACGAAC
CAAAAGCCATATAAGGAAACATACGGCATTTCATATACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAA
AAATGAAAAATATCAAGTTCCTGAATTCGATTTCGTCCACAATTAAAAATATCTCTTCTGCAAAGGCCTGGACGTTT
GGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGCTACCACATCGTCTTTGCATTAGCC
GGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGACAGCTG
GAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCAAAACAAG
AATGGTCAGGTTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTAC
GGCAAAACAAACACTGCAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGA
TTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCT
CAGGCGACAACCATAACGTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAAC
ACTGGAAGTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAAACAAAGCATACTATGGCAAAAGCACATATTCTT
CCGTCAAGAAAGTCAAAAACCTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGA
TTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATT
GAACGCGCGAACGTCTTTAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGA
CGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACA
AACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAA
GCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGC
GCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAA
CAGTTAACAAATAAactatgagttgaaacaatggcctatctcatatgaagatcttttgtgaatttcacttttgtcca
cgacctctgttgacgactctgctttccgaccggagcatacctttgttctatatgatttgtgtatgtatgtaggaa
cctatgttctcgagcatgcatacataattcctcataggtctatatacaccggctatccatatgcaaacctgtgtaa
tatttgttatatacaacacgcggaccattgtcttctgtgttattaattctttttcccgcaaaaaaggaaagtttct
ttatttggcactgcaatggatatgcctcacagctagtgggtggagaattcagtatattgacattaagattccctgatt
tgcaattgcaaatttcagtttctttacttatatcactacaaaagtcttattgtttcttttccacgtcattaccatct
gctccattggtttttctagtagaataaggatgaagcatggacacagattaactgagctcgagctcatatgagctcgg
gtgaacaataaaatctgaaaatacttagaagaattcaaaatcttctgtttttgtggcaaatgtgacaaatgtta

FIGURE 30 (CONT'D)

taaatgcttgcaagtttcatagacgacattcgtggatgtcatggcaaaaaacaaattcagcactctgaaat
aactttttgaagtatcggttgtgtcttcagattaacctccaaactttgattaaccaaaaaaattacaact
aacatgttcctttttctttagaaattctaacgaatttatctgattgaatacttaatttggtcat
tggatgccctttacaacctccttaccaaactcactatggcaaatatactatttccattgtaacataaatgtc
ataattgaattaattcgtgcagtacgaaaccactttgtccaaaaaaaccttaactaactatttactt
taatgtaaatatacctctactttgttttacaacctagctcaaaaaattattatttgcgataaaaaacat
tcgaaaaactcgatgattttttcttacgttaatgaaactaaatatagaaaaaaacagatgaacaa
ttttcacctatctaactacttaataatgattaaattggtaaagttgaaagttctttaggaatgtga
atattgatcacagttctattgctaaatcacaaaacgcatgtcgcattcataattatggttcacct
aactaggctaataagtaataagtagacaactagactcaggttgaaaaaccataaagccatatagcgtttc
attgaaactgcaacgatcgtgtgaatgttgcagttcagtttgatacaaaaaaacacaaattaac
ttagattaaaaaaaagaacggagcccactagccactccttcaaacgtcttaccaactctctctctct
caaattaggcttcacctcctctctccaacctctctctctctctctctctctctctctctctc
ccctaatttcttcacagaatcagaatactgcaaaaaacttATGGACTGCATCTAATTTCGGTCCAACT
TGCACAGGAAGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGACGAC
ATGCTGTCCTCAACTATCAACGGAAGCGACGACGACGACGACGACGACGACGACGACGACGACGAC
ATGATCGGCCTCTGGTGAGGTATCATCGCAGCAAGCTCATAGGCTGATCGAGGTGATAATCAT
GAGGCCAACGGCGGCTTATCTGAGGGAGGATCCACCTCGTTGCTCAACTGCATGGCGCGAACAGCT
TGCAGATTTCGTTGGCATATTTCGCCAAGTTACCCGACCAAGACCTTCATGAAGCGCCAAGGCAG
TTAAGCAGATGTGCACCCGTGCAGGCCATTCTATTATTCAAGAGTTGGTTATCTTTGGATGAACCTCGGCT
AGGCCCATTCTGAAGAGATCGATGGATATCGATATGCCATGTTGTTGCTAGCCAGAACCAGATCACGCAGAT
GCTATTGCAGCTTGACGCAATATGGAAGGTAAGTTGATAATGGGATCGCTCAGGAGTATTCATCCATGCGC
AACAGGAACAGAATTCCCCAAGTTAACGCAGCCGCTTTCGACGGATTCGAAGGTCATCCGTTCGGATGTATA
GTAAgtccgcaaaatcaccagtctctctacaaatctatctctctatttttccagaataatgtgtgagta
ttcccagataaggattaggttctaggttcgtcatgtgttgacataagaaaccctagtatgtat
tgtatttgtaaaatacttcataaaatttcaattcctaaaaccaaaatccagtgacct

FIGURE 31

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgttttagatcactacttctttacagagaatttccttccctccaaggggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccacccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagttagccagcaaattttatgataaaatgaaatattttgccca
gccaaactcagtcgcatcctcggacaatttggtatcaaggaactcacccaaaaacaagcaaagctagaaaaaggttgt
gtggcagccacctaataatgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaacgaggcaaccagcgaaactgagccatcccaaccacatctgtaccaagaaacgggg
ctatatataccgtggtgacccggcaatggggctcctcaactgtagccggcatcctcctctcctccgataatacaata
ccATGAAAGCCTTCACACTCGCTCTCTTTCTTAGCTCTTTTCTCTATCTCTGCCCCAATCCAGCCCCATTCCACTATT
AATGCAGACAATGTTAATGAAAATCAAACGTAGAAAGTAACGTAGTTCAGTAACAATGAAAATAATAAGCAAGT
AACTGAAAAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACCCAAGAAATCAAACGAAAATA
CAGAAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTT
ACCGCTGAAACACCTGTTGATAAAGTAGTAATAATAGTGATCAAAAGACAATAATGCTGCAACTACTGATACTAA
AAAAGATGATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAATAAGATAGTTCAG
TAAAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAAGGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTTGAT
ACTAGCAAGTTAACTAATGATCAAATTAATGAATTAAATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGGTACTCA
GTTAACTTACAACGACTTTAAAAAATTGCTAAACTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCA
ATGCAAGTAAATTAATAATATGCTGCTGCTAAACACTTGATGCTCAAAGTGGAAGTAGAAGATTGGAAATT
TGGGATTGATGGCTGTTCAAGATGCAAAACTGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTAT
GATGGGAGTTCCAAACGTCAATGATAACCACATTTATCTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATT
GGAAGAATGCCGCTCCTATTTTCGGTCTAGGTACTCCAGTTATTCACAATGGTCTGGATCAGCAACTTTAAATAAA
GATGGCTCAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCACCAAAAACCTCGCTAGTGC
AACTGTTTACTTAAATCTTGAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTG
AAGGTGATGGTTACCACTACCAAACCTTATGACCAATGGAAAGAACTAACAAGGGTCTGACAATATCGCAATGCGT
GATGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTTGTTGAAGCAAGTACTGGAACCGAAAATTATCA
AGGTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTTCTTCCAAATTT
TATCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAATTAATGATGATGTT
AAGAATCCAAGTGTGCAAGGTCTACAGCCCACTTATTAGTGCACCAATGGTAAGTGATGAAATTGAACGCCCTGA
TGTGTTAAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGG
TGGCAACTAACAAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATAACTTAACTCATGGTTATGTT
CCATTGAATGAATCTGGCGTTGTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCATACTATGC
AGTTCCAGTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGG
GTATGCATGCAACTTGGGCACCAAGTTTCTTGTTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAATG
ACTAACCAAGGGGATTGGATTGGGATGATAGTAGTGAATCCAGATATGATGGGTGACTTGAAAAAGATGCTCC
AAATAGTGCTGCCCTTCTTGGAATGGGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACCTGAAGCCAC
ACCAACCTGTAACCTCTATTCCAAATGTACCA :CCAACCACACCAGATAAGCCAGAGGTACCA

FIGURE 31 (CONT'D)

ACTACCCCTGAAGTTCCAACCACTCCAGAACTCCAACCTCCAGAAGCTCCAAAGAATCCAGTTAAGAAACTAGTCA
GTCTAAACTTCCAAAGGCTGGAGATAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAGTTCAATATTAGGTG
CTGTTGGTTTAAACAGGTGTTTCAAACGTAACGTAATAATTAAactatgagttgaaacaatggcctatctcatatg
aagatcttttgtgaatttcacttttgtccacgacctctgttgacgactctgctttccgaccggagcatcaccttttg
ttctatatgatttgtgtatgtatgtaggaacctatgttctcgagcatgcatacataattcctcataggtctatatac
accggctatccatattgcaaaacctgtgtaatatattgttatatacaacacgcggaccattgtcttgctgttattaatt
cttttttcccgcaaaaaaggaaaagtttctttatttggcactgcaatggatatgcctcacagctagtgggtggagaa
ttcagtatttgacattaagattccctgatttgcaattgcaaatttcagtttctttacttatatcactacaaaagtct
tattgtttcttttccacgtcattaccatctgctccattgggttttctgtagtagaataggatgaagcatggacacaga
ttaactgagctcgagctcatatgagctcgggtgaacaataaaatctgaaaatacttagaaagaattcaaaattttct
gttttttgtggcaaaatttgacaaatgttataaatgcttgcaaagtttcatcatagaacgacattcgtggatgtcat
ggcaaaaaacaaattcagcactctgaaaataacttttttgaagtatcg

FIGURE 32

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgttttagatcactacttctttacagagaatttccttccctccaagggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccacccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagttagccagcaaattttatgataaaatgaaatattttgccca
gccaaactcagtcgcacccctcggaacaatttggtatcaaggaactcacccaaaaacaagcaaagctagaaaaaggttgt
gtggcagccacctaataatgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaaacgaggcaaccagcgaacgtgagccatcccaaccacatctgtaccaaagaaacgggg
ctatatataccgtggtgacccggcaatggggctcctcaactgtagccggcatcctcctcctccgataatacaata
ccATGAAAGCCTTCACACTCGCTCTCTTCTTAGCTCTTTTCTCTATCTCTGCCCAATCCAGCCCATTCCACTATT
AATGCAGACAATGTTAATGAAAATCAAACGTAGAAAGTAACGTAGTTCAGTAAACAATGAAAATAATAAGCAAGT
AACTGAAAAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACACCAAGAAATCAAACGAAAATA
CAGAAACTACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTT
ACCGCTGAAACACCTGTTGATAAAGTAGTAATAATAGTGATCAAAGACAATAATGCTGCAACTACTGATACTAA
AAAAGATGATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAATGCTGAGGAAAATAAAGATAGTTCAG
TAAAGCCAGCTGAAAATGCTACTAAGGCTGAATTAAAGGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTTGAT
ACTAGCAAGTTAACTAATGATCAAATTAATGAATTAAATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGGTACTCA
GTTAACTTACAACGACTTTAAAAAATTGCTAAACTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCA
ATGCAAGTAAATTAATAATATGCCTGCTGCTAAACACTTGTATGCTCAAAGTGGAAGTAGAAGATTGGAAATT
TGGGATTCTAGGCTGTTCAAGATGCAAAACTGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTAT
GATGGGAGTTCCAAACGTCAATGATAACCACATTATCTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATT
GGAAGAATGCCGCTCTATTTTCGGTCTAGGTACTCCAGTTATTCACAATGGTCTGGATCAGCAACTTTAAATAAA
GATGGCTCAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCAACCAAAAACTCGCTAGTGC
AACTGTTTACTTAAATCTTGAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTG
AAGGTGATGGTTACCACTACCAACTTATGACCAATGGAAAGAACTAACAAGGGTGTGACAATATCGCAATGCGT
GATGCACACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTGAAGCAAGTACTGGAACCGGAAATTATCA
AGGTGATGATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTCTTCCAAATTT
TATCTAACTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAAATTAATGATGATGTT
AAGAATCCAAGTGTGCAAGGTCTACAGCCCACTTATTAGTGACCAATGGTAAGTGATGAAATTGAACGCCCTGA
TGTGTAAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGA
TGGCAACTAACAAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATAACTTAACTCATGGTTATGTT
CCATTGAATGAATCTGGCGTTGTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCATACTATGC
AGTTCAGTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGG
GTATGCATGCAACTTGGGCACCAAGTTTCTTGTTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAATG
ACTAACCAAGGGGATTGGATTGGGATGATAGTAGTGAATCCAGATATGATGGGTGACTTGAAAAAGATGCTCC
AAATAGTGCTGCCCTTCTGGAGAATGGGGAACCAAGTTGATTGGGATTTAATTGGTGGATACAACCTGAAGCCAC
ACCAACCTGTAACCTCTATTCCAAATGTACCA'CCAACCACACCAGATAAGCCAGAGGTACCA

FIGURE 32 (CONT'D)

ACTACCCCTGAAGTTCCAACCACTCCAGAACTCCAACCTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAAGTAGTCA
GTCTAAACTTCCAAAGGCTGGAGATAAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAGTTCAATATTAGGTG
CTGTTGGTTTAAACAGGTGTTTCAAAACGTAAACGTAATAATTAAactatgagttgaacaatggcctatctcatatg
aagatcttttgtgaatttcacttttgtccacgacctctgttgcacgactctgctttccgaccggagcatacctttt
ttctatatgatttgtgtatgtatgtaggaacctatgttctcgagcatgcatacataattcctcataggtctatatac
accggctatccatattgcaaaacctgtgtaattttgttatatacaacacgcgaccattgtcttgcgtgttattaatt
ctttttcccgcaaaaaggaaaagtttctttatttggcactgcaatggatattgctcacagctagtgggtggagaa
ttcagttattgacattaagattccctgatttgcattgcaatttcagtttctttacttatatcactacaaaagtct
tattgtttctttccacgtcattaccatctgctccattggtttttgcctagtagaataaggatgaagcatggacacaga
ttaactgagctcgagctcatatgagctcgggtgaacaataaaatctgaaaatacttagaaagaattcaaaattttct
gttttttgtggcaaaatttgacaaatgttataaatgcttgcaagtttcatcatagaacgacattcgtggatgtcat
ggcaaaaacaaattcagcactctgaaaataactttttgaagtatcggttgggtcttctagattaatcctccaaa
cttttgattaacccaaaaaattatcaaaactaacatgttctcctttttcttttagaaattctaacgaatttatcttta
tactgatttgaatatacttaatttgggtcatttggatgccctttacaacctccttaccaaactcactatggcaaatat
atactattttccattgtaacataaatgtccataatttgaattaaattcggtgcagtagcaaacatccaactttgtc
caaaaacaaatccttataactatttactttaattgtaaatatctctacttttgtttttacaacctagctcaaa
caaatattatttgcgataaaaaatcatatcgaaacaaactcgatgatttttttttcttacgttattaatgaaact
aaaatatagaaaaaacaagatgaaccaaattttcacctatctaactacttaaatataatatgattaaatttggtaa
agtttgaaaagtttcttttaggaatgtgaaatattgatcacagtttctatttgctaaaaatcaccaacaaaacgcaggt
cgccattcataattatgggtttcacacctacaactaggctaataagtaataagtagacaactagactcagggttgaa
aaaaccataaaaagccatatagcgttttctcattgaaactgcgaacacgatcgtgtgaatgttgcagtttctagttt
gatacaaaacaaacaaaaacacaatttaattcttagattaaaaagaaaaagagaacggagcccactagccactccttc
aaacgtgtctttaccaactctcttctagaaacaaattagggttcaccttcctcttccaacctctctctctctctct
ctctctttctcaaacctctctccataaagccctaatttcttcatcacagaatcagaagaatactgcaaaaaactt
ATGGACCTGCATCTAATTTTCGGTCCAACCTGCACAGGAAAGACGACGACCGCGATAGCTCTTGCCACGACAGG
GCTTCCAGTCCTTTCGCTTGATCGGGTCCAATGCTGTCTCAACTATCAACCGGAAGCGGACGACCAACAGTGGAAG
AACTGAAAGGAACGACGCGTCTCTACCTTGATGATCGGCCCTCTGGTGGAGGGTATCATCGCAGCCAAGCAAGCTCAT
CATAGGCTGATCGAGGAGGTGTATAATCATGAGGCCAACGGCGGGCTTATTCTTGAGGGAGGATCCACCTCGTTGCT
CAACTGCATGGCGCGAAACAGCTATTGGAGTGCAGATTTTCGTTGGCATATTATTCGCCACAAGTTACCCGACCAAG
AGACCTTCATGAAAGCGGCCAAGGCCAGAGTTAAGCAGATGTTGCACCCCGCTGCAGGCCATTCTATTATCAAGAG
TTGGTTTATCTTTGGAATGAACCTCGGCTGAGGCCATTCTGAAAGAGATCGATGGATATCGATATGCCATGTTGTT
TGCTAGCCAGAACCAGATCACGGCAGATATGCTATTGCAGCTTGACGCAATATGGAAGGTAAGTTGATTAATGGGA
TCGCTCAGGAGTATTTCATCCATGCGCGCCAACAGGAACAGAAATCCCCCAAGTTAACGCAGCCGCTTTCGACGGA
TTCGAAGGTCATCCGTTTCGGAATGTATTAGGTAAGtccgcaaaaatcaccagtcctctctctacaaatctatctctct
ctatttttctccagaataatgtgtgagtagttcccagataaggggaattaggggttcttataggggttcgctcatgtgt
tgagcatataagaaaccttagtatgtatttgtatttgtaaaatacttctatcaataaaatttctaattcctaaaaac
caaatccagtgacct

FIGURE 33

GATCCGGTGA CTCAAAAAAGAAGAGCCGCCATCTGTCCAAGCGCCACTCCTACGAGAACTAAAATCCTATTCCCTCC
GTAAATAAATATAAGAGTGTTTAGATCACTACTTCTTTACAGAGAATTTCTTCCCTCCAAGGGGAGGCGAATCCAT
AGGCACATCGACGGATATGGAGGGGGGAAACATATATTTTACTATGCTAGTTCAGTTAATTCTACCAAGAAAACATA
TATTTTATTTTGACAAACATTGTATAAATGTAGACATTACATACACGTATGTACACCACCCTCTATGATTGCACAC
CCGCACACTATATGCCTATGAGCATACTTTCAAGAGTGAGCCAGCAAATTTTATGATAAAATGAAATATTTTGCCCA
GCCAACTCAGTCGCATCCTCGGACAATTTGTTATCAAGGAACCTACCCAAAAACAAGCAAAGCTAGAAAAAGGTTGT
GTGGCAGCCACCTAATGACATGAAGGACTGAAATTTCCAGCACACACAATGTATCCGACGGCAATGCTTCTTCCACT
GATCCGGAGAAGATAAGGAAACGAGGCAACCAGGAACGTGAGCCATCCCAACCACATCTGTACCAAAGAAACGGGG
CTATATATACCGTGGTGACCCGGCAATGGGGTCTTCAACTGTAGCCGGCATCCTCCTCTCCTCCGATAATACAAATA
CCATGGAGTCCCCAAGCGCGTCTCCCCGGCACCACGGCGCGCTGCTTCTTATGCGTACGCGCGCTGCCGTCTG
TCCGCCGACGACGCGCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCTGCGCCGCGTCTGGCCGCATCGGCGTT
GGCGGTGGTCTGCTGGTCTGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGCGCGGCGGAACGAACCAAAAGC
CATATAAGGAAACATACGGCATTTCCTCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAATGAA
AAATATCAAGTTCCTGAATTCGATTCTGTCCACAATTAATAATATCTCTTCTGCAAAAGGCTGGACGTTTGGGACAG
CTGGCCATTACAAAACGCTGACGGCACTGTGCAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCGGAGATC
CTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAACTTCTATTGACAGCTGGAAAAAC
GCTGGCCGCTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTTCTATCCTAAAAGACCAACACAAGAATGGTC
AGGTTTCAGCCACATTTACATCTGACGGAATAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTACGGCAAAC
AAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATTATAAA
TCAATCTTTGACGGTGACGGAATAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGA
CAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAA
CTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAAACAAAGCATACTATGGCAAAAGCACATCTTCTCCGTCAA
GAAAGTCAAAAACCTTCTGCAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATTGAGCT
AAACGATGATTACACACTGAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCG
CGAACGTCTTTAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCATT
ACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCCTTAACTGGCCCATACAAGCCGCTGAACAAAACCTGG
CCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAGCGAAAG
GAAACAATGTCGTGATTACAAGCTATATGACAAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGCCAAGC
TTCCTGCTGAACATCAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAA
CAAATAAactatgagttgaaacaatggcctatctcatatgaagatcttttgtgaatttcacttttgtccacgacctc
tggtgcacgactctgctttccgaccggagcatatcttttgttctatgatgttgtgtatgtatgtaggaacctatgt
tctcgagcatgcatacataattcctcataggtctatatacaccggctatccatatgcaaaacctgtgtaatatgtt
tatatacaacacgcgaccattgtcttctgtgttattaattcttttttcccgcaaaaaggaaaagtttctttatttg
gcaactgcaatggatgatgcctcacagctagtgggtggagaattcagtatgttgacattaagattccctgatttgcaatt
gcaaatctcagtttctttacttatatcactacaaaagtcttattgtttcttttccacgtcattaccatctgtctcat
tggtttttgctagtagaataggatgaagcatggacacagattaactgagctcgagctcatatgagctcgggtgaaca
ataaaaatctgaaaatacttagaaagaattcaa; gtggcaaaatttgacaaatgttataaatgc

FIGURE 33 (CONT'D)

ttgcaaagtttcatcatagaacgacattcgtggatgtcatggcaaaaaacaaattcagcactctgaaaataactttt
ttgaagtatcg

FIGURE 34

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtataataatataagagtgttttagatcactacttctttacagagaatttccttccctccaaggggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccaccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagttagccagcaaattttatgataaaatgaaatattttgccca
gccaaactcagtcgcatcctcggacaatttggttatcaaggaactcacccaaaaacaagcaaagctagaaaaagggtgt
gtggcagccacctaattgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccgggagaagataaggaaacgaggcaaccagcgaacgtgagccatcccaaccacatctgtaccaagaacgggg
ctatatataccgtggtgacccggcaatggggctcctcaactgtagccggcatcctcctctcctccgataatacaata
ccATGGAGTCCCAAGCGCGTCTCCCCGGCACACGGCGCGCTGCTTCCTTATGCGTACGCGCGCTGCCGTCG
TCCGCGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCTGCGCGCGCTGCTGGCCGCATCGGCGTT
GGCGGTGGTCTGCTGGTCTGGGCTCCTCGCGGGCGGAGGGTGGATCGGGTCCCGCGCGCGGAACGAACCAAAAGC
CATATAAGGAAACATACGGCATTTCCTCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAATGAA
AAATATCAAGTTCCTGAATTCGATTTCGTCCACAATTAAAAATATCTTCTGCAAAGGCCTGGACGTTTGGGACAG
CTGGCCATTACAAAACGCTGACGGCACTGTGCGAACTATCACGGTACACATCGTCTTTGCATTAGCCGGAGATC
CTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAACTTCTATTGACAGCTGGAAAAAC
GCTGGCCGCTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCAACACAAGAATGGTC
AGGTTTCAGCCACATTTACATCTGACGGAATAATCCGTTTATTCTACACTGATTTCTCCGTAACATTACGGCAAAAC
AAACACTGACAACCTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATTATAAA
TCAATCTTTGACGGTGACGGAATAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGA
CAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACTGGAA
CTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAAACAAAGCATACTATGGCAAAAGCACATATTCTCCGTCAA
GAAAGTCAAAAACCTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATTGAGCT
AAACGATGATTACACACTGAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCG
CGAACGTCTTTAAATGAACGGCAAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAAAGACGATTGACGGCATT
ACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACAAAACCTGG
CCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAGCGAAAG
GAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGGCCCAAGC
TTCCTGCTGAACATCAAAGGCAAGAAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAA
CAAATAAactatgagttgaaacaatggcctatctcatatgaagatcttttgtgaatttcacttttgtccacgacctc
tggtgcaagactctgctttccgaccggagcataccctttgttctatatgatttgtgtatgtatgtaggaacctatgt
tctcgagcatgcatacataattcctcataggtctatatacaccggctatccatgcaaaacctgtgtaatatattgt
tatatacaacacgaggaccattgtcttctgtgttattaattctttttcccgcaaaaaggaaagtttctttatttg
gcactgcaatggatatgcctcacagctagtgggtggagaattcagtatatttgacattaagattccctgatttgcaatt
gcaaatttcagtttctttacttatatcactacaaaagtcttattgttttctttccacgtcattaccatctgtccat
tggtttttgctagtagaataggatgaagcatgacacacatttaactgagctcgagctcatatgagctcgggtgaaca
ataaaatctgaaaatacttagaagaattcaa :gtggcaaaatttgacaaatgttataaatgc

FIGURE 34 (CONT'D)

ttgcaaagtttcatcatagaacgacatttcgtggatgtcatggcaaaaaacaaattcagcactctgaaaataactttt
ttgaagtatcggtttgtgtcttctagattaatcctccaaacttttgattaaccaaaaaattatcaaactaacatgt
tctccttttttctttagaaattctaacgaatttatctttatactgatttgaatatacttaatttggtcatttggatg
ccctttacaacctccttaccaaactcactatggcaaatatatactattttcattgtaacataaatgtccataattt
gaattaaattcgttgcagtagcgaaccatccaactttgtccaaaaacaaatccttataactatttactttaatgta
aatatatcctctacttttgtttttacaacctagctcaaacaaatttattatttgcgataaaaaatcatatcgaaca
aactcgatgatttttttttcttacgattattaatgaaactaaaatatagaaaaaacagatgaaccaaattttcac
ctatctaactactttaatatataatgattaaatttggtaaagtttgaaaagtttcttttaggaaatgtgaaatattga
tcacagtttctattgtctaaaaatcaccaacaaaacgcagtgctgccatttcataattatggtttcacacctacaactagg
ctaataagtaaaataagtagacaactagactcaggtttgaaaaaacataaaagccatatagcgttttctcattgaaa
ctggaacacgacgtgtgtgaatgttgcagtttctagttttgatacaaaacaaaacacaatttaactcttagatt
aaaaagaaaaagagaacggagcccactagccactccttcaaacgtgtctttaccaactctctctctagaacaaatta
ggcttcaccttctcttccaacctctctctctctctctctctctctctcttctcaaacctctctccataaagcccta
ttcttcatcacaagaatcagaagaatactgcaaaaaacttATGGACCTGCATCTAATTTTCGGTCCAACCTGCACAG
GAAAGACGACGACCGCGATAGCTCTTGCCAGCAGACAGGGCTTCAGTCTCTTCGCTTGATCGGGTCCAATGCTGT
CCTCAACTATCAACCGGAAGCGGACGACCAACAGTGGAAGAACTGAAAGGAACGACGCGTCTCTACCTTGATGATCG
GCCTCTGGTGGAGGGTATCATCGCAGCCAAGCAAGCTCATCATAGGCTGATCGAGGAGGTGTATAATCATGAGGCCA
ACGGCGGGCTTATCTTGAGGGAGGATCCACCTCGTTGCTCAACTGCATGGCGCGAAACAGCTATTGGAGTGCAGAT
TTTCGTTGGCATATTATTGCGCCACAAGTTACCCGACCAAGAGACCTTCATGAAAGCGGCCAAGGCCAGAGTTAAGCA
GATGTTGCACCCCGCTGCAGGCCATTCTATTATTCAAGAGTTGGTTTATCTTTGGAATGAACCTCGGCTGAGGCCCA
TTCTGAAAGAGATCGATGGATATCGATATGCCATGTTGTTTGCTAGCCAGAACCAGATCACGGCAGATATGCTATTG
CAGCTTGACGCAAAATATGGAAGGTAAGTTGATTAATGGGATCGCTCAGGAGTATTTTCATCCATGCGCGCCAACAGGA
ACAGAAATCCCCCAAGTTAACGCAGCCGCTTCGACGGATTGGAAGGTCATCCGTTCCGAATGTATTAGGTAagtc
cgcaaaaatcaccagttctctctctacaaatctatctctctctattttctccagaataatgtgtgagtagttccag
ataaggggaattagggttcttatagggtttcgctcatgtgttgagcatataagaaacccttagtatgtatttgtattt
gtaaaatacttctatcaataaaaatttctaattcctaaaacaaaatccagtgcct

FIGURE 35

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgttagatcactacttctttacagagaatttccttccctccaaggggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccaccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagtgtgagccagcaaattttatgataaaatgaaatattttgccc
gccaactcagtcgcatcctcggacaatttggtatcaaggaactcacccaaaaacaagcaaagctagaaaaagggtgt
gtggcagccacctaataatgacatgaaggactgaaatttccagcacacacaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaaacgaggcaaccagcgaacgtgagccatcccaaccacatctgtaccaagaaacgggg
ctatatataccggtgacccggcaatggggctcctcaactgtagccgcatcctcctctcctccgataatacaata
cc**ATGGAGTCCCCAAGCGCCGTCGTCCCCGGCACACGGCGCCGCTGCTTCCTTATGCGTACGGCCGCTGCCGTG**
TCCGCCGACGACGCCCGTCAAAACCGGAGTGGCGGGAGGTGGCGCGCTGCGCCGCCGTGCTGGCCGCATCGGCGTT
GGCGGTGGTTCGTGCTGGTGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGGCCGGCGGAACTATTAATGCAG
ACAATGTTAATGAAAAATAAACTGTAGAAGTAAGTGTAGTTTCACTAAACAATGAAAAATAAAGCAAGTAAGTAA
AAAGATAGTGCAGATAAAAGTACTAGTGTGTGGCTGAAGATGCTAACACCAAGAAATCAAACGAAAATACAGAAAC
TACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTTACCGCTG
AAACACCTGTTGATAAAGTAGTAATAATAGTGATCAAAGACAATAATGCTGCAACTACTGATACTAAAAAAGAT
GATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAATAAGATAGTTTCACTAAAGCC
AGCTGAAAATGCTACTAAGGCTGAATTAAGGGCCAGTTAAAGATATCGTTGAAGAATCTGGTGTGATACTAGCA
AGTTAACTAATGATCAAATTAATGAATTAATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGGTACTCAGTTAACT
TACAACGACTTTAAAAAATTGCTAAACTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCAATGCAAG
TAAATTAATAAATATGCCTGCTGCTAAACACTTGATGCTCAAAGTGGAAGTAGAAGATTGGAAATTTGGGATT
CATGGCTGTTCAAGATGCAAAAACGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTATGATGGGA
GTTCCAAACGTCAATGATAACCACATTTATCTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATTGGAAGAA
TGCCGGTCTTATTTTCGGTCTAGGTACTCCAGTTATTAACAATGGTCTGGATCAGCAACTTTAAATAAAGATGGCT
CAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCACCAAAAACTCGCTAGTGCAACTGTT
TACTTAAATCTTGAAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTGAAGGTGA
TGTTTACCACTACCAAACTTATGACCAATGGAAGAACTAACAAGGTGCTGACAATATCGCAATGCGTGATGCAC
ACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTGAAGCAAGTACTGGAACCGAAAATTATCAAGGTGAT
GATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAATTTAGGTGATTTCTTCCAAATTTTATCTAA
CTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAATTAATGATGATGTTAAGAAATC
CAAGTGTGCAAGGTCTACAGCCACTTATTAGTGACCAATGGTAAGTGATGAAATTGAACGCCCTGATGTTGTT
AAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGGATGGCAAC
TAACAAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATACTTAACCTCATGGTTATGTTCCATTGA
ATGAATCTGGCGTTGTTTAACTGCATCTGTACCGCTAACTGGCGTACTGCAACTTATTCATACTATGCAGTTCCA
GTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGGGTATGCA
TGCAACTTGGGCACCAAGTTTCTTGTGTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAATGACTAACC
AAGGGGATTGGATTGGGATGATAGTAGTA**3GGTGTACTTGAAAAAGATGCTCCAAATAGT**

FIGURE 35A (CONT'D)

GCTGCCCTTCCTGGAGAATGGGGAAAACCAGTTGATTGGGATTTAATTGGTGGATACAACTTGAAGCCACACCAACC
TGTAACCTCTATTCCAAATGTACCAACTACTCCTGAAACCCCAACCACACCAGATAAGCCAGAGGTACCAACTACCC
CTGAAGTTCCAACCACTCCAGAACTCCAACCTCCAGAAGCTCCAAAGAATCCAGTTAAGAAACTAGTCAGTCTAAA
CTTCCAAAGGCTGGAGATAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAGTTCAATATTAGGTGCTGTTGG
TTTAACAGGTGTTTCAAAACGTAAACGTAATAATTAAactatgagttgaaacaatggcctatctcatatgaagatct
tttgtgaatttcactttttgtccacgacctctgttgacgactctgctttccgaccggagcataccttttgttctata
tgatttgtgtatgtatgttaggaacctatgttctcgagcatgcatacataattcctcataggtctatatacacgggt
atccatatgcaaaacctgtgtaatatattgttatatacaacacgcgaccattgtcttgctgttattaattctttttt
cccgcaaaaaaggaaaagtttctttatttggcactgcaatggatatgcctcacagctagtgggtggagaattcagta
tttgacattaagattccctgatttgcaattgcaatttcagtttctttacttatatcactacaaaagtcttattgtt
tcttttccacgtcattaccatctgctccattgggttttctagtagaataaggatgaagcatggacacagattaactg
agctcgagctcatatgagctcgggtgaacaataaaatctgaaaatacttagaaaagaattcaaaattttctgtttttt
gtggcaaaatttgacaaatgttataaatgcttgcaaagtttcatcatagaacgacattcgtggatgtcatggcaaaa
aaciaaattcagcactctgaaaataacttttttgaagtatcg

FIGURE 36

gatccggtgactcaaaaaagaagagccgccatctgtccaagcgccactcctacgagaactaaaatcctattccctcc
gtaaataaatataagagtgttagatcactacttctttacagagaatttccttccctccaagggaggcgaatccat
aggcacatcgacggatatggaggggggaaacatatattttactatgctagttcagttaattctaccaagaaaacata
tattttattttgacaaacattgtataaatgtagacattcacatacacgtatgtacaccaccctctatgattgcacac
ccgcacactatatgcctatgagcatactttcaagagtgagccagcaaattttatgataaaatgaaatattttgccc
gccaactcagtcgcatcctcggacaatttggtatcaaggaactcacccaaaaacaagcaaagctagaaaaagggtgt
gtggcagccacctaataatgacatgaaggactgaaatttccagcacacacaaatgtatccgacggcaatgcttcttccact
gatccggagaagataaggaaacgaggcaaccagcgaacgtgagccatcccaaccacatctgtaccaagaaacgggg
ctatatataccgtggtgacccggcaatggggctcctcaactgtagccggcatcctcctctcctccgataatacaata
c**ATGGAGTCCCCAAGCGCCGTCGTCCCCGGCACACGGCGCCGCTGCTTCCTTATGCGTACGCGCCGCTGCCGTCG**
TCCGCCGACGACGCCCGTCAAAACCGAGTGGCGGGAGGTGGCGCGCTGCGCCGCGTGGCGCGCATGGCGTT
GGCGGTGGTCTGTCGTGGTGGGCTCCTCGCGGGCGGCAGGGTGGATCGGGTCCCGGCCGGCGGAACTATTAATGCAG
ACAATGTTAATGAAAATCAAACCTGTAGAAGTAAGTGTAGTTTCAAGTAAACAATGAAAATAATAAGCAAGTAAGTAA
AAAGATAGTGCAGATAAAAGTACTAGTGATGTGGCTGAAGATGCTAACCAAGAATCAAACGAAAATACAGAAAC
TACAGAAAAGAATACTCAAACAGTTGTTACTAATGCGCCAGTAAGTGATGTGAAAAATACAAACACAGTTACCGCTG
AAACACCTGTTGATAAAGTAGTAAATAATAGTGATCAAAGACAATAATGCTGCAACTACTGATACTAAAAAAGAT
GATGTAAACAAGTTGAAAAGAAAGACTCAGTAGATAAAACAAATGCTGAGGAAAATAAAGATAGTTTCAAGTAAAGCC
AGCTGAAAATGCTACTAAGGCTGAATTAAAGGGCCAAGTTAAAGATATCGTTGAAGAATCTGGTGTTGATACTAGCA
AGTTAACTAATGATCAAATTAATGAATTAAATAAAATTAATTTCTCCAAAGAAGCAAAAAGTGGTACTCAGTTAACT
TACAACGACTTTAAAAAATTGCTAAACCTTTAATTGAACAAGATGCTCGTTATGCTATTCCATTCTTCAATGCAAG
TAAATTAATAAATATGCCTGCTGCTAAACACTTGATGCTCAAAGTGGAAGTAGAAGATTTGGAATTTGGGATT
CATGGCTGTTCAAGATGCAAAAACGGTTACGTATCTAACTGGAATGGCTACCAATTAGTGATTGGTATGATGGGA
GTTCCAAACGTCAATGATAACCACATTTATCTTCTTTACAACAAGTATGGTGATAATGACTTTAATCATTTGAAGAA
TGCCGGTCTCTATTTTCGGTCTAGGTACTCCAGTTATTCAACAATGGTCTGGATCAGCAACTTTAAATAAAGATGGCT
CAATTCAACTTTACTACACTAAGGTTGATACTAGTGATAATAATACTAACCAACCAAAAACCTCGTAGTGCAACTGTT
TACTTAAATCTTGAAAAGATCAAGATAAGATTTCTATTGCTCATGTTGACAACGACCATATTGTCTTTGAAGGTGA
TGTTTACCCTACCAACTTATGACCAATGGAAAGAACTAACAAGGGTCTGACAATATCGCAATGCGTGATGCAC
ACGTGATTGATGATGATAATGGTAATCGTTACCTTGTGTTGAAGCAAGTACTGGAACCGAAAATTATCAAGGTGAT
GATCAAATTTATCAATGGTTAAATTACGGCGGTACTAACAAGGATAAATTTAGGTGATTTCTTCCAAATTTTATCTAA
CTCCGATATTAAAGATAGAGCTAAATGGTCAAACGCTGCAATTGGTATCATTAATTAATGATGATGTTAAGAATC
CAAGTGTTGCAAGGTCTACAGCCCACTTATTAGTGACCAATGGTAAGTGATGAAATGAACGCCCTGATGTTGTT
AAATTAGGTAATAAGTATTACTTATTTGCTGCTACTAGATTAAACCGTGGTAGTAACGATGATGCTTGGATGGCAAC
TAACAAGCAGTTGGTGATAACGTAGCTATGATTGGTTATGTTTCTGATAACTTAACCTCATGGTTATGTTCCATTGA
ATGAATCTGGCGTTGTTTAACTGCATCTGTACCGGCTAACTGGCGTACTGCAACTTATTCATACTATGCAGTTCCA
GTAGAAGGAAGAGATGATCAACTTTTAATTACTTCATACATCACTAATCGTGGTGAGGTTGCTGGAAAGGGTATGCA
TGCAACTTGGGCACCAAGTTTCTTGTACAAATTAATCCAGATAACACTACTACTGTTTTAGCTAAAATGACTAACC
AAGGGGATTGGATTGGGATGATAGTAGTAA

GGTGTAAGTGAAGAAAGATGCTCCAAATAGT

FIGURE 36 (CONT'D)

GCTGCCCTTCCTGGAGAATGGGGAAAACAGTTGATTGGGATTTAATTGGTGGATACAACCTGAAGCCACACCAACC
 TGTAACTCCTATTCCAAATGTACCAACTACTCCTGAAACCCCAACCACACCAGATAAGCCAGAGGTACCAACTACCC
 CTGAAGTTCCAACCACTCCAGAACTCCAACCTCCAGAAGCTCCAAAGAATCCAGTTAAGAAAAGTAGTCAGTCTAAA
 CTTCCAAAGGCTGGAGATAAAAATAGCTTTGCAGCAGTTGTTTTAGGTGCTGTAAGTTCAATATTAGGTGCTGTTGG
 TTTAACAGGTGTTTCAAAACGTAAACGTAATAATTAAactatgagbttgaaacaatggcctatctcatatgaagatct
 tttgtgaatttcactttttgtccacgacctctgttgcacgactctgctttccgaccggagcatacctttttgttctata
 tgattttgtatgtatgttagaacctatgttctcgagcatgcatacataattcctcataggtctatatacaccggct
 atccatatgcaaaacctgtgtaatatgtttatatacaacacgcgaccattgtcttgcgtgttattaattctttttt
 cccgcaaaaaaggaaaagtttctttatgttgacgtgcaatggatagcctcacagctagtgggtggagaattcagta
 tttgacattaagattccctgatttgcaattgcaatttcagtttctttacttatatcactacaaaagtcttattgtt
 tcttttccacgtcattaccatctgctccattgggttttgcctagtagaataaggatgaagcatggacacagattaactg
 agctcgagctcatatgagctcgggtgaacaataaaatctgaaaatacttagaagaattcaaaatttctgtttttt
 gtggcaaaatttgacaaatgttataaatgcttgcaaatgttcatcatagaacgacattcgtggatgtcatggcaaaa
 acaaaattcagcactctgaaaataactttttgaagtatcggtttgtgtcttctagattaatcctccaaacttttga
ttaaccacaaaaaattatcaaaactaacatgttctccttttttctttagaatttctaacgaatttatctttatactgat
ttgaatatacttaatttggtcatttggtgacctttacaacctccttaccacaaactcactatggcaaatatatactat
tttccattgtaacataaatgtccataatttgaattaaattcggttgacgtacgaaacctccaactttgtccaaaaac
aaaatccttataactatttacttttaattgtaaataatcctctacttttgtttttacaacctagctcaaacaaattt
attatttgcgataaaaaatcatatcgacaaactcgatgatttttttttcttacgttatttaataaaactaaaatat
agaaaaaacagaatgaaccaaattttcacctatctaactacttaaatataatatgattaaatttggtaaagtgtga
aaagtgttctttaggaattgtgaaatattgatcacagtttctattgctaaaatcaccaacaaaacgcgatgtcgccatt
cataattatgggtttcacacctacaactaggctaataagtaaataagtagacaactagactcagggttgaaaaacca
taaaagccatatagcgtttttctcattgaaactgcgaacacgatcgtgtgaatgttgacgtttctagttttgatacaa
acaaacaaaaacacaatttaattcttagattaaaaagaaaaagagaacggagcccactagccactccttcaaacgtg
tcttaccacactctctcttagaacaatttaggtttcaccttccctcttccaaacctctctctctctctctctctt
tctcaaacctctctccataaaagccctaatttcttcatcacaagaatcagaagaatactgcaaaaaacttATGGACC
TGCATCTAATTTTCGGTCCAACCTGCACAGGAAAGACGACGACCGCGATAGCTCTTGCCACGACAGGGCTTCCA
GTCCTTTCGCTTGATCGGGTCCAATGCTGCTCCTCAACTATCAACCGGAAGCGGACGACCAACAGTGGAAGAACTGAA
AGGAACGACGCGTCTCTACCTTGATGATCGGCCTCTGGTGGAGGGTATCATCGCAGCCAAGCAAGCTCATCATAGGC
TGATCGAGGAGGTGTATAATCATGAGGCCAACGGCGGGCTTATTCTTGAGGGAGGATCCACCTCGTTGCTCAACTGC
ATGGCGCGAAACAGCTATTGGAGTGCAGATTTTCGTTGGCATATTATTGCCACAAGTTACCCGACCAAGAGACCTT
CATGAAAGCGGCCAAGGCCAGAGTTAAGCAGATGTTGCACCCCGCTGCAGGCCATTCTATTATTCAAGAGTTGGTTT
ATCTTTGGAATGAACCTCGGCTGAGGCCATTCTGAAAGAGATCGATGGATATCGATATGCCATGTTGTTTGCTAGC
CAGAACCAGATCACGGCAGATATGCTATTGCAGCTTGACGCAAAATATGGAAGGTAAGTTGATTAATGGGATCGCTCA
GGAGTATTTTCATCCATGCGCGCCAACAGGAACAGAAATCCCCCAAGTTAACGCAGCCGCTTTTCGACGGATTCCAAG
GTCATCCGTTTCGGAATGTATTAGGTAAGtcggcaaaatcaccagtctctctctacaaatctatctctctctat
tctccagaataatgtgtgagtagttccagat :cttatagggtttcgctcatgtgttgagcat

FIGURE 36 (CONT'D)

ataagaaacccttagtatgtatttgtaaaatacttctatcaataaaatttctaattcctaaaaccaaatac
cagtgcct

FIGURE 37

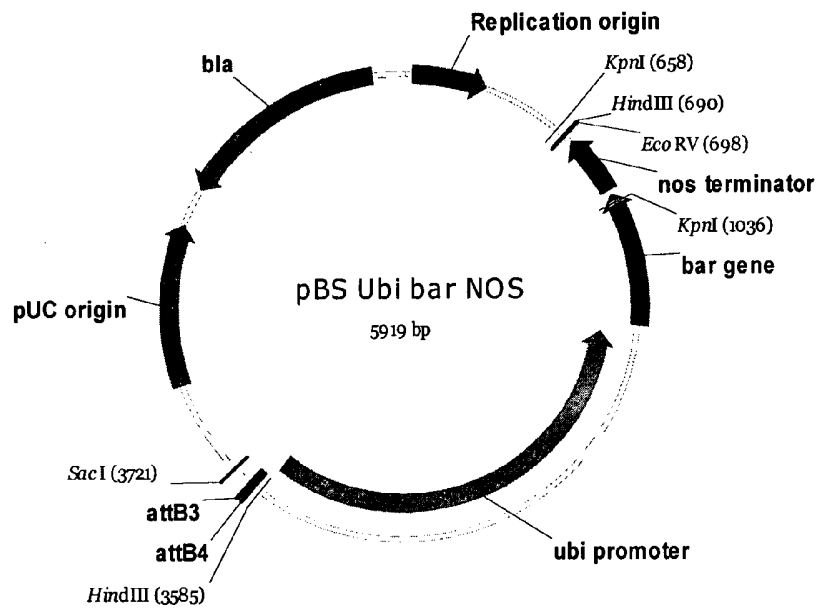
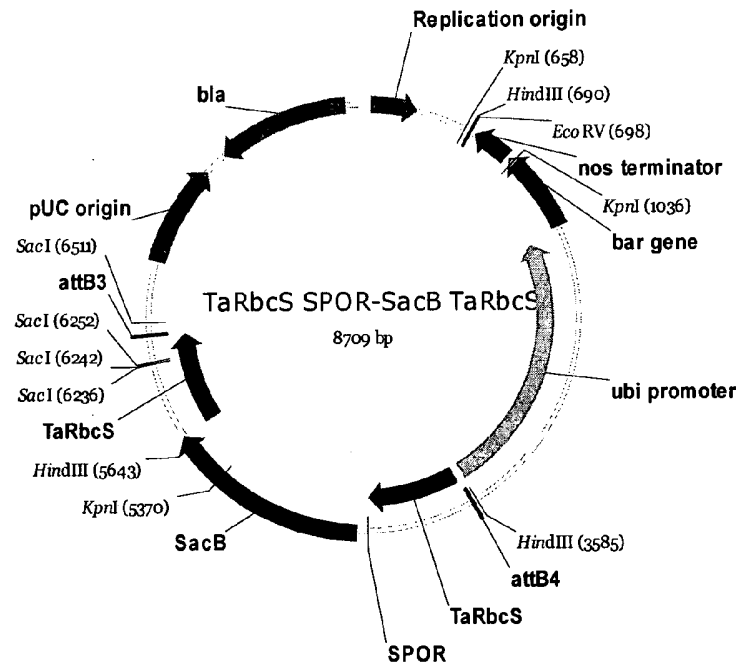


FIGURE 38

A



B

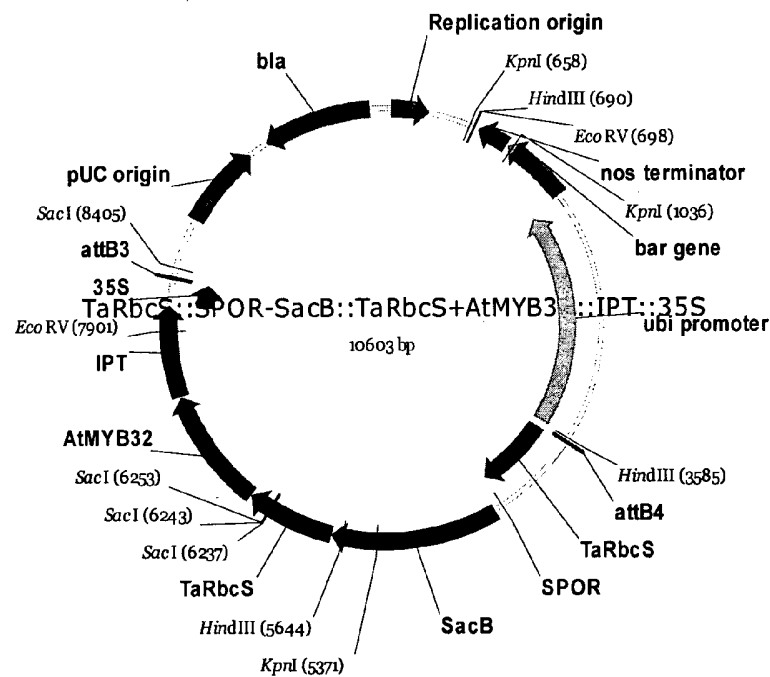
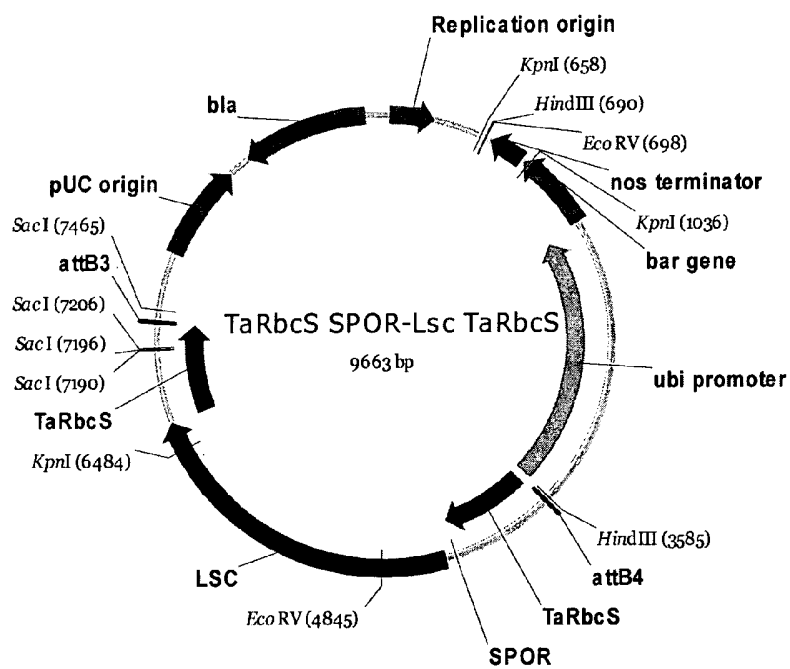


FIGURE 39

A



B

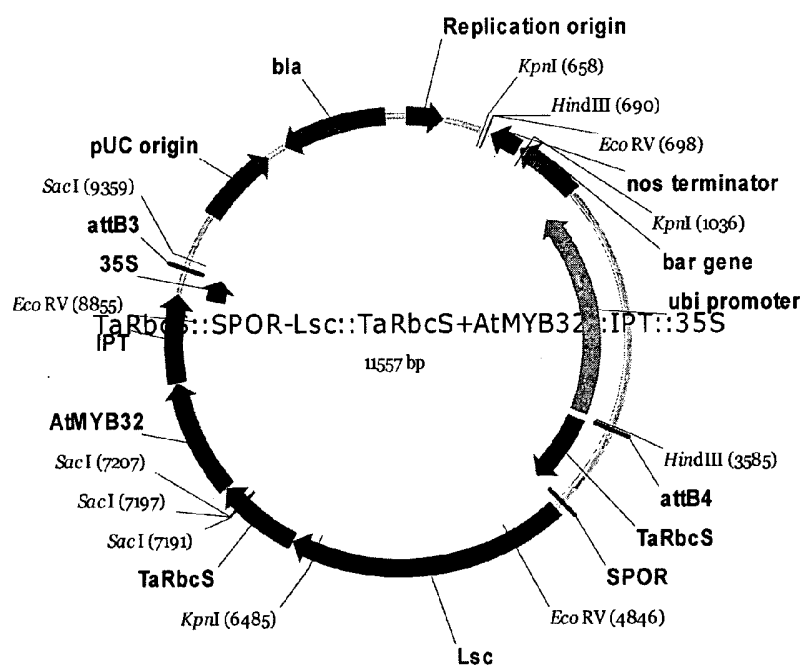
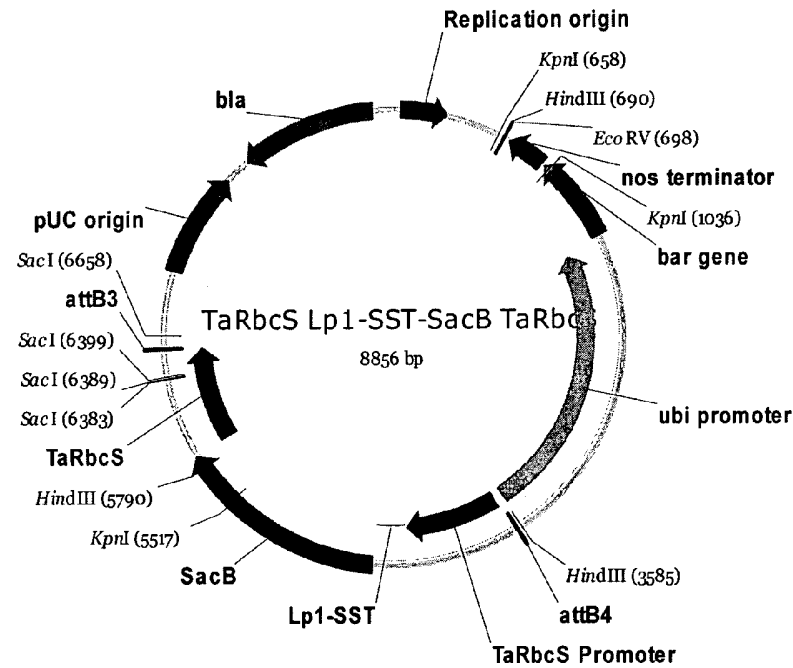


FIGURE 40

A



B

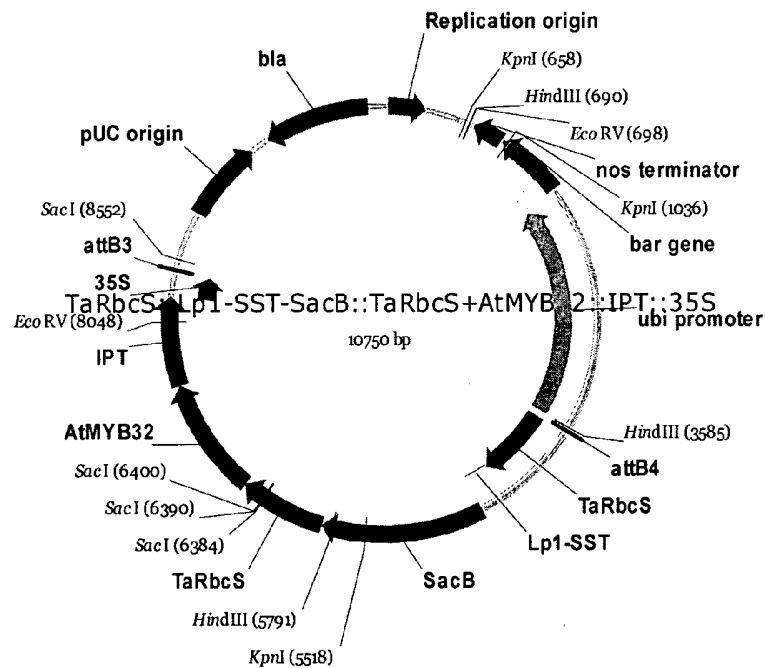
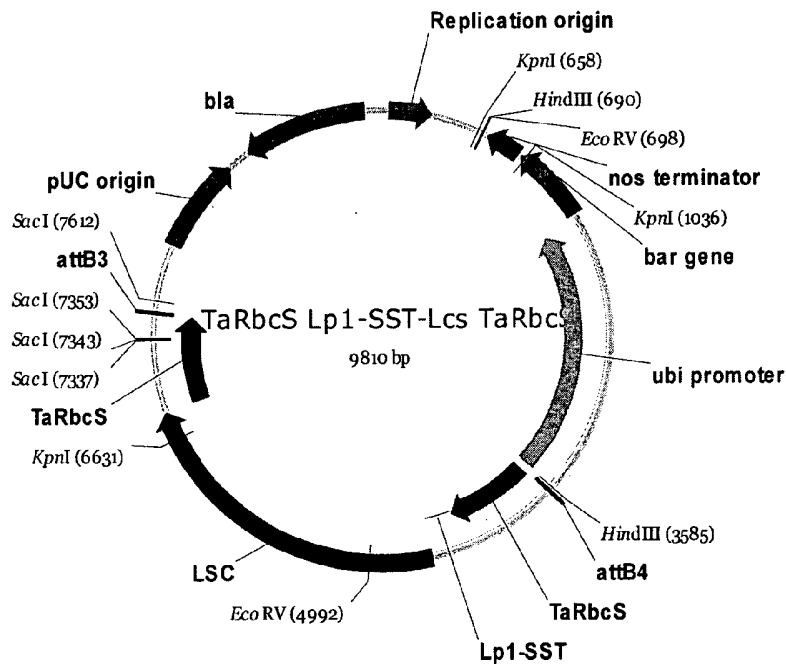


FIGURE 41

A



B

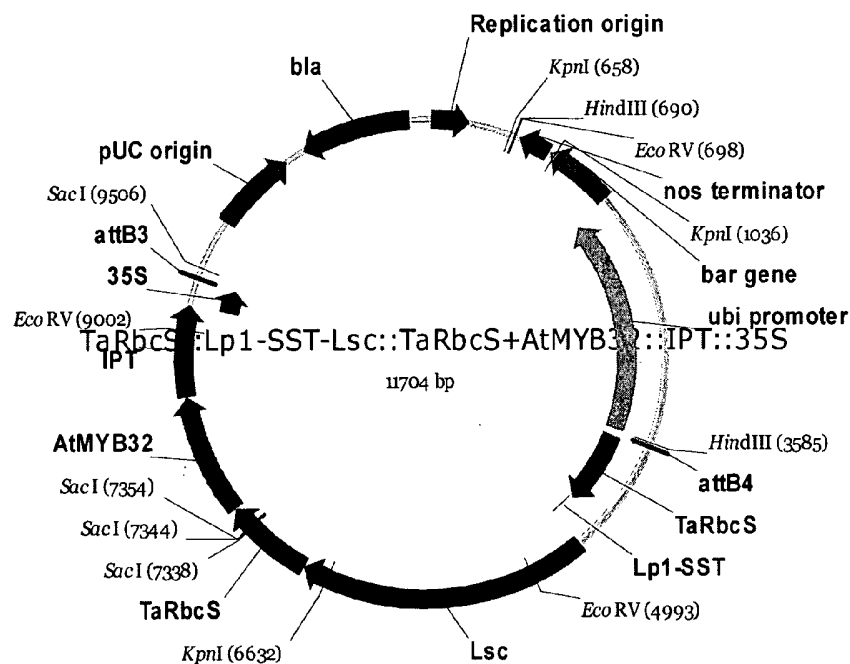


FIGURE 42

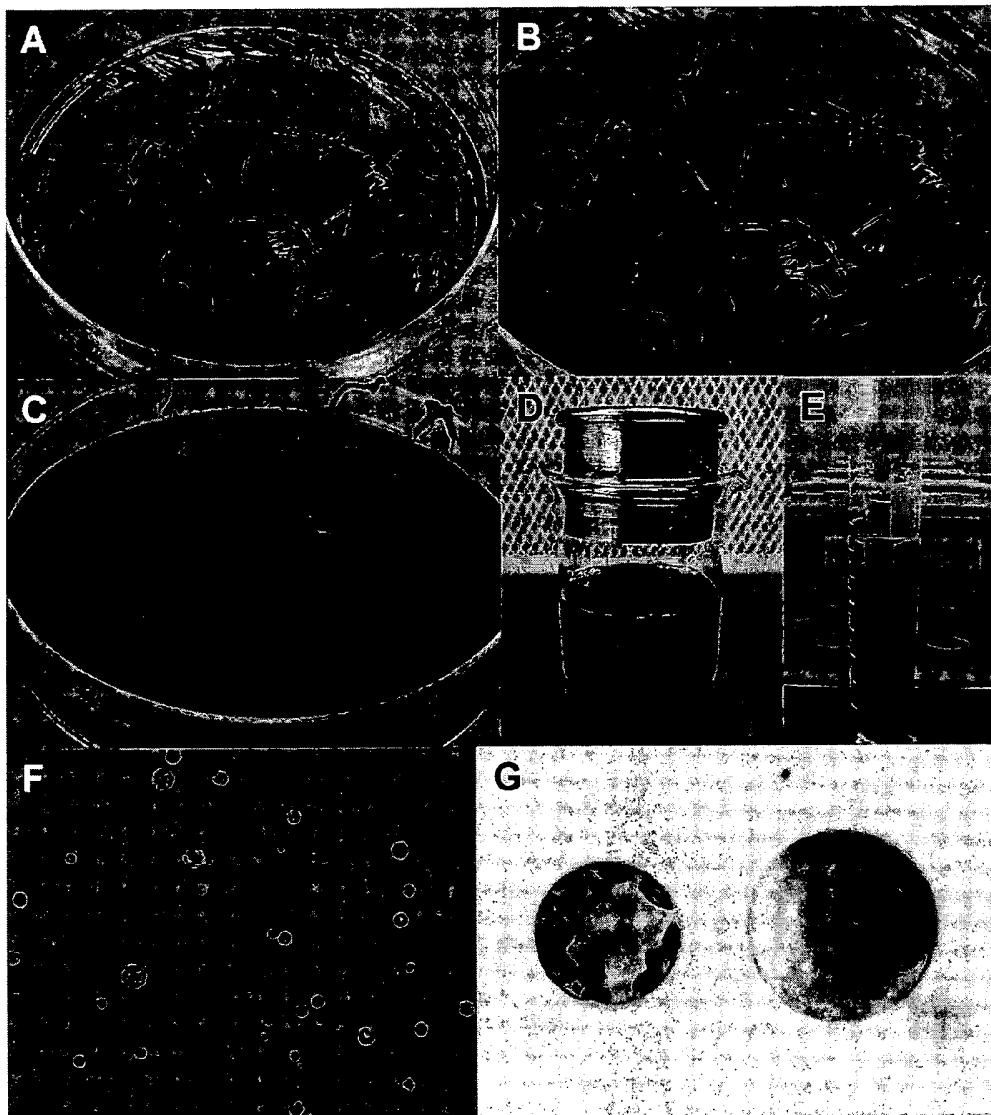


FIGURE 43

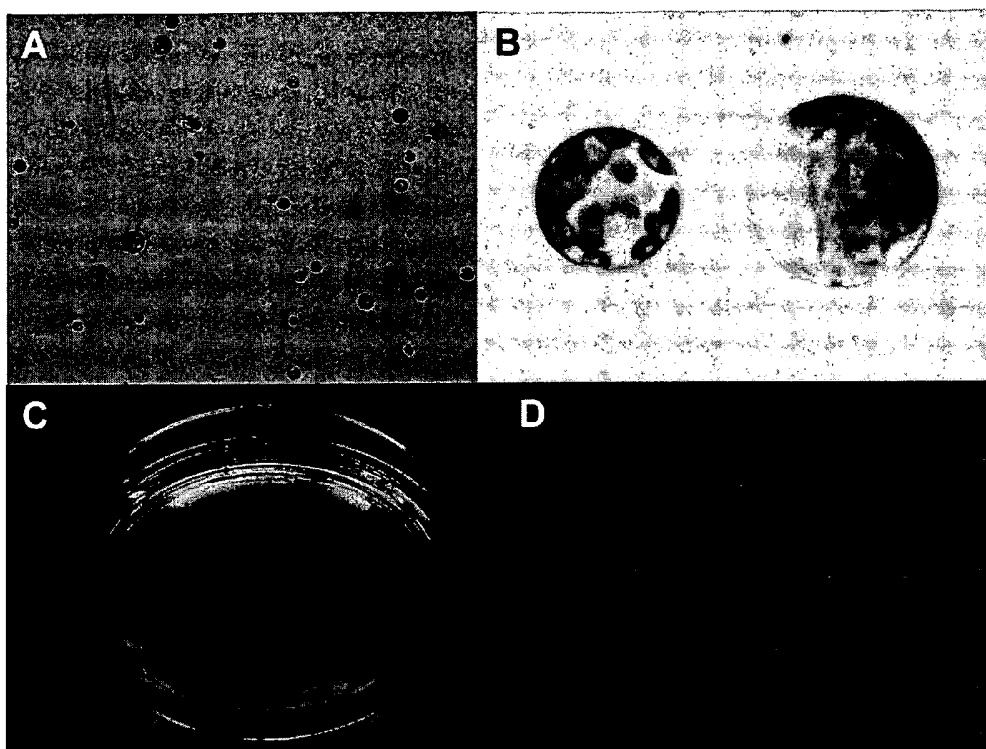


FIGURE 44

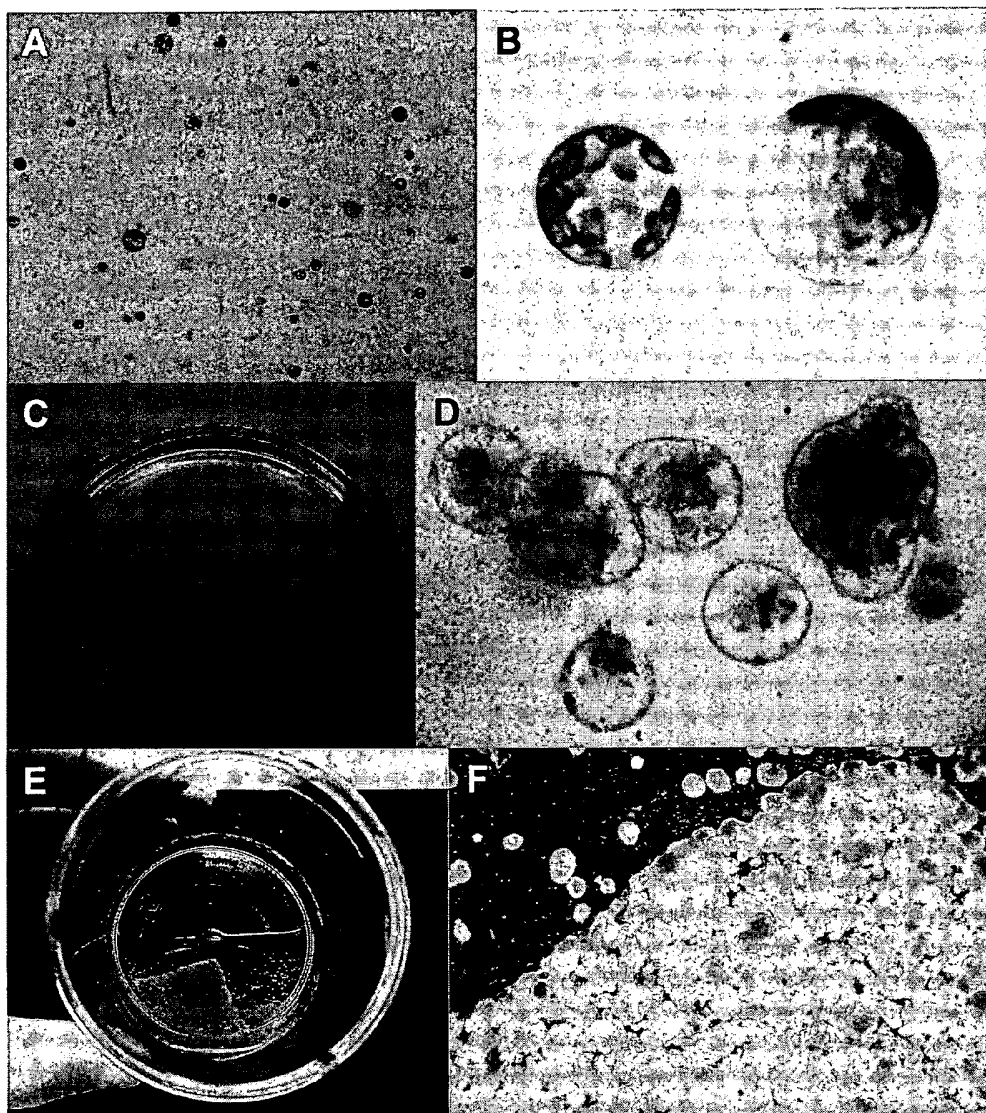


FIGURE 45

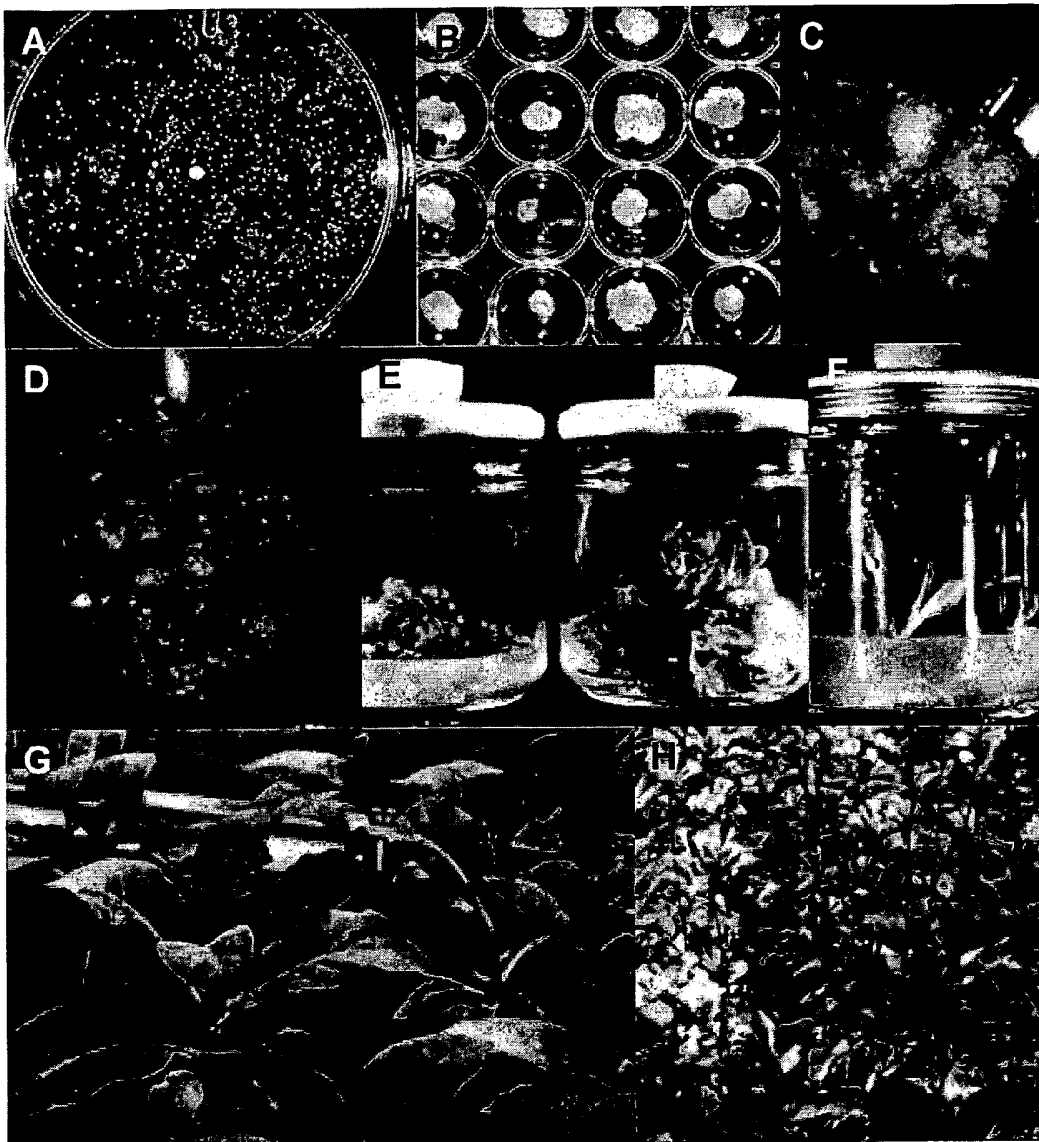


FIGURE 46

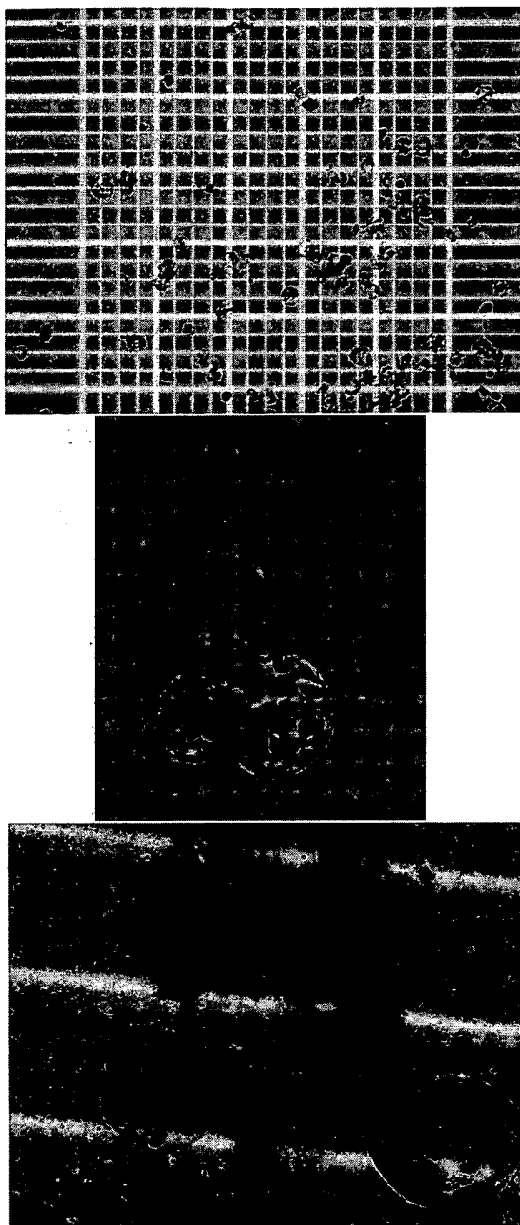


FIGURE 47

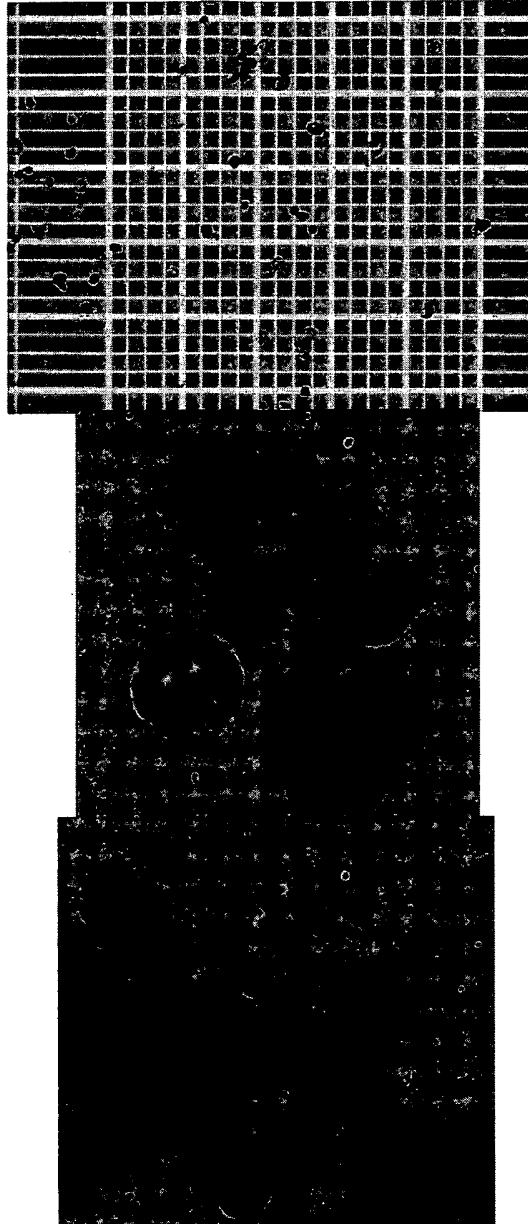


FIGURE 48

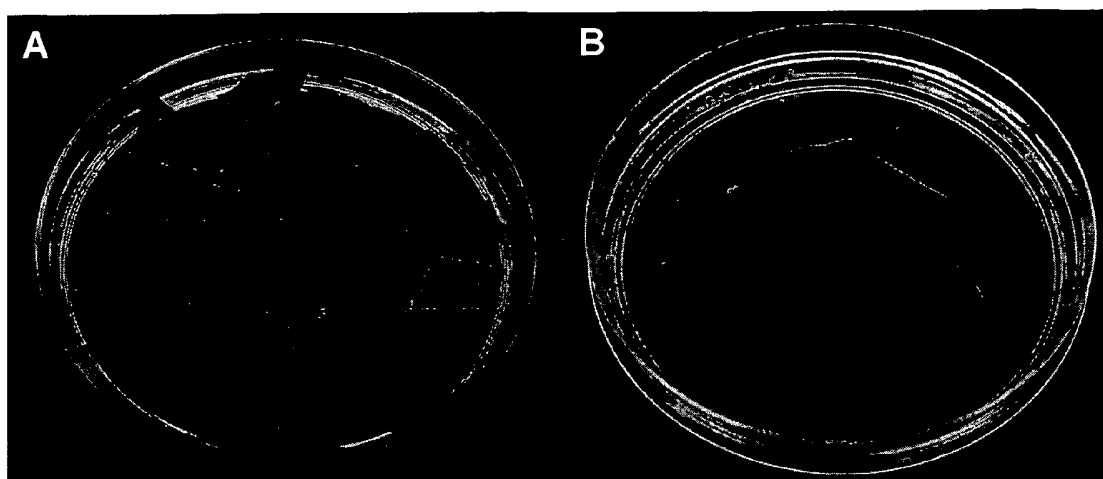


FIGURE 49

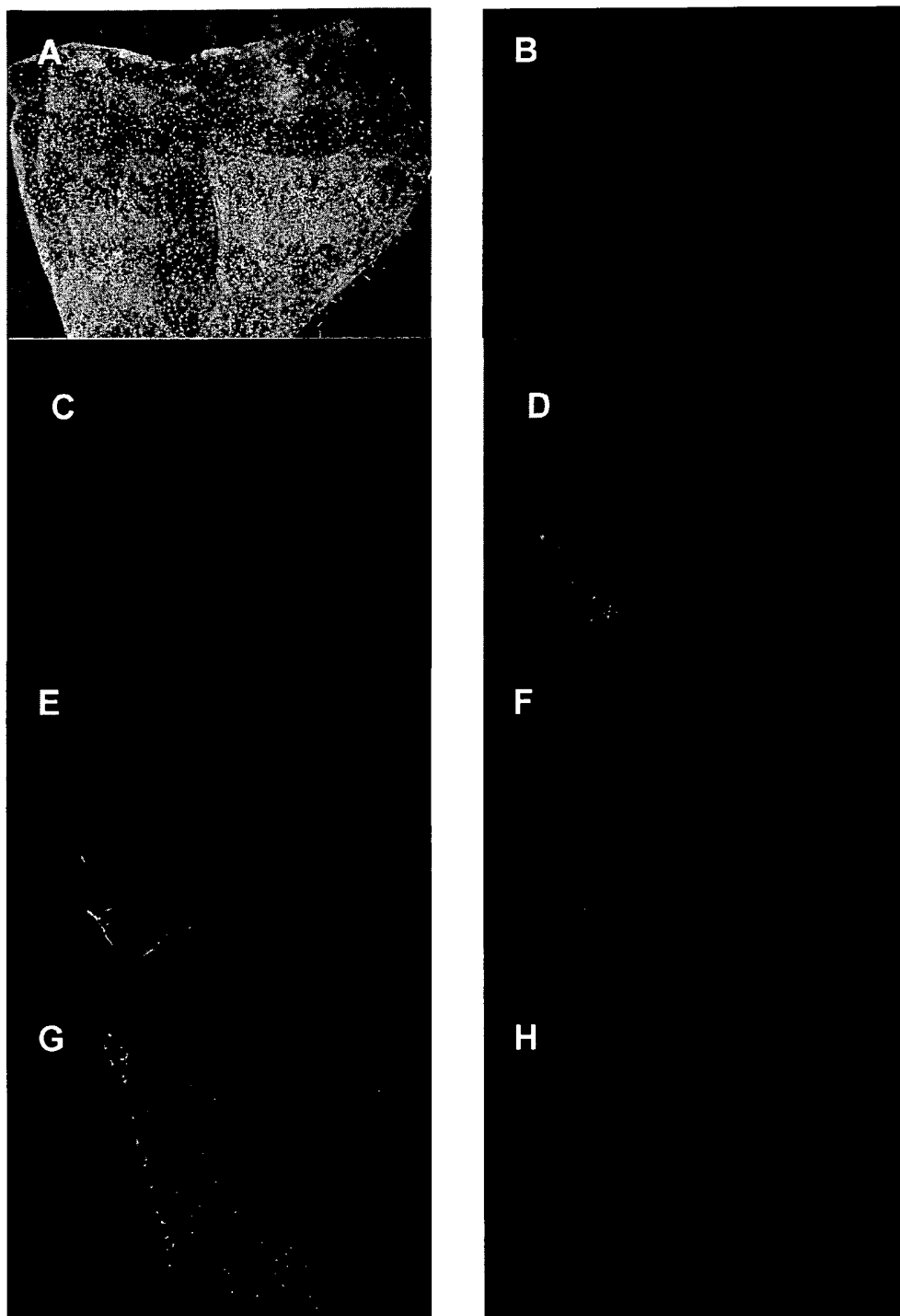


FIGURE 49 (CONT'D)

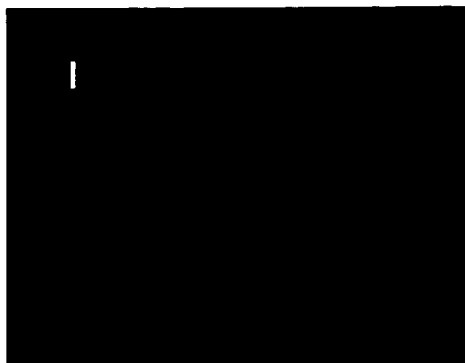


FIGURE 50

