REGULATION OF GENE EXPRESSION IN EUKARYOTES

This invention relates to a method for regulation of gene expression in eukaryotes.

This invention has particular but not exclusive application to regulation of highly specific expression in target organs or functions of plants, and for illustrative purposes reference will be made to such application. However, it is to be understood that this invention could be used in other applications, such as regulation of expression of other eukaryotic genes such as regulation of specific expression in target organs and functions of yeasts and animals.

Eukaryotic organisms of commercial benefit including plants, animals and lower organisms such as yeasts often divert growth resources into non commercial structures, or express other genes of economic detriment. Other commercial species may benefit from the introduction of characteristics which would enhance commercial worth.

For example, the development of reproductive structures on forest trees represents a significant burden on the resources of the trees, the reproductive effort occurring at the expense of wood growth. Seeds from mature trees produce an undergrowth of saplings which must be removed periodically to prevent competition with the tree crop for soil resources and to minimize the risk of damaging fire.

Australian Patent Specification No. 86224/91 discloses a method of enhancing vegetative growth in a plant. This disclosure exemplifies a method wherein a substantially tissue-specific promoter for reproductive-structure specific genes was selected on the basis of induction by the flowering regulator gibberellin A4/7, the early appearance of the gene product, and specificity of expression in both male and female reproductive buds. The gene and promoter were isolated and characterised, the gene was modified by fusing a portion of the gene including the tissue-specific promoter of the gene with a structural gene for a ribonuclease.

Whilst the transformants exhibit a lack of reproductive structures, promoter leakage tends to result in a low level of accumulation of the lethal ribonuclease in non target tissues, resulting in reduced growth and thus reduced commercial potential.

Gene expression promoters are to be generally regarded as falling in one of

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three broad classes. Constitutive promoters continuously express the gene in all tissues. Developmental promoters express the gene in specific tissues or in tissues at specific stages. Inducible promoters respond by switching expression on or off in response to the presence of metabolites, foreign compounds or other stimuli such as light, heat or pressure.

It is known that the expression of a lethal gene product such as Barnase under the control of a substantially flower-tissue specific promoter may be rendered more tissue specific by constitutive coexpression of an inhibitor (B* or "Barstar") for the gene product. The action of the expressed gene product in the target cell will occur where the inhibition is insufficient to suppress gene product action, and non target cell Barnase activity arising out of a degree of non-specificity of the promoter or promoter leakage is substantially suppressed.

However, the inserted promoters are often leaky and the preferred strong promoters may deleteriously express in other tissues with insufficient inhibition to prevent death of non target cells, which may adversely affect commercially important non-target tissues. Promoter leakage cannot be predicted and, for example, where the transformants such as tress have a long growth to first flowering, the resulting gene product activity in non target cells may negate or reduce the advantage of flower suppression. The generally preferred strong promoters limits the promoter choices.

The present invention aims to substantially alleviate at least one of the above disadvantages and to provide a method for regulation of eukaryotic gene expression which will be reliable and efficient in use. Other objects and advantages of this invention will hereinafter become apparent.

With the foregoing and other objects in view, this invention in one aspect resides broadly in a method of regulating a eukaryotically active gene, comprising transforming a cell with construct expressing a modulator gene product regulating the eukaryotically gene or its product and a further gene product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

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The eukaryotically active gene may comprise a native gene to the organism of interest or may comprise an introduced gene. The gene may be promoted by its natural promoter or comprise a heterologous construct with the gene. The eukaryotically active gene may code for an expression product of any selected cellular function. For example the gene may code for an active polypeptide including enzymes such as a ribonuclease having a lethal effect on a target tissue or a peroxidase conferring nematode resistance on root tissue. The gene may code for an enabling product for pathways for production of pigments and dyes such as anthocyanin, an insecticidal compound such as Bti toxin, or fragrances.

Expression of reporter genes under control of inducible promoters may display exact location of target cells in target tissues without any leakage to surrounded cells. This may be very useful for investigation of primary steps of interactions between pathogens and insects and plant cells. The GUS, Luc, anthocyanin gene and GFP may comprise candidates as reporters. Colourimetric or fluorescent analysis may be made of micropropagated materials before commecialisation.

As used herein, the expression "lethal gene" is taken to mean a gene or genes which encode a peptide or antisense or ribozyme or other non peptide which significantly disrupt a target cell leading thereby to the death of the target cell. The invention will be described hereinafter with reference to embodiments comprising a eukaryotically effective lethal gene.

The lethal gene may be any gene or combination of genes which encode a peptide or antisense or ribozymes which significantly disrupt a target cell leading thereby to the death of the target cell. For example, the lethal gene may be selected from those that encode ribonucleases such as Barnase from B.amyloliquefaciens, RNase T1 from A.aryzae, bovine RNase A, RNase I and RNase H from E.coli and set of plant RNases (family of S-proteins). Alternatively, the lethal gene may be selected from nucleases such as the family of restriction endonucleases, enzymes including glucanases such as 1-4-ß-glucanases or 1-3-ß-glucanases, ubiquitins, acid pyrophosphatases and inhibitors of plant cell wall synthesis.

Preferably, the lethal gene is an RNase. A chimaeric lethal gene may be

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constructed including the coding region of Barnase from B.amyloliquefaciens under the control of upstream (promoter) sequences of genes expressed in the male and female parts of plant reproductive organs at the early stages of development.

Restriction of expression of a lethal gene in target cells during first events of pathogen-host plant interaction in target cells may be achieved. For example, expression of Barnase or other toxins like ß-glucanase or chitinase in attacked cells will lead to necrosis of these cells which will prevent spreading of infection to surrounding cells. The critical stage in this artificial hypersensitive reaction project is blocking of possible promoter leakage in surrounding cells.

Expression of bacterial salicylate-hydroxylate gene may reduce level of salicylic acid which is responsible for systemic expression of cassettes under control of pathogen-inducible promoter in all plants. For example, a pathogen-inducible promoter may be isolated from barley which is strongly expressed after inoculation with powdery mildew Eresify graminis (Mouradov et al.,1993). The genomic clone of this gene was isolated and characterised (Mouradov et al.,1994). This promoter region may be fused to cassettes including a lethal or other functional gene.

Three eucalypt genes designated EGM1, 3 & 2 with homology to other plant MADS-box genes have been cloned and sequenced. Phylogenetic analysis has been performed to examine the relatedness of the eucalypt genes to other MADS-box genes. This revealed that EGM2 is likely to be homologous to the GLOBOSA MADS-box gene group which are involved in development of petals and stamens, EGM3 and EGM1 both fall in the AGL2 group of MADS-box genes. These genes are most commonly expressed in the inner three whorls of the flower (petals, stamens and carpels).

The pattern of expression of all three EGM genes has been characterised. Expression of these genes was not detected in any vegetative tissue. The EGM1 gene is expressed in the petals, stamens and the carpel of eucalypt flowers. EGM3 is expressed in the floral meristem and in sepals. EGM2 is expressed in petals and stamens. The EGM3 and EGM2 genes have been shown by Southern analysis to be single genes. EGM1 is also likely to be a single gene.

The promoter regions of all three of these genes may be used in sterility gene

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constructs may be isolated from a eucalypt genomic library and engineered into lethal gene constructs.

The eukaryotically active gene, modulator gene and further gene and their respecive promoters may comprise a transformation cassette with which the target organism may be transformed by any known means. The promoters of the eukaryotically active gene, modulator gene and further gene or genes are selected in combination such that the specificity of expression of the eukaryotically active gene is enhanced. For example, the eukaryotically active gene may be promoted by a promoter which is inducible or developmental whereas the modulator gene may be constitutive. Specificity and the usefulness of even weak promoters may then be enhanced by promoting the futher gene or genes with a promoter with the same specificity as that used to promote the eukaryotically active gene.

Alternatively, a tissue specific promoter for the eukaryotically active gene may be inhibited by a constitutively-promoted inhibitor gene product, which in turn is controlled by an inducibly-promoted further gene product.

In a yet further alternative, the eukaryotically active gene product may be contitutively expressed subject to the modulating effect of a modulator gene expressing under the control of a tissue specific promoter, the modulator gene or product being itself controlled by the expression of a further gene under the control of a promoter specific for a different or complementary tissue. By this means for example, a constitutively-promoted gene conferring insect resistance may be localized in its effect to exclude the seeds by expressing a gene inhibitor under the control of a seed specific promoter, the gene inhibitor itself being controlled by a further gene product expressed under the control of a promoter specific for non-seed tissues.

In a further aspect, this invention resides broadly in a method of regulating expression of a eukaryotically active gene, comprising transforming a cell with a transformation cassette comprising said eukaryotically active gene, a modulator gene expressing a product regulating the eukaryotically gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible

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promoters and developmental promoters for the same or complementary tissues.

In a further aspect this invention resides broadly in a transformation cassette comprising a eukaryotically active gene, a modulator gene expressing a product regulating the eukaryotically gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

In a yet further aspect this invention resides broadly in a method of transformation of a propagatable eukaryotic cell with an expression cassette including:-

a lethal gene expressing a cytotoxic peptide in a target tissue under the control of a promoter substantially specific to said target tissue;

an modulator gene constitutively expressing an inhibitor of the production or action of said cytotoxic peptide, and

a further gene under the control of said substantially specific promoter and functioning to block said inhibitor or modulator gene.

In a further aspect, this invention resides broadly in an expression cassette including:-

a gene expressing a peptide cytotoxic to a target tissue under the control of an inducible promoter substantially specific to said target tissue;

an inhibitor gene expressing an inhibitor of the production or action of said cytotoxic peptide, and

a repressor gene under the control of said inducible promoter and functioning to block said inhibitor or inhibitor gene.

In a further aspect, this invention relates to primers for PCR isolation of the Barnase and Barstar genes from an extract of B.amyloliquefaciens, designated P1 and P2 (for Barstar) and P3 and P4 (for Barnase) and comprising:

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
EcoRI

P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'

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HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

The cytotoxic effect of the lethal gene expression product may be increased by coupling a localisation signal sequence to the construct. For example, the cytotoxic effect of the preferred RNase may be increased by targeting into the nucleus where most of the pre-RNAs and mature RNAs exist in non-protected form. For this purpose the coding region of, for example, the Barnase gene may be modified by fusion of a nuclear localisation signal (NLS) sequence to the RNase gene.

Accordingly in a further aspect this invention provides a set of PCR primers for fusing the NLS sequence from VirD2 gene of A.tumefaciens to the Barstar gene and comprising:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

The tissue-specific or developmental promoter may be selected from those tissue specific promoters that are controlling genes in plant reproductive organs during the early stages of flower development. These genes are maintained in the repressed state in all plant organs during the non-flowering period and are induced substantially in reproductive organs during the flowering period.

The developmental promoter can be isolated by any known means. For example,

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mRNA only present during the development of the target tissue may be identified and isolated, cDNA from these specific mRNAs prepared and used to probe the genomic library, followed by identifying the portion of the plant genome which contains promoter region of these genes. The tissue-specific promoters may be homologous (native) or heterologous (foreign) in origin. For plant reproductive organ tissues, the promoters may be selected from those that are expressed in cells of different floral tissues in both male and female organs, or only in specific tissues such as sepals, petals, ovary, style, stigma, ovule, corola and others. For the generation of the sterile plants, promoters which express in tissues at early stages of both male and female organ development are preferable.

For example, the gene family encoding homeotic MADS-box genes which play an important role in flower development express at the early stages of the developing floral primordia. We have isolated MADS genes from P.radiata which display strong homology to the Arabidopsis homeotic MADS-box genes known as the AGAMOUS AGL-2 and AGL-4 genes. These genes express in young floral primordia and are not expressed in the inflorescence meristem.

The gene encoding an inhibitor of the lethal gene product may comprise a gene repressing expression of the lethal gene, a gene transcribing an antisense RNA, or may comprise a gene expressing a protein capable of deactivating the lethal protein. The inhibitor gene will generally be under the expression control of a constitutive promoter which expresses the inhibitor in non target tissues, although it is envisaged that a packaged operator-repressor system may be used.

For example, a Barnase gene/reproductive organ-tissue specific promoter system may be associated with an anti-Barnase (B* or Barstar) gene/promoter system.

In the alternative, negative regulation in expression of bacteriophage lambda N gene and SOS system in E.coli could be used in expression cassettes. Expression of the bacteriophage lambda N gene is tightly regulated via the P^L promoter and controls the level of expression from this promoter. This system may be used to provide the requisite negative control of the expression of an inhibitor gene in the target tissue.

The SOS system is regulated by the interplay of two proteins. A transcriptional

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repressor (LexA) inhibits the initiation of transcription through specific binding to its operator targets. A RecA protein blocks LexA protein and thus prevents inhibition at its specific site. This would be a one component lethal gene system and would not require a toxin blocking gene.

The repressor gene under the control of the inducible promoter may be selected to repress transcription of the inhibitor gene, translation of the RNA transcript or action of a peptide product thereof. Preferably, the repressor gene codes for a peptide product which directly represses expression of the inhibitor gene. Accordingly, it is preferred that the repressor gene and inhibitor gene be adapted to form a specific repressor/operator couple. For example, the E.coli laclq gene under control of the same tissue specific promoter as the lethal gene construct may be used to repress the action of the repressor gene promoter modified by insertion of a lac operator sequence.

The repressor gene promoter may be a modified 35S-RNA-CaMV promoter having at least one lac operator between the promoter and coding region of the repressor. In the selection of the modified 35S-RNA-CaMV promoter exemplified hereinafter, the present inventors have developed primers for amplification of the modified 35S-RNA-CaMV sequences.

Accordingly, in a further aspect this invention provides primers for PCR amplification of modified 35S-RNA-CaMV sequences, designated 35S1 and 35S2 and comprising:

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

For the introduction of lac operators into both the 3' and 5' region of the modified 35S-RNA-CaMV promoter, the sequence may be amplified by PCR primers:

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

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35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

The molecular mechanism of the lactose operon, interaction between the DNA-binding protein (repressor, laclq) and the target promoter sequence (operator) is understood in great detail. Expression of the lac operon is under the strong and tightly negative control from the laclq repressor. Purified repressor is a tetramer, formed from four identical subunits, each with 360 amino acids (MW 38,350 kDa). The operator sequence constitutes 35 base pairs, including 28 base pairs of symmetrical sequence.

For the maximum repression of the expression of the inhibitor gene, the repressor protein is preferably targeted into the nucleus of the target cells. This may be achieved by fusion of a the nuclear localisation signal sequence to the C-end of the repressor.

In a further aspect this invention relates to a set of primers suitable for isolation of the laclq gene and having suitable restriction enzyme cleavage sites for cloning into the plant vectors, the two primers corresponding to the 5' and 3' ends of the gene and designated AM 1 and AM 2:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
Kpn I

In a yet further aspect of the present invention, there is provided a set of PCR primers for use in fusing the NLS sequence to the 3' end of the laclq gene and comprising:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'

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Xba I

LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of laclq 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of laclq

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

If desired, the method of the present invention may be a reversible method. For example, the gene cascade may use molecular elements which will allow the reversal of the engineered sterility to fertility thus permitting seed production. The reversal mechanism may consists of placing an anti-repressor gene under the control of a chemically switchable promoter. When, for example, restoration of fertility is required plants may be sprayed or otherwise administered a chemical which induces expression of anti-repressor RNA which will block inhibition of expression of the modified inhibitor gene. Accumulation of the inhibitor product in floral organs will inhibit lethal gene function, thus allowing normal flower formation.

For example, for the lac/laclq system described, a chemical may be sprayed on the plant which induces expression of anti-laclq RNA which will block inhibition of expression of a modified Barstar gene. Accumulation of the Barstar product in floral organs will inhibit lethal gene function, thus allowing normal flower formation. The promoter region of the maize glutathione-S-transferase (GST II) gene could be used for chemically-induced expression. Expression of the GST II promoter can be switched on by chemicals such as dichloramin (N,N-diallyl-2,2-dichloracetamide), or fluorazole (benzyl-2-chloro-4-trifluoromethyl-5-thiazole-carboxylate).

In the case of Eucalyptus, the EGM3 promoter, comprising the upstream sequences of the EGM3 gene, may be used to express the lethal gene specifically in eucalypt floral tissue. Using this promoter, the cytotoxic Barnase gene may be specifically expressed in the floral meristem at a very early stage of development. The inhibitor Barstar may be expressed under the control of 35S-RNA-CaMV promoter

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carrying the lac-operator sequences.

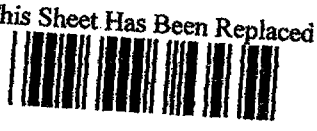
The E. coli laclq gene under the control of the EGM3 promoter could be used as repressor gene to prevent expression of Barstar in the floral meristem tissue during flowering initiation. During flowering, the EGM3 promoter would express Barnase and laclq in the floral meristem while the modified 35S-RNA-CaMV promoter would express Barstar in all tissue except the floral meristem, giving protection from promoter leakage. During non-flowering period, the 35S-RNA-CaMV promoter would express Barstar generally, giving protection from leakage.

Nematodes causes annual losses well in excess of US$100 billion to world agriculture. The most important pathogens by far are the nematodes Meloidogyne incognita and Meloidogyne javanica which attack a wide range of plants. Control measures include use of hazardous chemicals, cultural practices such as rotation and the use of resistant varieties in a limited range of crops. The advantages of engineering resistance to nematodes include no requirement for specific cultural practices and reduced environmental risks.

The lethal gene/inhibitor/repressor approach can be used to engineer resistance to nematodes. The ideal site to activate a plant defence against nematodes is in the feeding cells in the vascular region of the plant root. In several plant-nematode interactions, gene promoters are being identified by differential screening which could be used to express the Barnase gene specifically at the site of nematode feeding. Recently two genes (Lemmi9 and LemmilO) have been identified that are expressed at high levels in giant cell feeding sites in tomato roots infected with Meloidogyne incognita. (Van der Eycken et al., (1996) Plant Journal 9, 45-54).

Expression of a lethal gene in feeding cells would rapidly kill the cell and disrupt the feeding process which is very critical particularly for the development of the females. Expression of the inhibitor gene in non-target tissue would be required to prevent damage to other parts of the root due to promoter leakage. This is particularly important since the promoter which shows high level expression in the giant feeding cell is also expressed at lower levels in non target tissue. Expression of the repressor gene under the feeding site promoter should block the expression of the inhibitor gene sufficiently

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in the target tissue to allow the lethal gene to destroy the cell.

In order that this invention may be more readily understood and put into practical effect, reference will now be made to the following Examples and the accompanying drawings which illustrate preferred embodiments of the invention and wherein:

FIG. 1 represents the construction of the Barnase/NLS gene localised lethal gene system;

FIG. 2 represents constructs including lac operon- modified 35S-RNA-CaMV operator/Barstar inhibitor gene system;

FIG. 3 represents the construction of laclq/NLS localised repressor gene system;

FIG. 4 represents the mode of action of Barnase/Barstar:lac/laclq cascade in accordance with the present invention;

FIG. 5 represents an alternative cascade Barnase:op/LexA/RecA in accordance with the present invention;

FIG. 6 represents the mode of action of a reversible Barnase/Barstar:lac/laclq/antisense-laclq cascade in accordance with the present invention;

FIG. 7 is a diagrammatic illustration of the control cassette and modes of action of the lethal gene/NLS system of FIG. 1;

FIG. 8 is expression of PrMADS1, PrMADS2, PrMADS3, PrFL1 and PrCON1 genes in reproductive and vegetative tissues of *P. radiata*.

FIG. 9 is expression of PrMADS3 gene in male and female cones.

Fig. 10 is the gel mobility shift assay of PrMADS1, PrMADS2, and PrMADS3.

FIG 11 is the nucleotide sequence of the EGM1 promoter;

FIG. 12 is the nucleotide sequence of the EGM2 promoter;

FIG. 13 is the deduced sequence of the promoter of the eucalypt floral-specific MADS gene EGM3;

Fig. 14 is a graphical representation of Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM1 cDNA (6 hours exposure);

Fig. 15 is a graphical representation of Northern blot of total RNA extracted from Eucalypt tissue probed with EGM3 cDNA;

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Fig. 16 is a graphical representation of Northern blot of total RNA extracted form Eucalypt tissue probed with EGM2 cDNA (6 hour exposure);

Fig. 17 is an alignment of the predicted protein sequence of the MADS-box region of several plant MADS-box genes;

FIG. 18 is a phylogenetic tree showing relatedness of several plant MADS-box genes;

FIG. 19 is a Southern blot of Eucalyptus grandis DNA digested with EcoRI and HinDIII and then probed with the EGM3 and EGM2 genes;

FIG. 20 is a Northern blot of total RNA extracted from eucalypt tissue probed with the EGM3 cDNA (6 hour exposure).;

FIG. 21 is a diagrammatic illustration of plasmid 7 prom Barstar;

FIG. 22 is a diagrammatic illustration of plasmid pBR Barnase prom Barstar containing V1-promoters;

FIG. 23 is a diagrammatic illustration of a plasmid containing V2-promoters;

FIG. 24 is a diagrammatic represenation of agarose seperation of a *Pst1* digest of an EGM3 double bin. Given that the *EcoRI* cloning site is at base 6772 in EGM3 bin, the predicted fragment sizes from the map for this digestion are 4.9, 4.8, 4.4, 3.3, 1.9, 1.1, and 0.6 kb.

FIG. 25 is a diagrammatic representation of two gel exposures of a *Pst1* digest of an EGM3 bin showing the other possible orientations of the cloned insert. The sizes of fragments expected from this digest are 7.4, 4.9, 4.4, 1.1, 0.7, and 0.6 kb.

FIG. 26 is a plasmid diagram of EGM3 double bin.

FIG 27 is a plasmid diagram of EGM3 V1 sense.

FIG 28 is a plasmid diagram of plasmid V1 (Barnase-Barstar).

FIG. 29 is a plasmid diagram of plasmid V2 (LaclqNLS-35s promoterOp-Barstar).

FIG. 30 is a representation of a promoter finder strategy;

FIG. 31 is the nucleotide sequence of PrMADS2 promoter;

FIG. 32 is the nucleotide sequence of PrMADS3 cDNA;

FIG. 33 is the amino acid sequence of PrMADS3 protein;

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FIG. 34 is the nucleotide sequence of PrMADS promoter;

FIG. 35 is the nucleotide sequence of PrFL1 cDNA clone;

FIG. 36 is the amino acid sequence of PrFL1 protein;

FIG. 37 is the nucleotide sequence of PrFL1 promoter;

FIG. 38 is the nucleotide sequence of PrCon1 gene (partial); and

FIG. 39 is a Northern blot of total RNA extracted from eucalypt tissue probed with the EGM2 cDNA (3 day exposure).

EXAMPLE 1

A chimaeric reproductive organ-specific expression cassette was constructed including a reproductive organ-specific promoter and Barnase gene from B.amyloliquefaciens, under the positive control of a gene encoding an inhibitor of Barnase, Barstar (B*), from B.amyloliquefaciens under control of the modified constitutive promoter (35S RNA CaMV) with introduced lac-operator sequence, and under the negative control of the E.coli laclq gene under control of the same reproductive organ-specific promoter as used to express the lethal gene as per FIG. 4.

The reproductive organ specific promoter comprises the upstream sequences of genes expressed in reproductive organs at the early states of flower development as described hereinafter. The cytotoxic effect of the Barnase was increased by targeting it into the nucleus where most of the pre-RNAs and mature RNAs exist in non-protected form. For this purpose the coding region of the B gene was modified by fusion of the nuclear localisation signal (NLS) sequence from VirD2 gene of A.tumefaciens strain C58 to the 3'-region of the B gene, as illustrated in FIG. 1.

The inhibitor Barstar (B*) gene portion vectors carrying lac-operator sequences in 5'-, 3'- and 5'- plus 3'- regions of the 35S-RNA-CaMV promoter region were constructed as per FIG.2.

The E.coli laclq gene under control of the same reproductive organ-specific promoter as used to express the lethal gene has its laclq protein targeted into the nucleus by fusion of the NLS from VirD2 gene of A.tumefaciens to the C-end of the laclq

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protein as per FIG. 3.

EXAMPLE 2

An alternative chimaeric reproductive organ-specific expression cassette was constructed as per FIG. 5, wherein the reproductive organ-specific promoter and Barnase gene is under the positive transcriptional control of the LexA product under control of 35S-RNA-CaMV promoter, and under the negative control of the RecA gene product under control of the same reproductive organ-specific promoter as used to express the lethal gene, as per FIG. 5.

EXAMPLE 3

A reversible system was constructed substantially in accordance with Example 1. In addition a chemically switchable promoter from the maize glutathione-S-transferase (GST II) gene regulating an antisense LacIq RNA gene was included. This allowed seed production as per FIG. 6. Restoration of fertility may be thus achieved by spraying with an inducer such as dichloramin (N,N-diallyl-2,2-dichloracetamide) or fluorazole(benzyl-2-chloro-4-trifluoromethyl-5-thiazole-carboxylate). This chemical induced expression of anti-lacIq RNA blocks inhibition of expression of modified 35S-RNA-CaMV-B* gene. Accumulation of the B* in floral organs will inhibit lethal gene function, thus allowing normal flower formation.

EXAMPLE 4

Modification and insertion of the lacIq repressor gene into plant expression vectors.

The pRT99GUS vector was digested with XbaI and KpnI to release the coding region of the GUS gene and become a suitable vector for expression in plants.

The lacIq repressor is available on almost all commercial E.coli strains. Some commercial plasmids also contain the repressor gene. In order to express this gene in plants, the prokaryotic translational initiation codon (GTG) was changed into ATG. In order to isolate the lacIq gene two primers corresponding to the 5' and 3' ends of the gene were designed (AM 1 and AM 2). Both primers have suitable restriction enzyme cleavage sites for cloning into the plant vectors.

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'

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Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
Kpn I

After amplification the PCR fragment was digested with XbaI and KpnI and ligated into the pBR-35SGUS vector digested with same enzymes in order to replace the GUS gene (pBR-35SlacIq).

In order to fuse the NLS sequence to the 3' end of the lacIq gene several PCR were performed.

Primers:

AM 1 as mentioned above.

LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of lacIq 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of lacIq

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

Final PCR fragment containing NLS from VirD2 gene from A.tumefaciens fused to the coding region of the lacIq gene was digested with Xba I and Kpn I and ligated into the plant expression vector pBR322-35SGUS digested with the same enzymes (pBR-35SlacIq-NLS). This vector was transformed from the XL1 Blue into the JC 8697(recA-) E.coli strain in order to eliminate possible homologous recombination.

EXAMPLE 5

Barnase and Barstar genes.

These genes were isolated by PCR from the crude extract of the B.amyloliquefaciens using P1 and P2 (for Barstar) and P3 and P4 (for Barnase) primers.

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
EcoRI

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P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'
HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

The P1-P2 PCR fragment (Barstar) was digested with EcoRI and HindIII and ligated into the Bluescript SK vector digested with same enzymes (BS-B*). The BamHI-HindIII fragment of the BS-B* vector was introduced into the protein expression vector pQE32 (pQE-B*).

The P3-P4 PCR fragment (Barnase) was ligated into the PCR cloning vector p/GEM-T (pGEM-T-B). In order to fuse NLS sequence to the 3' end of the B gene several PCR were performed.

Primers:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

Final PCR fragment containing NLS from VirD2 gene from A.tumefaciens fused to the coding region of the Barnase gene was ligated into the PCR cloning vector pGEM-T (pGEM-T-B-NLS).

EXAMPLE 6

Introduction of lac-operator sequence into the 35S-RNA-CaMV promoter.

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The commercial plasmid pQE-32 contains the ideal combination of two lac-operators (operators I and II). As a first step the HindIII fragment from the pRT99GUS vector was introduced into the Bluescript SK vector (A 1). The EcoRI-SalI fragment of the A 1 vector carrying the GUS gene fused into the 35S-RNA-CaMV promoter and terminator regions was introduced into the pQE32 vector digested with the same enzymes.

The resulting vector contains 35S-RNA-CaMV promoter with two lac operons in the upstream region (Op+ 35S-GUS).

In order to introduce the lac operator between the 35S-RNA-CaMV promoter and coding region of the GUS gene initially a BamHI-SalI fragment of the A1 vector carrying promoterless GUS gene was ligated into the pUC19 vector digested with same enzymes (pUC19-promoterGUS). In the next step the pUC19-promoterGUS vector was digested with EcoRI and SalI enzymes and ligated into the pQE32 vector digested with the same enzymes (pQE-promoterGUS). The 35S-RNA-CaMV promoter region was amplified by PCR using the 35S1 and 35S2 primers, corresponding to the 5' and 3'-region of the 35S-RNA-CaMV promoter.

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

The PCR fragment was digested with XhoI and SalI and introduced in to the pQE-promoterGUS digested with XhoI. Clones containing the promoter in the correct orientation were selected and resulting plasmid (35S- GUS) was transformed from the XL1 Blue into the JC 8697 (recA-) E.coli strains in order to eliminate possible homologous recombination.

In order to introduce lac-operators into both, the 5'- and 3'-region of the 35S-RNA-CaMV promoter the operator-35S promoter region of the -35S-GUS vector was amplified by PCR using the OP1 and 35S2 primers, corresponding to the 5'- and 3'-region of the -35S promoter.

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

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The PCR fragment was digested with XhoI and SalI and introduced into the pQE-promoterGUS digested with XhoI. Clones containing the promoter in the correct orientation were selected and resulting plasmid (-35S-promoterGUS) was transformed from the XL1 Blue into the JC 8697 (recA-)E.coli strains in order to eliminate possible homologous recombination.

Barstar gene was cloned into the pQE-promoterGUS vector after digestion of both with EcoRI+KpnI.

One difficulty with the tissue ablation approach is the effect of promoter leakage or expression in other tissues. To overcome this we have developed a control system (gene cascade) whereby expression of a lethal gene(s) is countered in non-target tissues (Fig. 7). Two different vectors carrying Barnase (V1) and Barstar plus repressor (*lacIq*) (V2) parts of these cascade were designed.

In order to clone promoter regions into the vectors V1 and V2 they have been amplified using sets of primers with *SalI* restriction sites on the ends for V1 (*SalI* primers) and *EcoR*I restriction site for V2 (*EcoR*I primers). In order to check the orientation of the promoters in V1 and V2 vectors an *EcoR*I restriction site was designed in the forward *SalI* primer downstream from *SalI* site and a *SalI* site was designed in the *EcoR*I primer downstream from *EcoR*I:

SalI primers:

PrMADS2 promoter:

Forward primer:

5' CCGGTCGACGAATTCCGACAGTGGAGTCCACAAAGAAAGATGCG3'
SalI *EcoR*I

Reverse primer: 5' CCGGTCGACTTCTTTCCTTCTTTTCTTTCTGC 3'
SalI

PrMADS3 promoter:

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Forward primer: 5' ACGCGTCGACGAATTCAAGATTTCAAATCAGTCC 3'
SalI *EcoRI*

Reverse primer: 5' ACGCGTCGACCAAGATCCCTCTGCTTCTTCACC 3'
SalI

PrFL1 promoter:

Forward
primer: 5' ACGCGTCGACGAATTCGAACTTCTGGAATAAGCTGC 3'
SalI *EcoRI*

Reverse primer: 5' ACGCGTCGACTTCATCTTACGTCACGCGAGG 3'
SalI

***EcoRI* primers:**

PrMADS2 promoter:

Forward primer:

5' CCGGAATTCGTCGACCGACAGTGGAGTCCACAAAGAAAGATGCG3'
EcoRI *SalI*

Reverse primer: 5' CCGGAATTCTTCTTTCCTTCTTTTCTTTCTGC 3'
EcoRI

PrMADS3 promoter:

Forward primer: 5' CGGAATTCGTCGACAAGATTTCAAATCAGTCC 3'
EcoRI *SalI*

Reverse primer: 5' GCGAATTCCAAGATCCCTCTGCTTCTTCACC 3'
EcoRI

PrFL1 promoter:

Forward primer:

5' CGGAATTCGTCGACGAACTTCTGGAATAAGCTGC 3'
EcoRI *SalI*

Reverse primer: 5' GCGAATTCTTCATCTTACGTCACGCGAGG 3'
*Eco*RI

PCR fragments of *Sal*I primers were digested with *Sal*I restriction enzyme and introduced into the V1 vector digested with *Sal*I. The orientation of promoters was checked using digestion with *Eco*RI enzyme.

PCR fragments of *Eco*RI primers were digested with *Eco*RI restriction enzyme and introduced into the V1 vector digested with *Eco*RI. The orientation of promoters was checked using digestion with *Sal*I enzyme. Maps of the resulting plasmids are shown in Figs. 21 and 22.

These vectors were linearised with HindIII enzyme and introduced into the Bin19 binary vector digested with HindIII. Colonies carrying modified Bin 19 vectors were selected on plates with kanamycin and ampicylin antibiotics. As a next step Bin19-V1-promoter and Bin19 V2-promoter vectors were transformed into several agrobacterium strains. *Arabidopsis thaliana* *Eucalyptus grandis* and *Pinus radiata* embryos and explants were co-transformed with *Agrobacterium* strains.

The HindIII fragment from the plasmid pRT 99 gus (Töpfer *et al.* Nucleic Acids Research (1988) 16 (17): 8725) was cloned into the HindIII site of pBR 322. This insertion resulted in two plasmids corresponding to the insert being cloned in both orientations. The inserted region contains the Cauliflower Mosaic Virus (CaMV) 35S RNA promoter, beta glucoronidase (GUS) gene, and CaMV 35S terminator region. These plasmids were called pBrGUS1 and pBrGUS2 and provided the basis for the lethal gene constructs. The orientation of the insertion event was checked with a BamHI digest. pBRGUS 2 results in BamHI fragments of 2.5kb and 4.4kb, pBRGUS 1 in fragments of 6.1kb and 0.8kb.

The DNA encoding the lacIq nuclear localisation signal peptide was amplified from the plasmid pGEM lacIqNLS by PCR using the 5' primer Lac I (this has an EcoR1 site) and the 3' primer D23 which has a KpnI site. The amplified DNA fragment was restricted with EcoRI and KpnI and cloned into pBRGUS 2 cut with the same enzymes. The resulting plasmid was called pBRLac. The pBRLac plasmid was then cut with SphI and run on an agarose gel to allow the purification of the plasmid fragment containing

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the LacIq gene, Ampicillin resistance gene, and origin of replication away from the small SphI fragment, which contained restriction sites that we wished to remove. The resulting plasmid was called pBRLac BH-.

The second stage in the plasmid construction was the preparation for cloning of the Barstar gene under the regulation of the modified CaMV plus lac operator promoter. This was amplified from the plasmid p35S-op-Barstar EcoRI- which contained the promoter/gene sequence which had been modified to remove the EcoRI site between the promoter and coding sequence. This modification was accomplished by cutting the 35S-op-Barstar plasmid with EcoRI, carring out a blunting reaction with T4 DNA polymerase, and then religating the blunted plasmid. The PCR was carried out using the 5' primer pQE-F and the 3' primer Bar-3'. The PCR fragment was cut with XhoI and KpnI. This was cloned into the 35S-op-Barstar EcoRI- plasmid also cut with XhoI and KpnI, and from which the unwanted gene had been purified away by gel electrophoresis. This enabled the removal of restriction sites at the three prime end of the Barstar coding region. This plasmid was called p35S-op-Barstar EcoRI- 2.

The Barstar gene was then cut out of the p35S-op-Barstar EcoRI- 2 plasmid with XhoI and SalI and cloned into pBrLac BH- cut with Sal1. The Barstar gene could go into the SalI site in either orientation but only one orientation was found. The orientation of the insert was ascertained by a KpnI digest. Only a 1kb band was seen which was indicative of the orientation of the insert seen in plasmid pBRLac Op Barstar 1. The resulting plasmid was called V2.

The plasmid was then ready for the cloning of, in this case, a flower specific promoter, into the unique EcoRI cloning site 5 prime of the lacIq gene. The EGM3 promoter from the Eucalyptus MADS gene EGM3 was used for this purpose. This was cut from plasmid pEGM3 with EcoRI. This promoter was cloned in both orientations, and the resulting plasmids were called V2 EGM3 sense and V2 EGM3 antisense. The plasmid V2 EGM3 sense was used as a source the EGM3 regulated lacIq and CaMVop Barstar genes for the plant transformation vector. The orientation of the EGM3 promoter was determined by an XbaI PstI digest. The presence of a 2.3 and 0.9 kb band was indicative of the correct orientation.

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The second construct V1, containing the promoterless Barnase gene, was constructed using pBrGUS 2 as the starting point. A barnase gene was amplified from genomic *Bacillus amiloliquefaciens* using the primers Barnase 5 prime Sal, and Barnase 3 prime Kpn. The resulting PCR product was restricted with Kpn I and Sal I as was pBrGus 1 and a ligation reaction performed. The plasmids from the resulting colonies were used as templates for sequencing using the amplification primers. Plasmid SB4 was found to contain a Barnase fragment of the same sequence as the *B. amiloliquefaciens* Barnase gene and this called pBRBarnase and was used for further constructions.

Previous work using Barnase as a lethal gene in plants had shown that the anti-toxin gene for Barstar was required to be present and expressed from the same plasmid as the Barnase before a promoter region could be cloned 5 prime of the barnase gene (Paul *et al.* 1992 Plant Molecular Biology 19: 611-622). This was achieved by using a plasmid that contained the Barstar gene and promoter from *B. amiloliquefaciens* in cis with the barnase sequence. To this end the promoter plus barstar DNA region was amplified from the *B. amiloliquefaciens* genomic DNA using primers 5 prime Barstar promoter and Bar 3 prime. The PCR fragment was restricted with KpnI as was pGEM 3f and a ligation reaction performed. The resulting white colonies were screened for inserts. The DNA from colony 7 was used for seqencing and found to contain the Barstar promoter plus Barstar sequence as in the genomic *B. amiloliquefaciens* DNA. This plasmid was called 7 prom Barstar, as shown in Figure 21.

The 7 prom Barstar plasmid was resticted with Kpn I as was pBR Barnase and the Promoter Barstar fragment was cloned into the Kpn I site of the PBR Barnase DNA. This resulted in a plasmid known as V1. The EGM3 promoter was cut out of pEGM3 using EcoRI, blunted using DNA polymerase I (Klenow fragment) and cloned into the unique SalI cloning site of the V1 DNA which had also been restricted and blunted. The resulting plasmid with the promoter inserted in the correct orientation was called EGM3 VI sense (FIG. 27)

The EM3 V1 sense DNA was linearised with HindIII and cloned into the plant

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transformation vector Bin19 also linearised with HindIII. This resulted in two plasmids, EGM3 V1 Bin 1 and 2, corresponding to the two orientations of insertion. In orientation 2, the two EcoRI sites present in EGM3 V1 Bin are approximately 80 bp appart. The EGM3 V1 Bin 2 was cut with EcoRI and blunted using DNA polymerase I (Klenow fragment). The EGM3 promoter laclq gene, and the CaMV 35S op Barstar genes were cut out of EGM3 V2 sense using AatII and HindIII. This fragment was also blunted and ligated into the EcoRI cut and blunted EGM3 V1 Bin 2 to create the plasmids EGM3 Double Bin I and II (FIG. 26), again corresponding to the two possible orientations. These plasmids were used for transformation of plants via A. Tumefaciens.

The proceedure was then repeated with further *Eucalyptus* MADS promoters. These were EGM2 long and short, and a shorter version of EGM3.

Primers.

Barnase 5 prime Sal

5' CCGTCGACATGGCACAGGTTATCAA 3'
SalI

Barnase 3 prime Kpn

5' CGGGTACCTTATCTGATTTTTGTAAAGG 3'
KpnI

Five prime Barstar promoter

5' CGGGTACCGTCCAATCTGCAGCCGTCCGA 3'
KpnI

Bar 3'

5' GCGGTACCTTAAGAAAGTATGATGGTG 3'
KpnI

D23

5' CGGGTACCTATCTCCTATTTCCCCCACG 3'
KpnI

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pQE-F

5' GCGTATCACGAGGCCCTTTC 3'

LacI

5' GCGAATTCAACATGGAACCAGTAACGTTATA 3'

EcoRI

EXAMPLE 7

Isolation of reproductive organ-specific promoters

Five cone-specific genes displaying strong homology to *Arabidopsis thaliana* and *Anthirrhinum majus* floral meristem and organ identity genes were isolated from *P. radiata* cDNA library prepared from immature female and male cones. Three of them, PrMADS1, 2 and 3, belong to the family of MADS-box genes showing homology to *Arabidopsis AGL-2, AGL-4* and *AGL6* genes and *dal1* gene from another non-angiosperm, *Picea abies* (Norway spruce), respectively. The PrFL1 gene is the pine ortholog of *Arabidopsis Leafy* (*Lfy*) and *Floricaula* (*Flo*) gene from *Anthirrhinum.* The *PrCon1* shows strong homology to *Arabidopsis CONSTANS* (*co*) gene. To elucidate the function of these genes we took the approach of characterising their expression pattern in male and female cones during different stages of cone development.

A significantly lower level of expression was detected in vegetative tissues: vegetative buds, needles, stem and roots. *In situ* hybridisation showed that expression of these genes is detectable only in reproductive tissue cells.

Expression analysis revealed that all five genes show different patterns of expression in different stages of development of male and female cones (Figs. 8 and 9). PrMADS1, 2 and 3 genes are cone-specific: expression of both genes was restricted to reproductive organ primordium tissues. No detectable expression of these genes was observed in vegetative tissues: vegetative buds, needles, stems, roots. For PrFL1 and PrCon1 low detectable expression was observed in vegetative buds.

In reproductive organs low level of expression of both genes was detected at early stages of cone development (5 mg cones) which was increased during cone development (50 mg cones). In male cones expression of both genes was restricted to

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microsporangium containing primary sporogenous cells. In female cones expression of both genes was restricted to premature ovules.

To characterise MADS proteins as DNA-binding proteins *in vitro*, we expressed both proteins in *E. coli* and characterised their DNA-binding properties. PrMADS1,2,3 proteins are sequence-specific DNA-binding proteins. Their DNA-binding consensus sequence is similar to that of the *AGAMOUS* protein. All three proteins bind a DNA sequence matching the consensus sequence of CArG box TT(A/T)CC(A/T)(A/t)$_2$(T/A)NNGG(-G)(A/T)$_2$ (oligo A) for PrMADS1 and PrMADS2) (Fig. 32). Mutation of these consensus sequences (oligo B) significantly decreases their binding of PrMADS 1,2 or 3 proteins. Competition with non-radioactive oligos did not decrease binding of any of the proteins to the CArG consensus. This indicates that we are dealing with specific DNA-protein interactions.

Upstream sequences were isolated using a 'Promoter finder' strategy (Fig. 30). A special adaptor was ligated to the ends of DNA fragments generated by digestion of genomic DNA from *P. radiata* with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I separately. The enzymes used were selected because they have six-base recognition sites and generate blunt ends. Following adaptor ligation, these DNA fragments were used as a template for PCR using first adaptor primers AP1, AP2 and gene-specific primers GSP-1,2.

The sequences of adaptor (first sequence), adaptor-primers and polymerase blocking primer shown below. It is noted that adaptor primer AP1 corresponds to bases 1 to 22 of 22 of the adaptor, adaptor primer AP2 corresponds to bases 13 to 31, and the polymerase block corresponds to bases 41 to 48.

Adaptor:

5'GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGG-CTGGT-3'

Polymerase block:

AP1 _____ 3'-NH_2-CCCGACCA-PO_4-5'

AP2

Adaptor primer 1, (AP1): 5'-GTAATACGACTCACTATAGGGC-3'

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Adaptor primer 2, (AP2): 5'-ACTATAGGGCACGCGTGGT-3'

The presence of the amine group on the 3' end of the lower strand blocks polymerase catalysed extension from free adaptor molecules that have not been ligated, thus preventing the generation of the primer binding site unless a defined, gene specific primer extends a DNA strand opposite the upper strand of the adaptor.

Primary PCR was performed using Avantage Tth Polymerase Mix (Clontech) and adaptor primer AP1 and GSP-1 for PrMADS2,3 and PrFL1 genes.

GSP-1 sequences:

PrMADS2: 5' CGGCGCTTCCGAACTCATAGAGTTTTCCTC 3'
PrMADS3: 5'TTAGCGCCACTTCGGCATCGCACAGC 3'
PrFL1: 5' CAAGGGACTTCAAATCCTTTCTCCCATTCATGG 3'.

PCR two step cycle parameters:

7 cycles:	94°C	25 sec
	72°C	4 min
32 cycles:	94°C	25 sec
	67°C	4 min

67°C for an additional 4 min after the final cycle.

The 1 ml of primary PCR was used in secondary PCR using the same cycling parameters and a second set of GSP primers with AP2 adaptor-primer.

GSP-2 primers sequences:

PrMADS2: 5' CGCCTTTTTCAGCAGACCATTCCGGC 3'

PRMADS3: 5' CAGCAGTCCGTTTCCGGCGCTTCG 3'

PrFL1: 5' CGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGG 3'

The 1-2 kb PCR fragments were cloned into the TA-type cloning vector and sequenced. Sequences of cDNAs, proteins and promoter regions for PrMADS2, PrMADS3 and PrFL1 are shown in Figs 31, 34 & 37.

EXAMPLE 8

Introduction of the chimaeric DNA sequences into the plants

Chimaeric DNA sequences were co-transferred into the plant tissue using the A.tumefaciens system. Transformants were retained by growing on selective medium. Presence of two plasmids was proved using standard molecular biology techniques (Southern, northern hybridizations, PCR). Plasmid A has Barnase gene and plasmid B has Barstar gene under control of plant promoters. This creates an additional selection for co-transformation.

The vector system designed involves a cascade of genes expressing under the positive control of the reproductive organ-specific promoters and negative control of the E.coli lactose (lac) repressor-operator system. During the non-flowering period, in the absence of the repressor protein, the operator sequence will permit constitutive expression of the Barstar gene or antisense RNA in all organs. Because of possible leakage of the reproductive organ-specific promoter, low level of the lethal gene product will exist in different plant organs and cells.

Accumulation of the Barstar protein in cytoplasm or antisense RNA in nuclei of these cells will block the cytotoxic effect of the lethal gene product. At the early stages of flowering, expression of the B (or other RNases) and lacIq genes in the appropriate cells of the reproductive organ will dramatically increase. Accumulation of the lacIq protein in the nucleus of these reproductive organ cells will block expression of the Barstar gene (or antisense RNA) via the lac repressor-operator interaction which will

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increase the level of lethal gene products in the target reproductive organ cells.

EXAMPLE 9

Three eucalypt genes (EGM1, 3 & 2) with homology to other plant MADS-box genes have been cloned and sequenced (Figures IO-13 and 17). Phylogenetic analysis has been performed to examine the relatedness of the eucalypt genes to other MADS-box genes (Figure 18). This revealed that EGM2 is likely to be homologous to the GLOBOSA MADS-box gene group which are involved in development of petals and stamens. EGM3 and EGM1 both fall in the AGL2 group of MADS-box genes. These genes are most commonly expressed in the inner. three whorls of the flower (petals, stamens and carpels) but their function in flower development is yet to be determined.

The pattern of expression of all three EGM genes has been characterised (Figure 14, 15 & 16). Expression of these genes was not detected in any vegetative tissue. The EGM1 gene is expressed in the petals, stamens and the carpel of eucalypt flowers. EGM3 is expressed in the floral meristem and in sepals. EGM2 is expressed in petals and stamens. The EGM3 and EGM2 genes have been shown by Southern analysis (Figure 19) to be single genes. EGM1 is also likely to be a single gene.

The promoter regions of all three of these genes may be used in sterility gene constructs and have been isolated from a eucalypt genomic library. The EGM3 promoter has been engineered into lethal gene constructs.

Northern blots were performed on a range of tissues in order to determine the tissue specificity of expression of the three EGM MADS-box genes. Tissue used for isolation of RNA from roots, seedlings stems shoots leaves and mature flowers was obtained from *Eucalyptus grandis* plants. Tissue for isolation of RNA from floral tissues including receptacles, petals, stamens, carpels, and styles was collected from *E. globulus* flowers. This species was used because it produces very large flowers, making collection of sufficient amounts of RNA much easier. Ten micrograms of total RNA was used in the blots which included the different floral tissues and 20 micrograms was used in blots of *E. grandis* RNA which included the vegetative tissue. Blots were probed with the three EGM genes which had been digested with appropriate restriction enzymes to remove the MADS-box.

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The northern blots indicated that all three EGM genes are expressed at a high level in eucalypt flowers. When northerns were exposed to film for long periods, weak expression of the EGM2 and EGM1 genes was detected in vegetative tissue. The EGM3 three gene was specifically expressed in vegetative tissue. Within the flower, the EGM2 gene was observed to be expressed in stamens and petals. The EGM3 gene is expressed in receptacles, petals, stamens, carpels and styles.

Example 10

The first part (component 1) of the cascade contains the reporter GUS gene under control of floral meristem identity gene promoter (ie. *LEAFY* from *Arabidopsis thaliana*) with *lac*-operator (*LEAFY-op*). The second construct (component 2) contains the repressor *laclq* gene under control of maize *GST-27* gene promoter. This promoter is up-regulated by treatment of plants with the safeners, dichlormid and R-29148. The *Arabidopsis* plants were co-transformed with two components.

An alternative strategy is for two transgenic *Arabidopsis* lines carrying components 1 and 2 separately to be crossed. Expression from component 1 alone produces blue flowers after staining with X-gal. Chemically-inducible induction of expression from component two, in plants expressing component one, eliminate of the blue colour seen in flowers.

Example 11

The first component of the cascade contains the barnase gene under control of floral meristem identity gene promoter (ie. *LEAFY* from *Arabidopsis thaliana*) with *lac*-operator. The second component contains the repressor *laclq* gene under control of maize *GST-27* gene promoter. *Arabidopsis* plants were co-transformed with the two components. In non induced plants expression of only component one produces sterile *Arabidopsis* plants. Induction of expression from component two, in plants expressing component one, stopped expression from *LEAFY-op* promoter and restored the fertility in initially sterile *Arabidopsis* plants.

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Example 12

A number of abbreviations are used in the following text. These are in common use in the field of plant tissue culture.

BA: benzyladenine also known as 6-benzylaminopurine.
IBA: indole-3-butyric acid.
NAA: 1-naphthaleneacetic acid
TDZ: thidiazuron also known as 1-phenyl-3-[1,2,3-thiadiazol-5-yl] urea.

The following is a detailed description of the preferred steps for producing transgenic *Eucalyptus* from shoot and seedling explant material.

Shoot explants

1. Subculture shoots monthly on solid KG medium containing 0.2mM BA. Keep in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) and 22.5°C.
2. Use whole shoots 3-4 weeks after subculture.
3. Remove lower leaves leaving the top 4-6 leaves.
4. Wound leaves 5 to 6 times with a needle (e.g. 25G) and place whole shoots in an *Agrobacterium* suspension (approximately $1x10^8$ cfu ml^{-1}) containing 10-100mM acetosyringone for 10 minutes to 2 hours. An *Agrobacterium* suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Best results when wound near base of leaf. Alternatively, instead of leaving the wounded shoots in an *Agrobacterium* suspension for 1 hour, the shoots can be vacuum-infiltrated with the *Agrobacterium* for 10-30 mins at 40-100 Kpa. The shoots do not need to be wounded for the vacuum infiltration procedure. However, callus formation is greater when wounding is effected with a needle rather than by vacuum-infiltration.
5. Blot shoots between sterile filter papers and insert shoots vertically into KG medium containing 0.2mM BA. Co-culture for 2 days in the dark.
6. Transfer shoots (still upright) to KG medium containing 0.2mM BA and 200 mgL^{-1}

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cefotaxime. Keep in low light for 5 days (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR).

7. Excise 4-6 upper leaves and place them, adaxial face up, on solid callus induction medium G22 containing 2mM BA, 2.5mM NAA, 200 mgL^{-1} cefotaxime and 5-10 mgL^{-1} geneticin. Incubate for 2 weeks in the dark.

8. Transfer explants to G22 medium containing 2mM BA, 2.5mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 15 $mg.L^{-1}$ geneticin. Subculture every 2 weeks in the dark. Brown phenolic compounds are produced when incubated in the light.

9. After 6 weeks transfer explants to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin. Leave in dark for 5-6 days then move into light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

10. After 8-10 weeks on GBA with 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin transfer pieces of callus with buds and callus formed on the original explants to GBA with 200 mgL^{-1} cefotaxime and 30 mgL^{-1} geneticin. Subculture every 2 weeks.

11. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks. The liquid cultures are shaken at 100-120 rpm with 8 ml of liquid in a 70 ml container.

12. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

13. Assay putative transformed shoots for marker gene(s) activity.

14. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

The protocol detailed above will take between 1 and 8 months from transformation to regenerated shoots.

Seedling explants

1. Disinfest seeds and germinate on KG containing 0.2 mM BA.

2. Using 10-12 day old seedlings, remove roots and place them in an overnight-grown *Agrobacterium* suspension (approximately $1x10^8$ cfu ml) containing 50mM acetosyringone. An *Agrobacterium* suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Wound the cotyledons and hypocotyl by gently stabbing with a 30 gauge syringe needle under a dissecting microscope. Incubate for 1 hour then remove the seedlings from the suspension and blot them between sterile filter papers to remove excess liquid. Instead of leaving the wounded cotyledons in an *Agrobacterium* suspension for 1 hour, the cotyledons can be vacuum-infiltrated with the *Agrobacterium* for 20 mins at 95 KPa (28mm Hg). The cotyledons do not need to be wounded for the vacuum infiltration procedure. However, the hypocotyls require wounding even when using vacuum infiltration.

3. Co-cultivate on KG medium (containing 0.2 mM BA) for 2 days in the dark making sure that the seedlings are standing upright in the medium.

4. Transfer the seedlings (still upright) to KG medium (containing 0.2mM BA) and containing 200 mgL^{-1} cefotaxime. Continue incubation in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) for 5 days.

5. Excise the hypocotyls and cotyledons. Transfer the hypocotyls to callus induction medium G22 containing 0.5mM BA, 1.0mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Transfer the cotyledons to G22 medium containing 1.0mM BA, 1.0mM NAA, 0.3mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Continue incubation in the dark for 2 weeks.

6. Transfer explants to the same medium containing 15 $mg.L^{-1}$ geneticin and 200 $mg.L^{-1}$ cefotaxime. Continue dark incubation. Subculture every two weeks until about 6 weeks have elapsed.

7. After 6 weeks, transfer to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 15 mgL^{-1} geneticin. Leave the cultures in the dark for 5-7 days then transfer them to the light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$

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PAR).

8. After about 10 weeks on the GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime, a number of explants will have produced shoots. These shoots are excised and transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime. Meanwhile, the callus that has also formed on the original explants is subcultured back to GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime and left for a further month, by which time more shoots may develop which can then be transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime.

9. All shoots and callus are now transferred to fresh KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime for another month or so.

10. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks.

11. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

12. Assay putative transformed shoots for marker gene(s) activity.

13. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

Seedlings of 12 days old or younger are best for transformation but seedlings up to 20 days old can be used for regeneration.

Shoot regeneration frequencies for non-transformed cotyledon and hypocotyl explants without selection are approximately 80%.

Experience has shown that removal of cefotaxime from the medium, even after 3 months, will result in rapid overgrowth by *Agrobacterium*. The concentration of cefotaxime varies between liquid and solid media.

Shoot material grew faster in liquid medium and formed more shoots when

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compared to shoots grown on solid medium. This liquid selection step has the advantages of reducing false positives by increasing selection pressure, reducing residual *Agrobacterium* and increasing the amount of shoot tissue compared to solid grown cultures.

The regenerated rooted transgenic plants are then moved to a soil based medium and grown into trees of a size and form suitable for planting. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

The media used in this study were G22, GBA and KG as described by Laine and David (1994). However, various basal media, including MS, B5 and P24, have been tested and found to support shoot regeneration. The plant growth regulator regimes were generally different from those of Laine and David (1994), which were in the combination of 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for callus induction from leaves; 1-3 mM BA, 0.05-1 mM TDZ and 0.5-2.5 mM NAA for cotyledons; and 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for hypocotyls. The differentiation medium was generally a GBA medium, which was a G22 but supplemented with 2.5 - 5 mM BA and 0.5 mM NAA (Laine and David, 1994). A KG medium containing 0.2 mM BA is generally used as subculture medium for clone materials. All media were solidified with 0.25% Gelrite or Phytagel. pH was adjusted to 5.7 - 5.8 using potassium hydroxide before autoclaving for 15 minutes at 121 C.

A preferred method for the preparation of *Agrobacterium* inoculum is described below.

1. Streak out the *Agrobacterium* strain containing the construct onto plates with selection, eg YEP + rifampicin (50 mg/L) + kanamycin (100 mg/L) for LBA4404, EHA105 and AGL1 containing the pBin GUSINT construct.
2. Incubate plate at 280C for 2 days.
3. Pick a single colony into YEP broth with selection and grow on shaker overnight at 280C.
4. Use the overnight culture to inoculate (1% inoculum) fresh medium with selection and

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grow on shaker overnight at 280C. This additional step will produce a more consistent inoculum for plant transformation.

5. Harvest *Agrobacterium*, wash and resuspend in tissue culture medium. Dilute to an appropriate concentration ready for transformation.

6. Streak out the resuspended *Agrobacterium* on to lactose yeast medium plates and incubate plate at 280C for 2 days. Do a Benedict's test (Bernaerts and Deley, 1963) on the colonies to confirm they are *Agrobacterium*.

This strategy will give good thick cultures of *Agrobacterium* on the day of transformation. A culture grown overnight which was inoculated from a plate or glycerol stock will give variable results.

An AGL1 culture grown as described above and diluted to an optical density (600nm) of 0.98 had a viable count of 2.37×10^9 cfu/ml.

It will of course be realised that while the above has been given by way of illustrative example of this invention, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as claimed in the claims appended hereto.

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CLAIMS

1. A method of regulating a eukaryotically active gene, comprising transforming a cell with a construct expressing a modulator gene product regulating the eukaryotically gene or its product and a further gene product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

2. A method of regulating a eukaryotically active gene according to claim 1, wherein said eukaryotically active gene is selected from a native gene to the organism of interest or an introduced gene.

3. A method of regulating a eukaryotically active gene according to claim 2, wherein said eukaryotically active gene codes for an expression product selected from an active polypeptide, an enzyme or an enabling gene product for a synthetic pathway.

4. A method of regulating a eukaryotically active gene according to claim 3, wherein said synthetic pathway is selected for production of pigments and dyes.

5. A method of regulating a eukaryotically active gene according to claim 4, wherein said dye is such as anthocyanin.

6. A method of regulating a eukaryotically active gene according to claim 4, wherein said synthetic pathway is selected for production of an insecticidal compound.

7. A method of regulating a eukaryotically active gene according to claim 6, wherein said insecticidal compound is Bti toxin.

8. A method of regulating a eukaryotically active gene according to claim 3, wherein said enzyme is selected from a ribonuclease having a lethal effect on a target tissue

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and a peroxidase conferring nematode resistance on root tissue.

9. A method of regulating a eukaryotically active gene according to claim 8, wherein said ribonuclease gene is that of Barnase from B.amyloliquefaciens.

10. A method of regulating a eukaryotically active gene according to claim 9, wherein said gene is constructed including the coding region of Barnase from B.amyloliquefaciens under the control of promoter sequences of genes expressed in the male and female parts of plant reproductive organs at the early stages of development.

11. A method of regulating a eukaryotically active gene according to any one of the preceding claims, wherein said eukaryotically active gene is constructed with a promoter selected from those promoting eucalypt genes designated EGM1, 3 & 2 in *Eucalyptus grandis*.

12. A method of regulating a eukaryotically active gene according to any one of claims 1 to 10, wherein said eukaryotically active gene is constructed with a promoter selected from those promoting pine genes designated PrMADS1, 2 and 3 in *Pinus radiata*.

13. A method of regulating a eukaryotically active gene according to any one of the preceding claims, wherein said eukaryotically active gene, modulator gene and further gene and their respective promoters may comprise a transformation cassette with which the target organism may be transformed.

14. A method of regulating a eukaryotically active gene according to claim 13, wherein said the eukaryotically active gene is promoted by a developmental promoter and wherein the promoter of said modulator gene is constitutive.

15. A method of regulating a eukaryotically active gene according to claim 13,

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wherein said promoter for said eukaryotically active gene is tissue specific and is inhibited by a constitutively-promoted inhibitor gene product, which in turn is controlled by an inducibly-promoted further gene product.

16. A method of regulating a eukaryotically active gene according to claim 13, wherein said eukaryotically active gene product is contitutively expressed subject to the modulating effect of a modulator gene expressing under the control of a tissue specific promoter, the modulator gene or product being itself controlled by the expression of a further gene under the control of a promoter specific for a different or complementary tissue.

17. A method of regulating expression of a eukaryotically active gene, comprising transforming a cell with a transformation cassette comprising said eukaryotically active gene, a modulator gene expressing a product regulating the eukaryotically gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

18. A method of transformation of a propagatable eukaryotic cell with an expression cassette including:-

a lethal gene expressing a cytotoxic peptide in a target tissue under the control of a promoter substantially specific to said target tissue;

an modulator gene constitutively expressing an inhibitor of the production or action of said cytotoxic peptide, and

a further gene under the control of said substantially specific promoter and functioning to block said inhibitor or modulator gene.

19. A method of regulating a eukaryotically active gene according to any one of the

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preceding claims 1 to 16, wherein said eukaryotically active gene is selected from Barnase and Barstar genes from an extract of B.amyloliquefaciens, isolated by PCR by the primers designated P1 and P2 (for Barstar) and P3 and P4 (for Barnase) and comprising:

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
EcoRI

P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'
HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

20. A method of regulating a eukaryotically active gene according to any one of the preceding claims 1 to 16, wherein said eukaryotically active gene is a lethal gene wherein the cytotoxic effect of the lethal gene expression product is increased by coupling a localisation signal sequence to the construct.

21. A method of regulating a eukaryotically active gene according to claim 20, wherein said eukaryotically active gene codes for an Rnase targeted into the nucleus by fusion of a nuclear localisation signal (NLS) sequence to the RNase gene.

22. A method of regulating a eukaryotically active gene according to claim 21, wherein the gene is the Barnase gene and said fusion is by PCR, wherein a set of PCR primers for fusing the NLS sequence from VirD2 gene of A.tumefaciens to the B gene are used and comprising:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

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BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

23. A method of regulating a eukaryotically active gene according to any one of the preceding claims 1 to 22, wherein said repressor gene and inhibitor gene are adapted to form a specific repressor/operator couple.

24. A method of regulating a eukaryotically active gene according to claim 23, wherein said repressor/operator couple comprises the E.coli lacIq gene under control of the same promoter as the eukaryotically active gene construct and used to repress the action of the repressor gene promoter modified by insertion of a lac operator sequence.

25. A method of regulating a eukaryotically active gene according to claim 24, wherein said repressor gene promoter is a modified 35S RNA CaMV promoter having at least one lac operator between the promoter and coding region of the repressor.

26. A method of regulating a eukaryotically active gene according to claim 25, wherein said modified 35S-RNA-CaMV sequences are amplified by PCR using primers designated 35S1 and 35S2 and comprising:

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

27. A method of regulating a eukaryotically active gene according to claim 25,

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wherein said modified 35S-RNA-CaMV sequences are amplified by PCR using primers designated OP1 and 35S2 for the introduction of lac operators into both the 3' and 5' region of the modified 35S promoter:

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

28. A method of regulating a eukaryotically active gene according to claim 24, wherein said laclq gene is isolated with enzyme cleavage sites for cloning into the plant vectors, using two primers corresponding to the 5' and 3' ends of the gene and designated AM 1 and AM 2:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
Kpn I

29. A method of regulating a eukaryotically active gene according to claim 28, wherein the 3' end of the laclq gene is fused to the NLS sequence by a set of PCR primers:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of laclq 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of laclq

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

30. A transformation cassette comprising a eukaryotically active gene, a modulator

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gene expressing a product regulating the eukaryotic gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

31. An expression cassette including:-

a gene expressing a peptide cytotoxic to a target tissue under the control of an inducible promoter substantially specific to said target tissue;

an inhibitor gene expressing an inhibitor of the production or action of said cytotoxic peptide, and

a repressor gene under the control of said inducible promoter and functioning to block said inhibitor or inhibitor gene.

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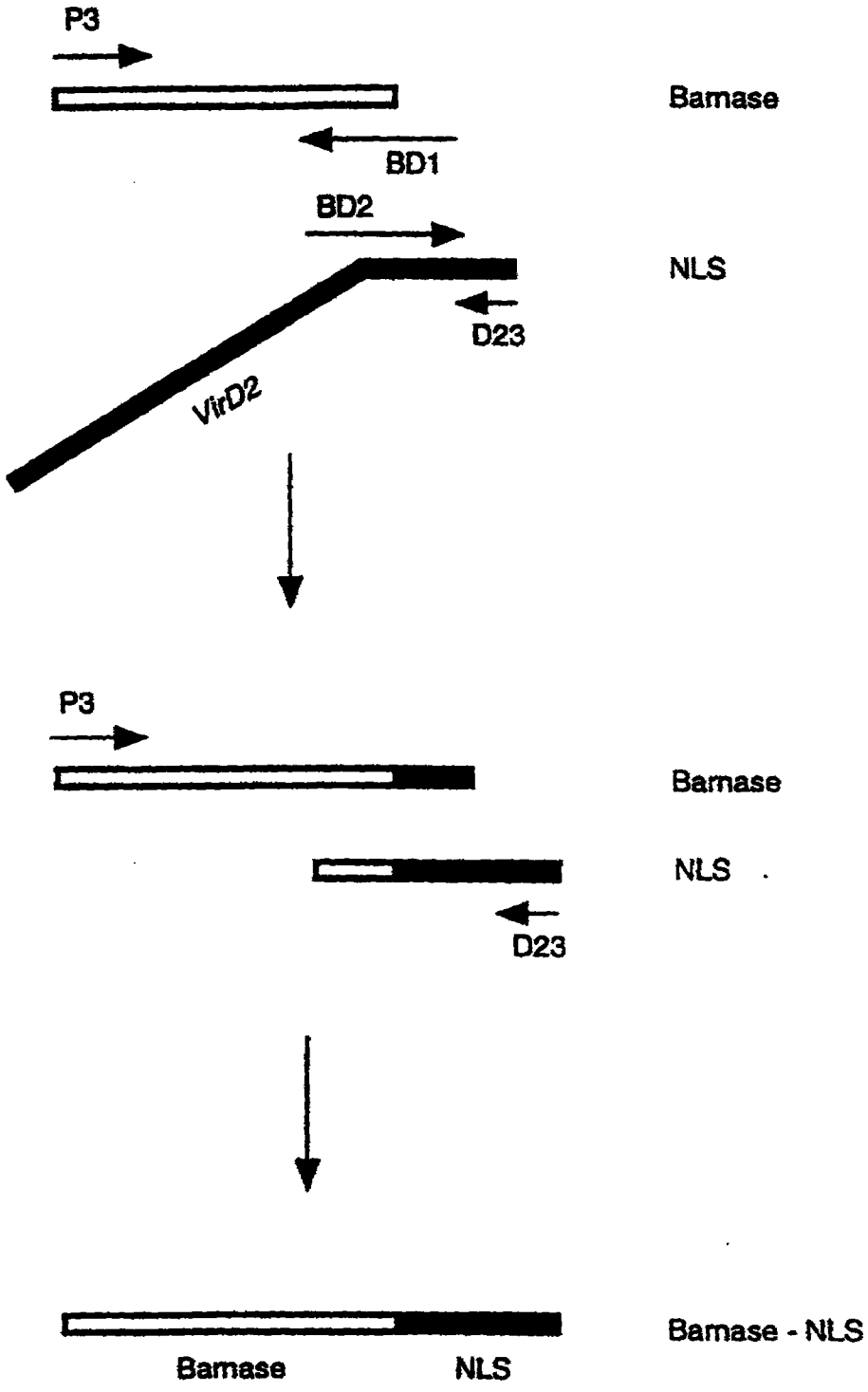


Figure 1

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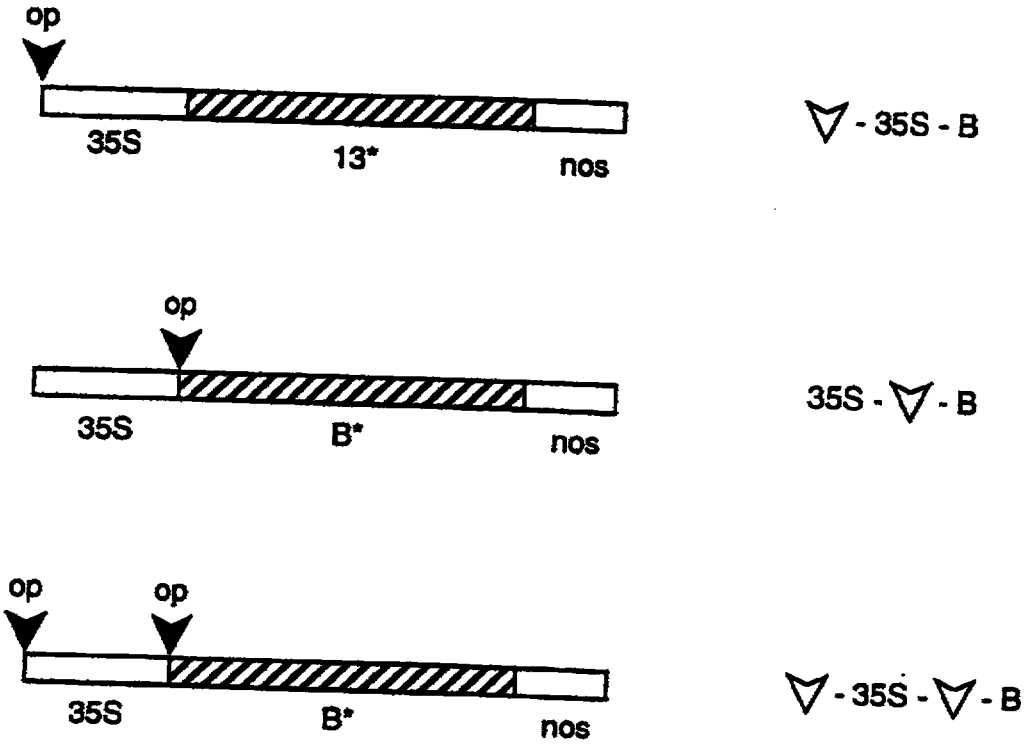


Figure 2

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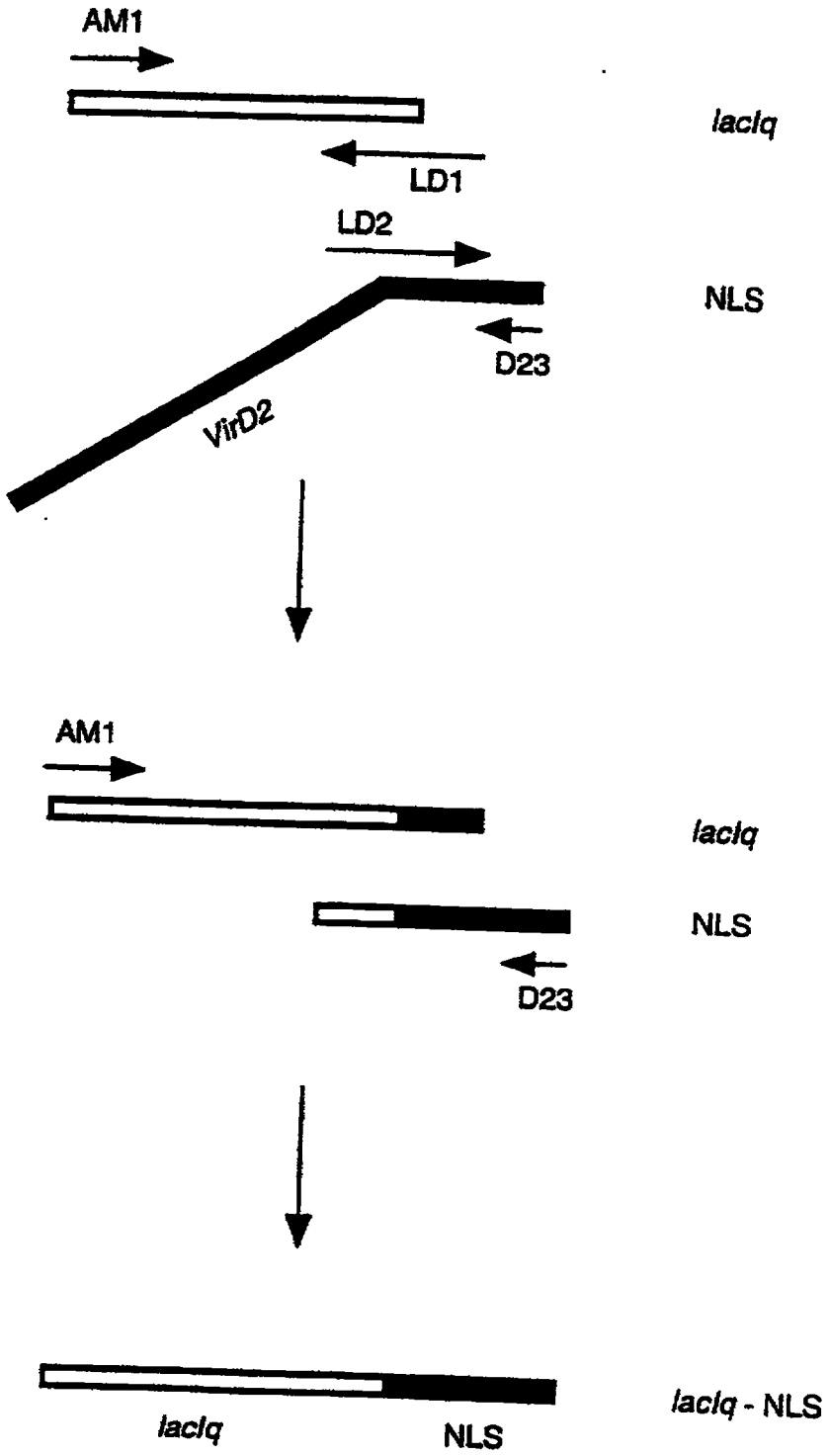
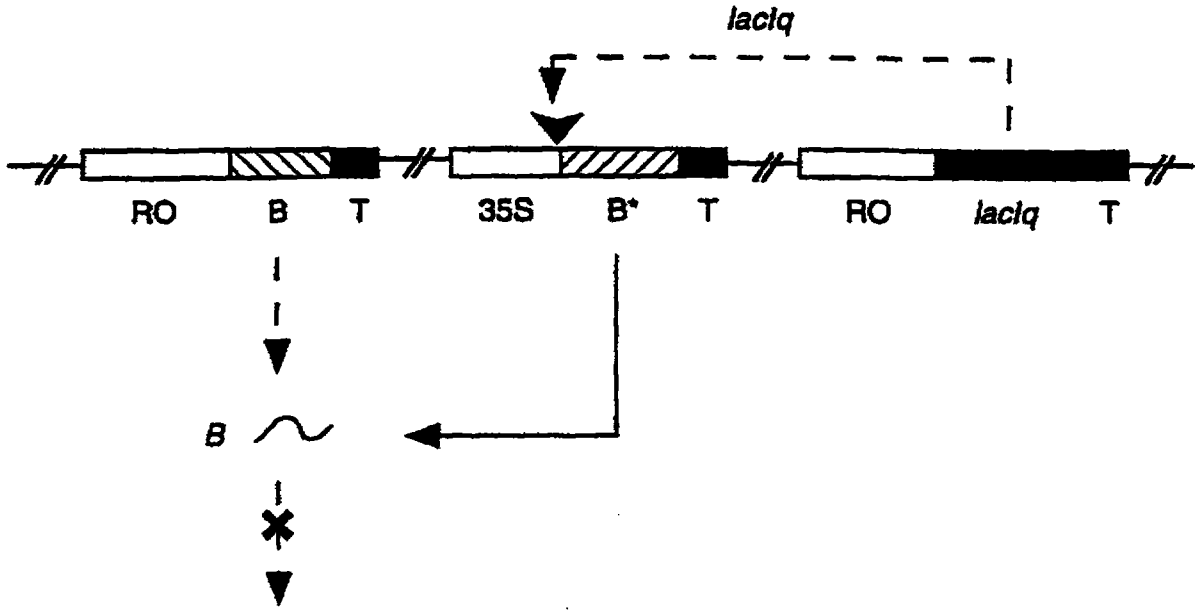


Figure 3

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A. non - flowering period



B. flowering period

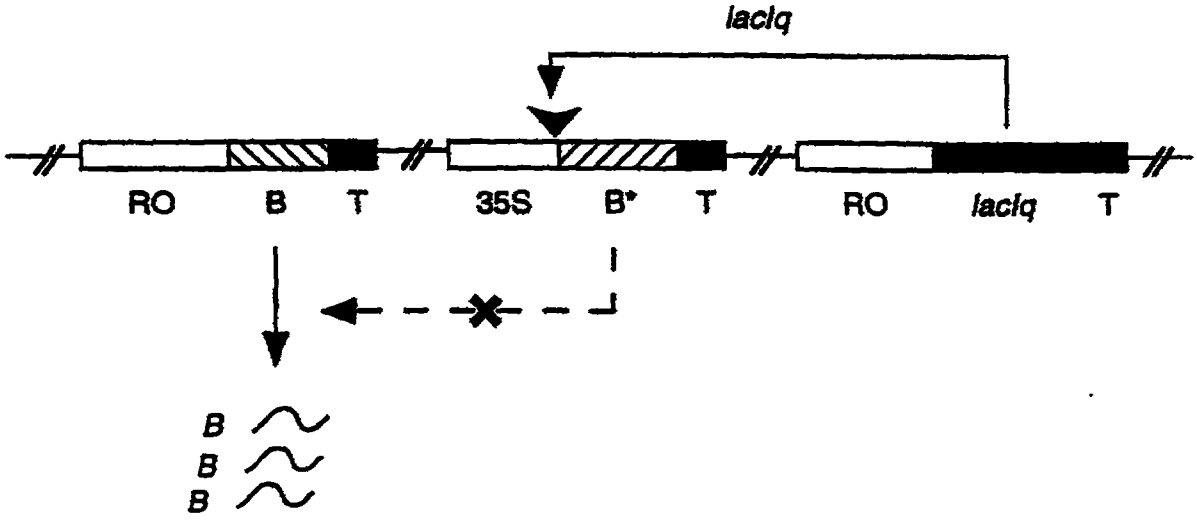
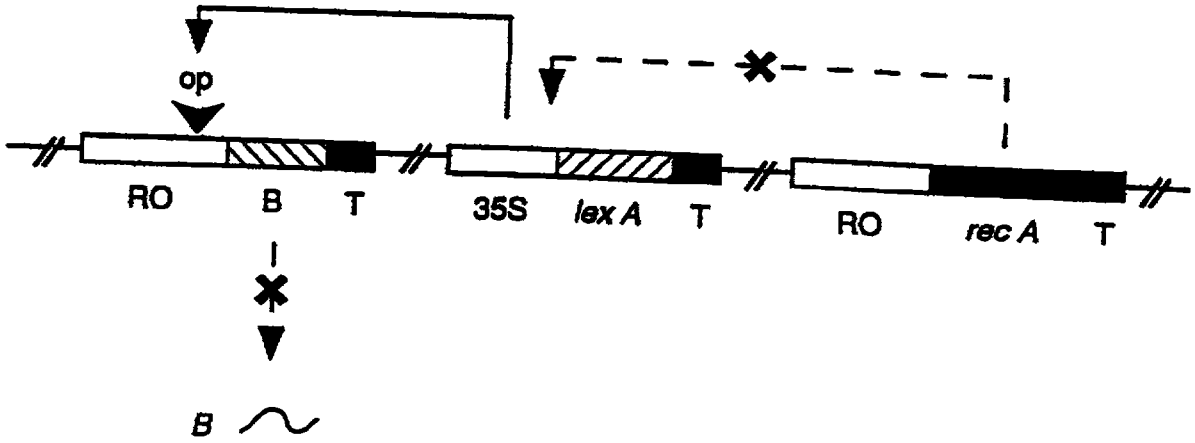


Figure 4

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A. non - flowering period



B. flowering period

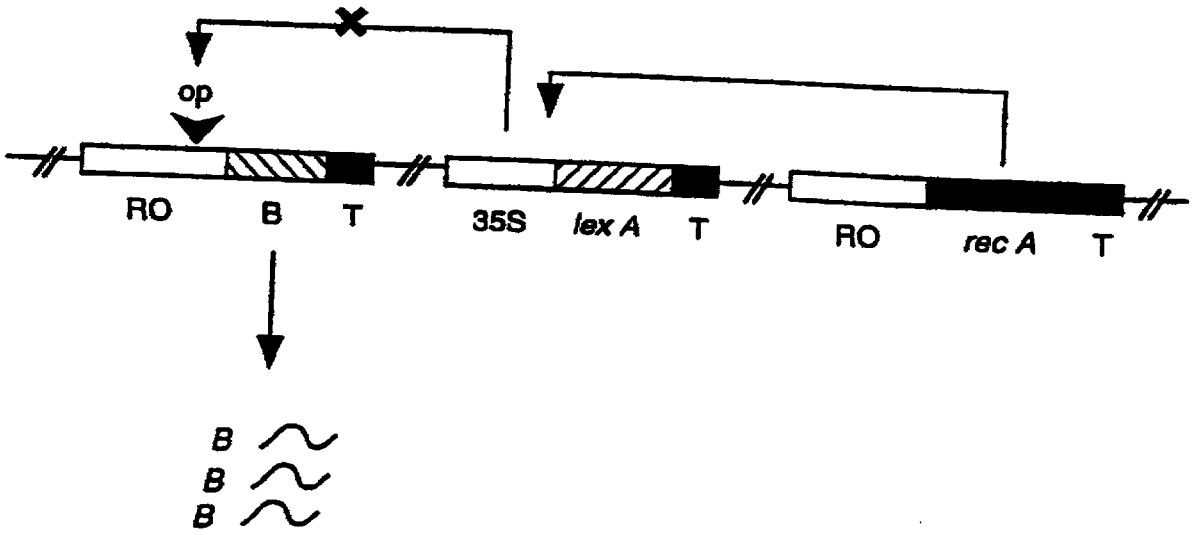


Figure 5

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A. non-flowering period



F flowering period

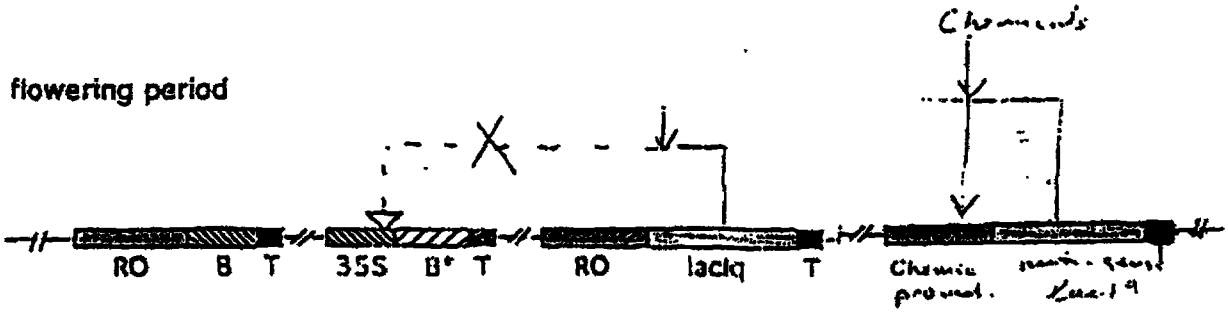
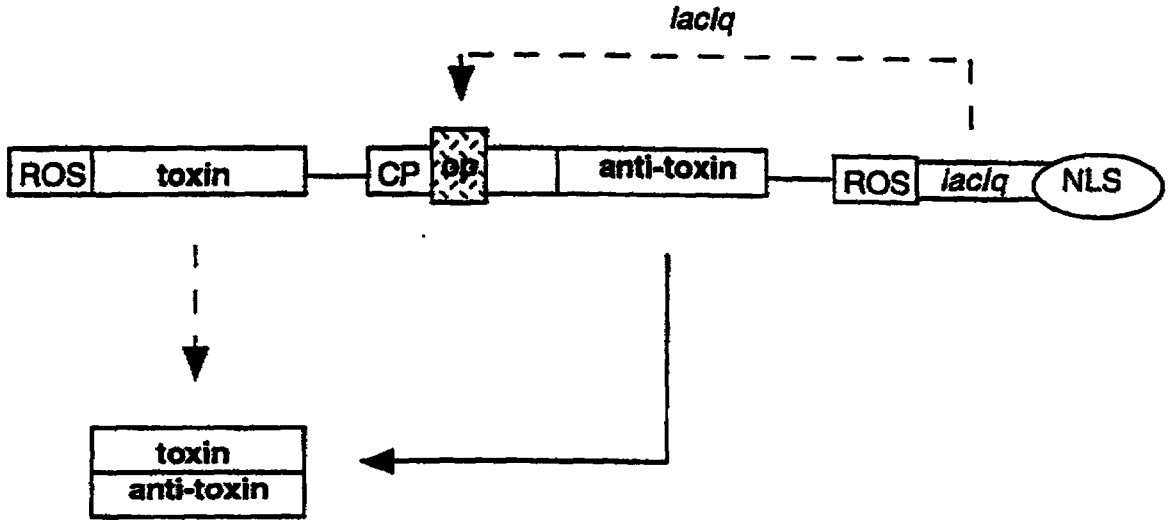


Figure 6

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A. non - flowering period
(in all tissues)



B. flowering period
(in reproductive tissues)

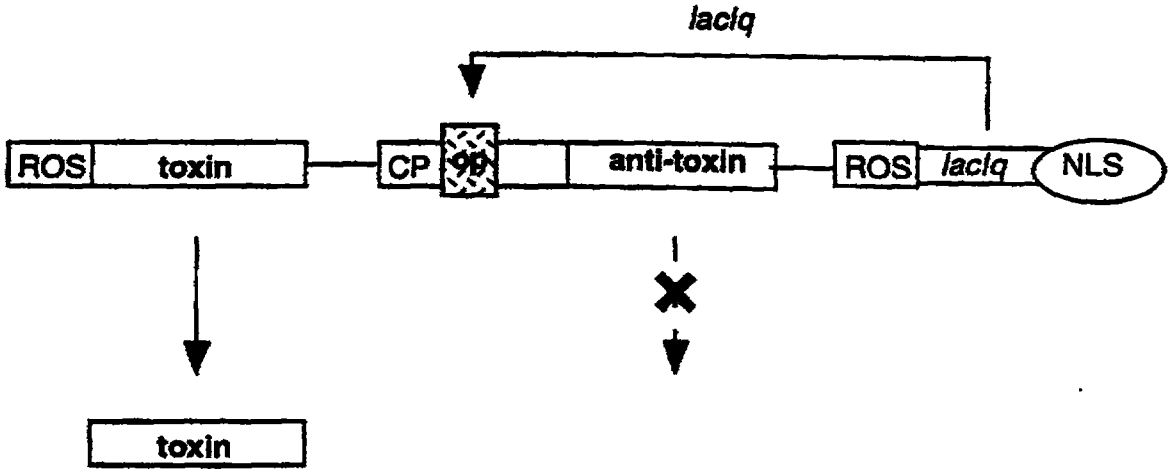
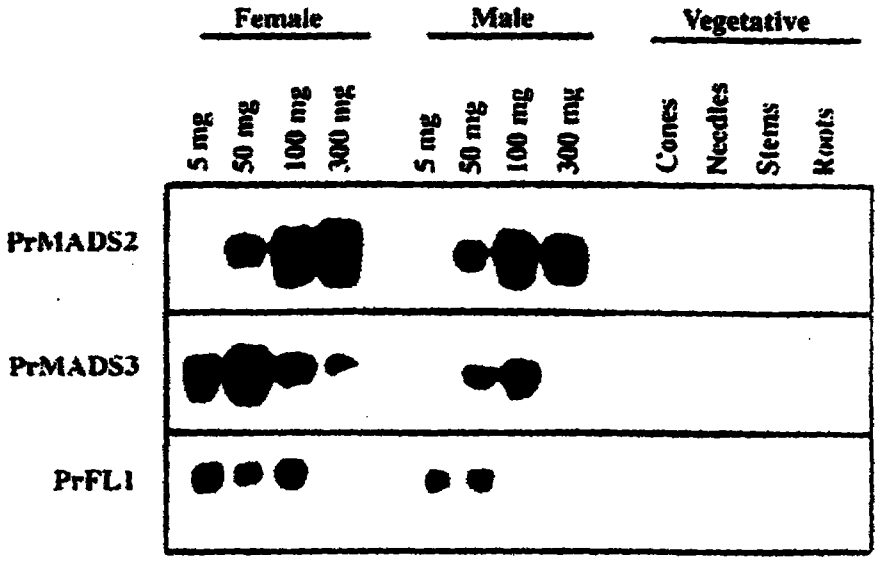


Figure 7

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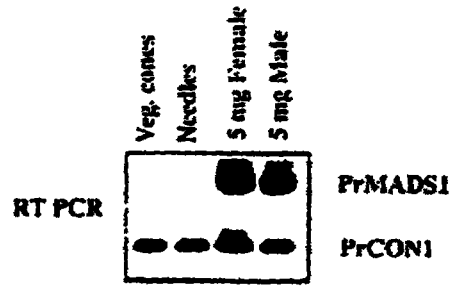


Figure 8

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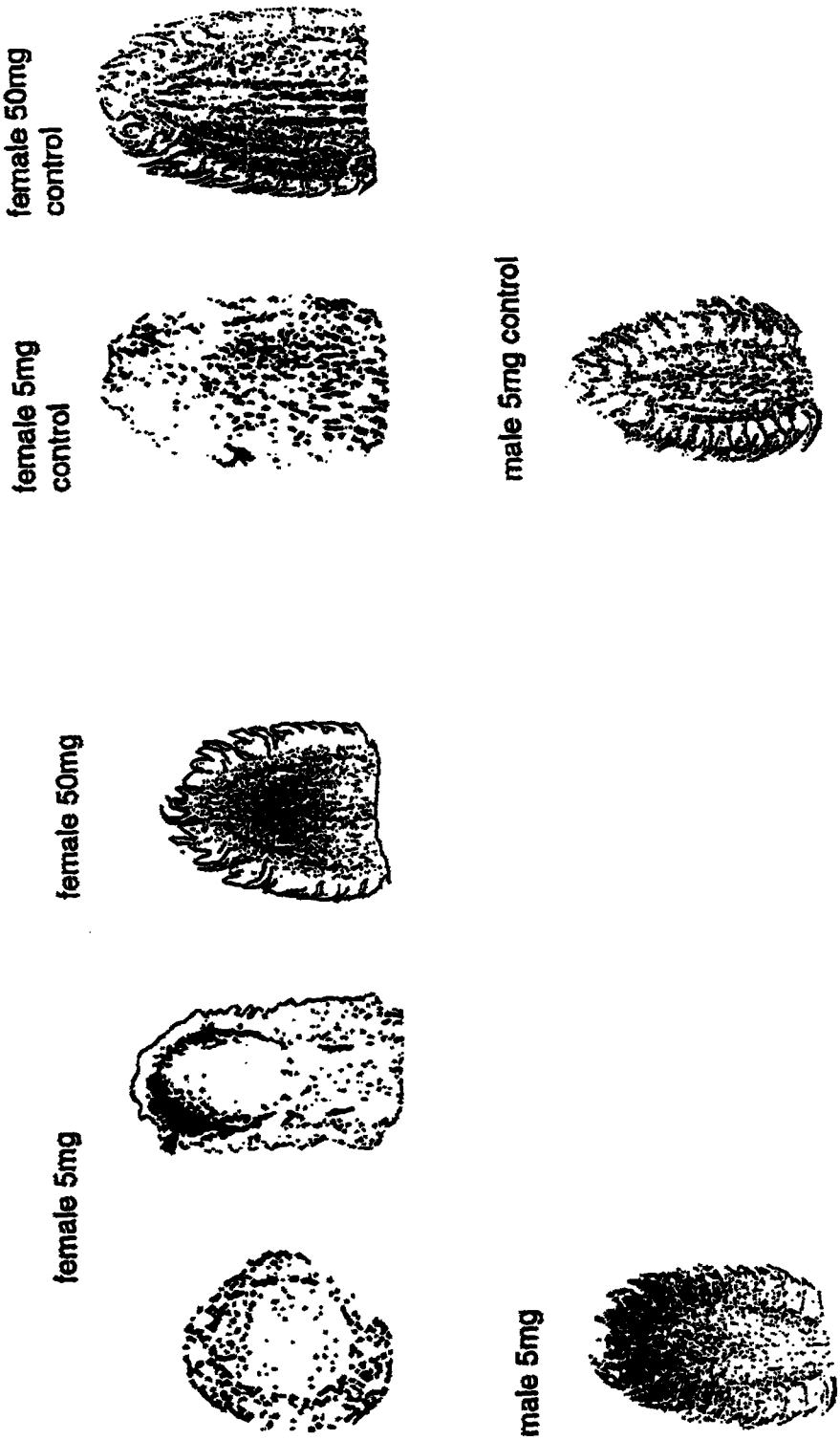
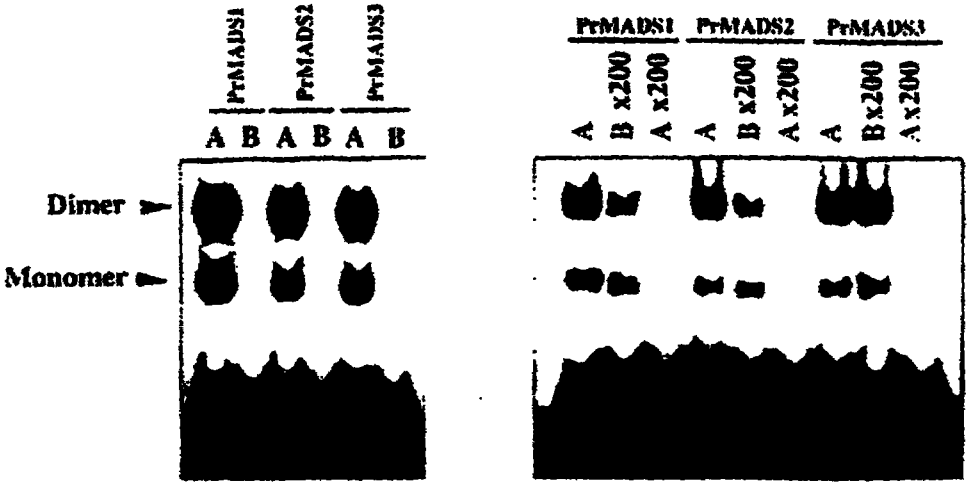


Figure 9

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AG consensus CArG-box
NTT(A/T)CC(A/T)NNGG(-G)(A/t)2N

A: TTACCAAAAAAGGAAA

B: TTAGGAAAAAACCAAA

Figure 10

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-373 TTTTACTTGGCATAATACATGCACGAACCACTATGCAATCAACCAATTGGGAAGGTTTTTTTTTTGTTTTTTTTTTTTTTTT

-293 TGGGGGGGGGGTGTTCTGGTTTATTGGGTGGTTCTGGTCTCTTGACATTGTAATTTCCCCTTAAAATTCCGCTTCATGACA

-213 AAAAGTACATAGAGGGGCTACTATCTTTGTTGACTTTTACCGTGTAGAGGGTTCCTAAGAGAGAGGGGGGAGGGAAGAGA

-133 GAGACCATGCATAAGTATGGCTAGACACAAGAAAGGTTGCAGAACCGAGAAGAGATCTCTTTTGAAGAAGTGAACAAATT

-53 TGTGGGATTGTGGGTTGGTGACGAAAACAGCAAAGACAAGAGAAAGGGGGAAAGATG

Figure 11

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-940 ATAGAGCAGTACACTTTAAGGGAGCACGCACCCCAATTTGATATAAGGTACAAAGTTTTGTTCCAAGATGTGGGTGACAT

-860 CATTAAATGAGATTTACCATCTTTCAACAGGTTCAAAGGGTACCCTGTACTATAAAGCCCACGAAGCTATTTGTATGTTA

-780 AAAAAAAATTCACATAGACTAGGTACGATCGAAAAGCTCATTAAGGAGCTGCCAACGAAAAGTTCACTTTGTTAACCTCT

-700 CCTCTTATCTTATATTCAATAAGAATATGCACATCGGTTGTGATTTGATGCTCAATTGAACTTGACATTTGGTTATTGGT

-620 TCTACCCACTTAAACCCTAAGCAAAAAGGCCAGAAATGTGCACACATTATTCATCAAGCCGGGTGCCACGTGTCCAGTAA

-540 GACAAACTAAATAAGGAGAAAATACTGGTAAGGAAGCCTATGTTGTGTATGTAAGATACCCAATGATAGTCCGTGGTTTT

-460 GATAAGTTCAAACTTAACATTTGAAAAAGGGACAATATGACGAAGCTGCAGTTAGAACAGGAGAACACCCCAACCCGAGA

-380 AAATATCCGGTCGTTTAATATCAATAGCTCACGTCAGTGCAGGATGTCTGAAAATGACCGATGGTCACACGCGCGTGACA

-300 TGGAAGAGAAGAACCAATAACCAGAAAGCCCTAATAATGAACAGTAGACTTTAGCATGAAACCTCTCACCTTTTCTTTTG

-220 TGCCAAGAAAGTCGCAGTTCCCCTTAACCCTAAATGGAATGTTGTGTCTTTACTTCTGTACCTCTCCTTTTTTTCAGCCT

-140 CTCTCTCTCTCTCTCTCTCTCTCTCAGTCTCAGCTATTCAGCTATTCTGCTCCTCAGCTTTCATTTGCAAACAAGAGC

-60 AGATAAGGAAGATAATACGAGAGAGAACAAGAGAGAAAGAGAAGGCCCGGAAAAAGAAAG<u>ATC</u>GGTCGAC

(mutated ATG)

Figure 12

GATTGTTACGATCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGG

CCGCCATGGCCGCGGGATTTAATACGACTCACTATAGGGAGCTCGATCTCTT

GAAAGGGAAAATCCGTGTCGTTTTTCTTCGTAAACAAAAGTCGATGAAATAATA

TGTATTGTTACTATATATAAAACAAAAGTGCTCGTGAACATTGTCAAATACTATA

AGGTATAGACAGGTTTATGACTCACAATTACGAAGGGGCCTAAAAGCCGAGAG

GGAAAAGGTAATGCTAGGTATAGTACATCTAGACTATTAATTGATATAATAAGTA

AGTGGATCATGTTCAATTTCAGATAAAAATTTCTATGTCACGTTGTTTAATATTTG

AATTTAATTATTGATTTTATTATATTATTCTTTGTCATATTAATTAATTAGATTGTG

GATATTTGCCGATTCTCATGAGAGAAAATTGATCTTTAATTAATTTCAAAATACC

ATGGCAAGAAATCATAAGAAAACTCACTCTTCCCCACACGCACGCCACATCG

TGCTCTAGGTTAAATAGAGATGTGATCTCCAAATTTTTTGTCTGGAGCAATGCT

AAAGATGCTAAAAAAGTTTAGCAAGTAGGATGACTAACTTGAAATAGGGCAGTC

AATAAACCAAGTCTAACGATCAATTACTACATTACACGTAATGAGAAACATTTC

GAGAAAATCTCTCTATTTATTGTGGTTACATAAGTAGCCTACCTATTTGTAATTA

TGCAAATTAACCTAGGACGAGCCATATATGCAGTCGACATTCATAAGGCTTTAT

AGTTTGTGCTTCCGGAAATAGCTTAGAAAACCACATACACTTTTCATCAAGACT

AATCTGACAGTAAAAAAAGGAAAAAGAAAAGAACACCATCAAGACTAATGTAAT

GAAAAGAAAACCCTATTACTAATTTTGAGAGTTTTCTTGTAGACCCATCACGTC

ATCCATCTAAAACGATGCAATGCACACAACTAATGACAAGGGACATGTTAATAT

ACAGCAAACCTACCAGGTTTCCCCTTCCTTCATTAGAGTATAATAACTGTTCCC

TTTTCTACCTCCTTTTTGTACCCTAGCTCTCGAGCTCCTTAAGAAAGTTTGCTC

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CAGAGATATCGGAATTC

Figure 13

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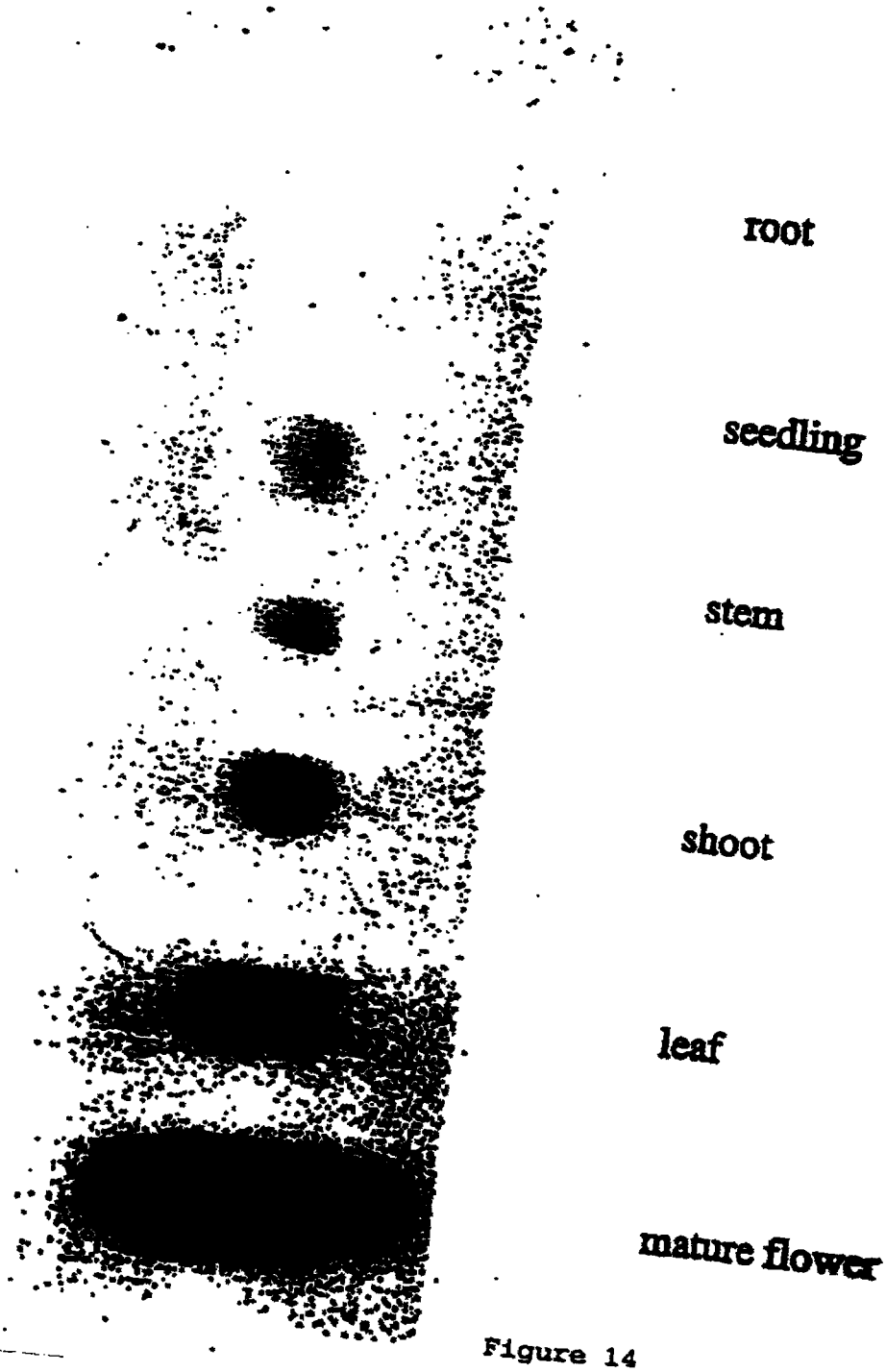
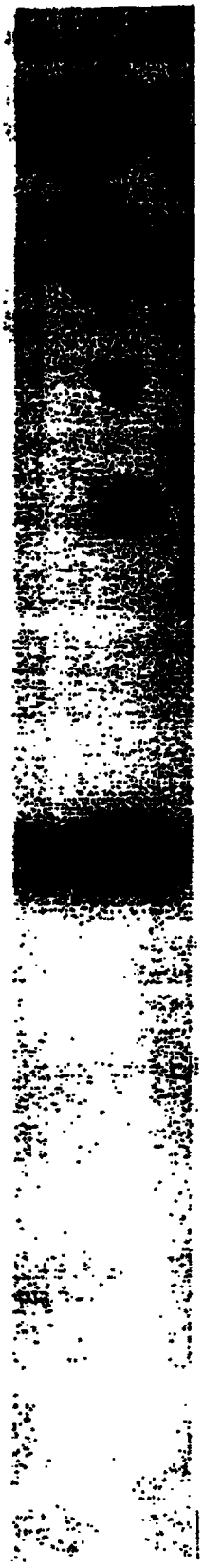


Figure 14

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receptacle

petal

stamen

carpel

style

mature flower

root

seedling

stem

shoot

leaf

Figure 15

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Figure 16

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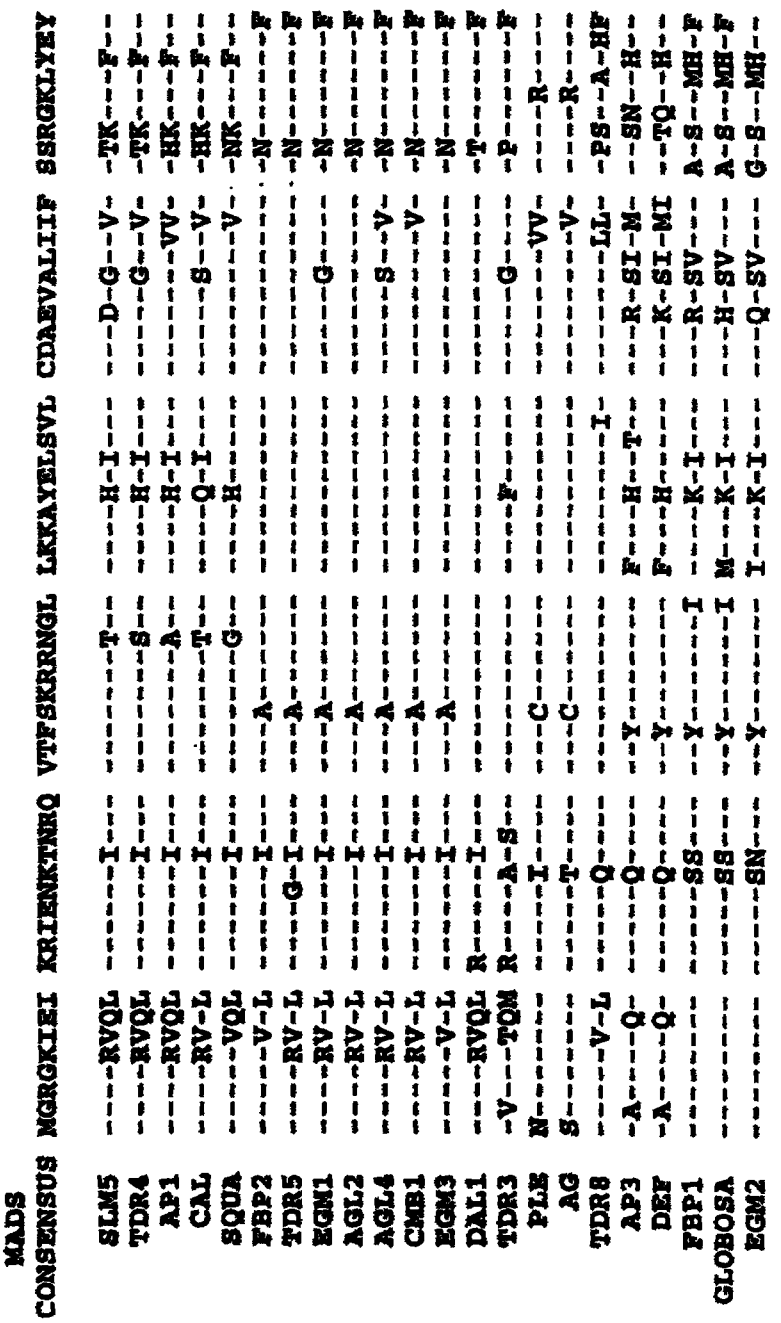


Figure 17

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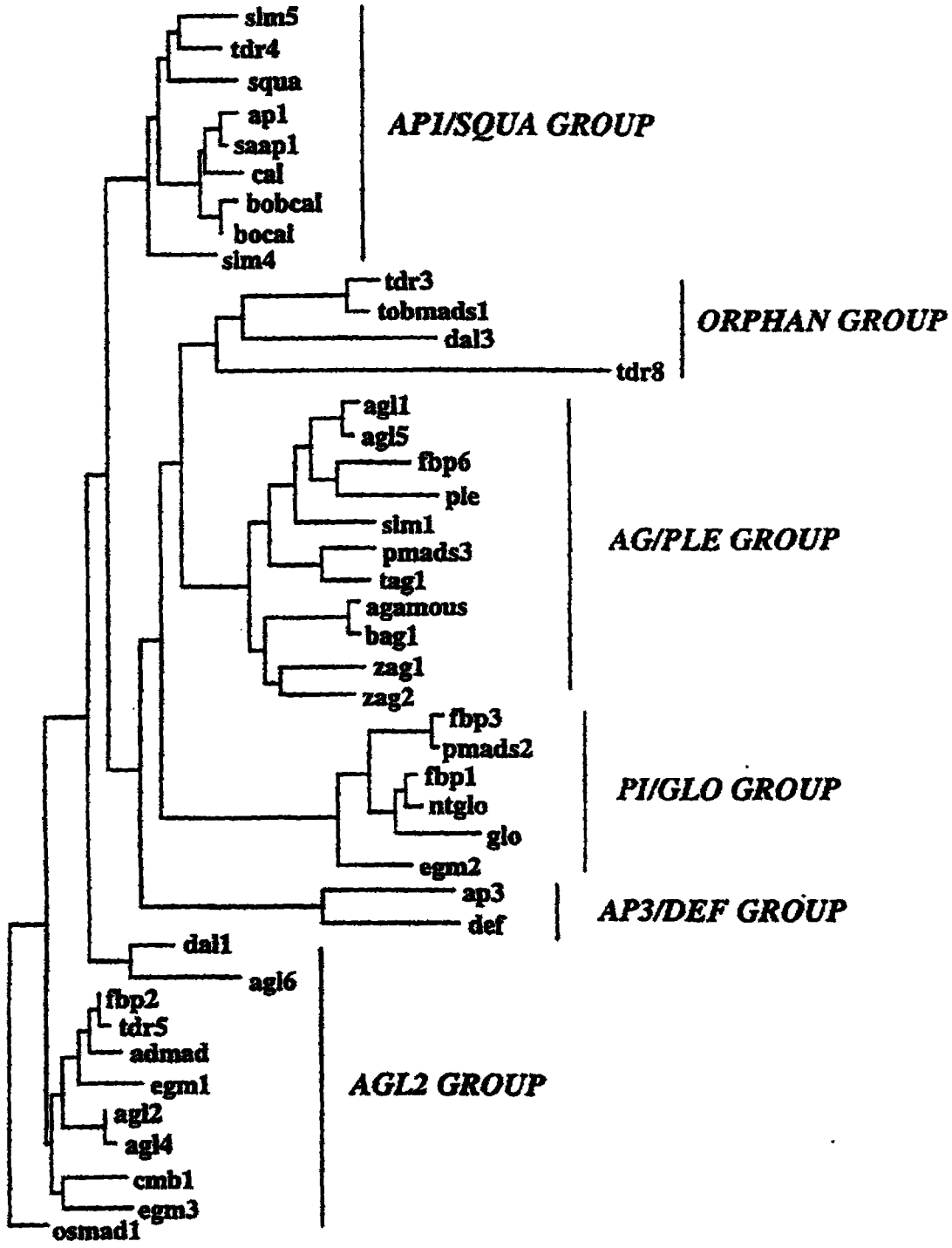


Figure 18

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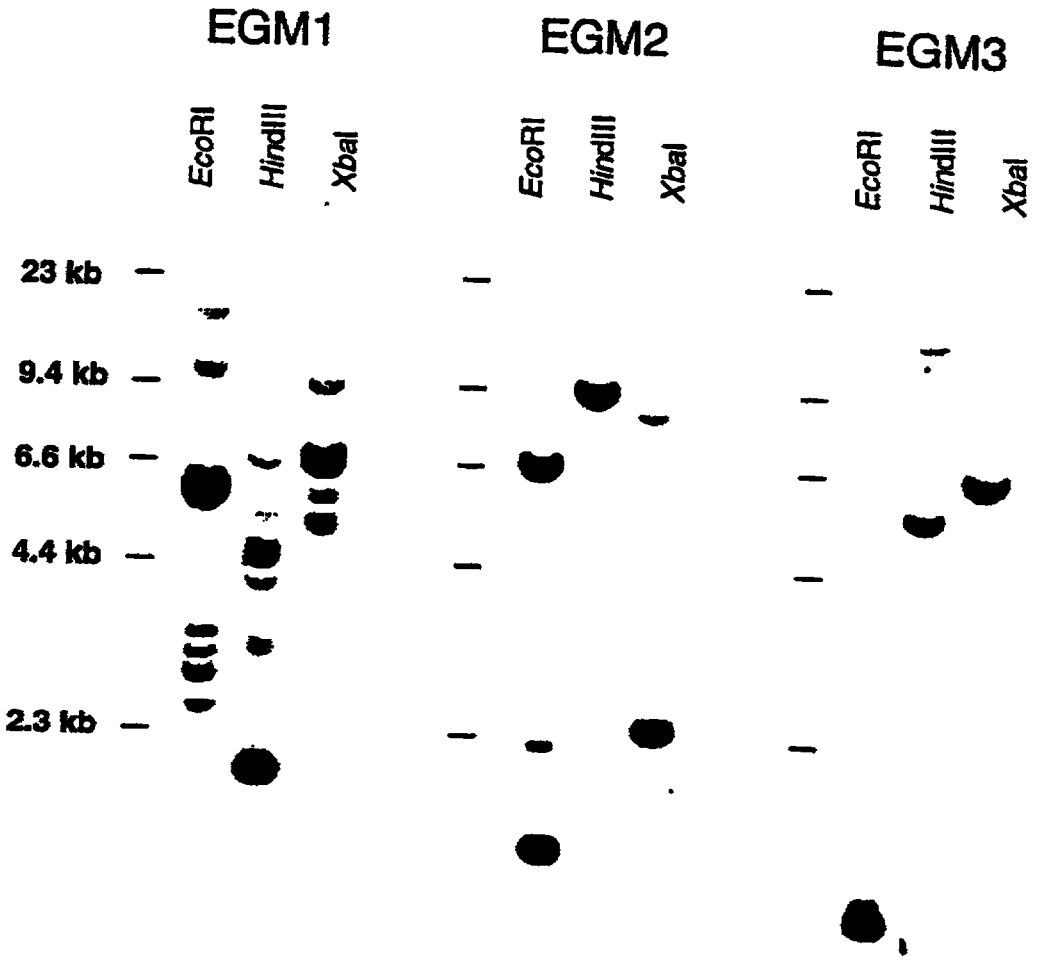


Figure 19

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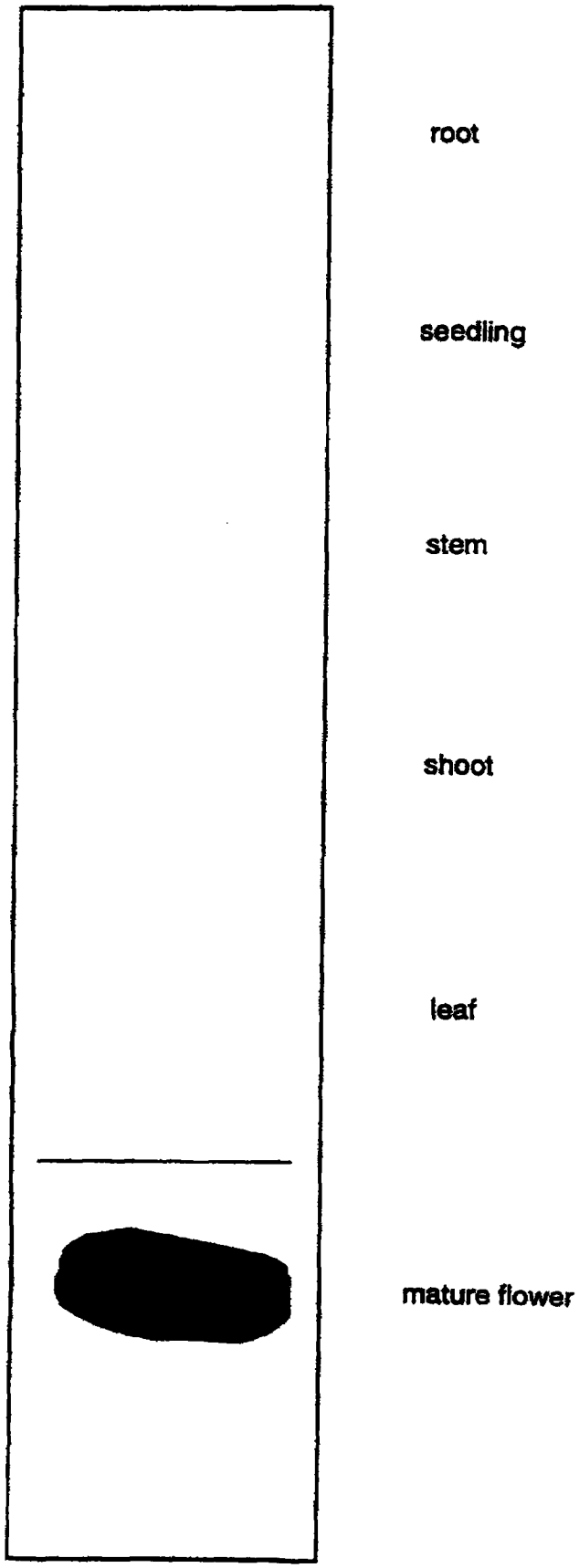


Figure 20

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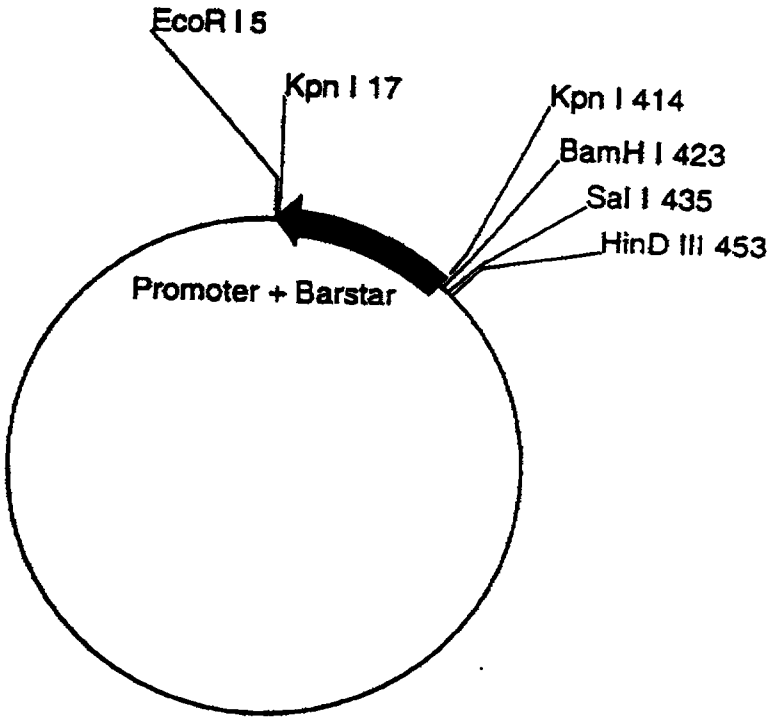


Figure 21

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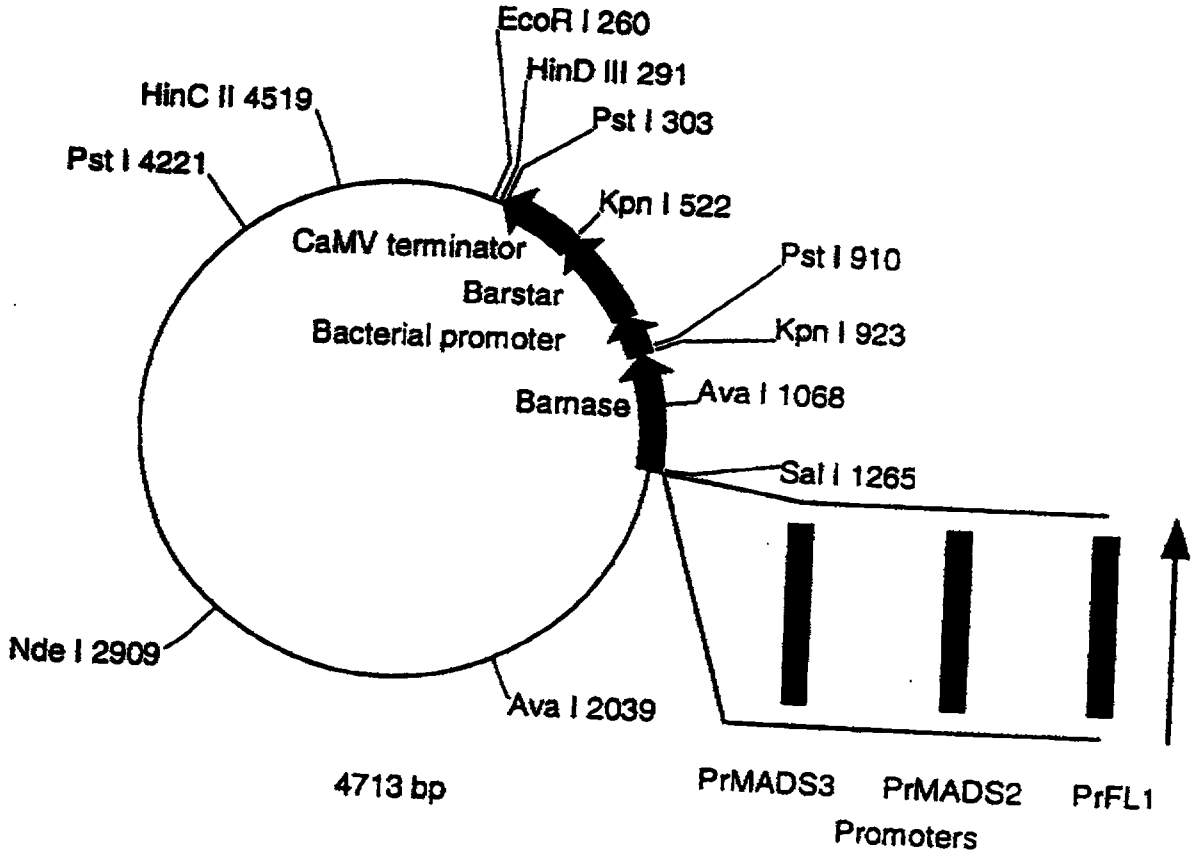


Figure 22

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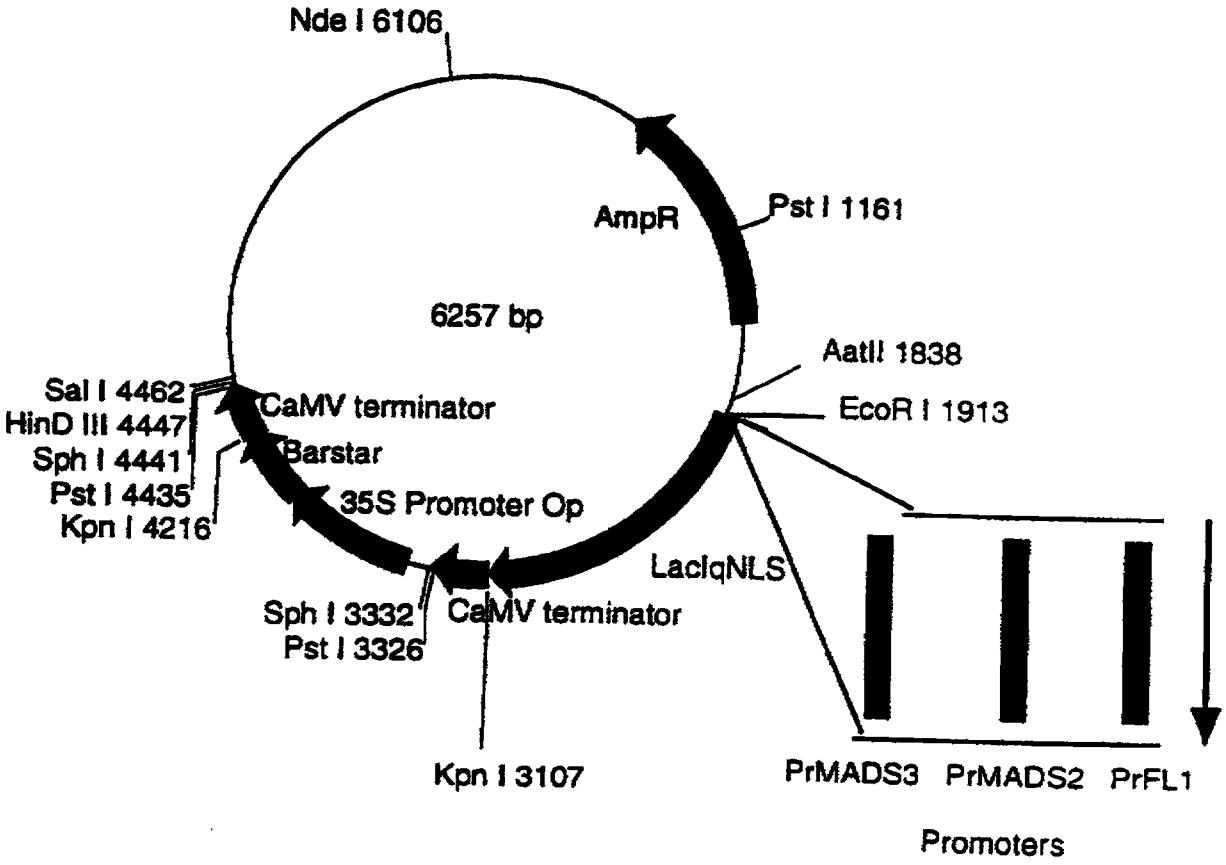


Figure 23

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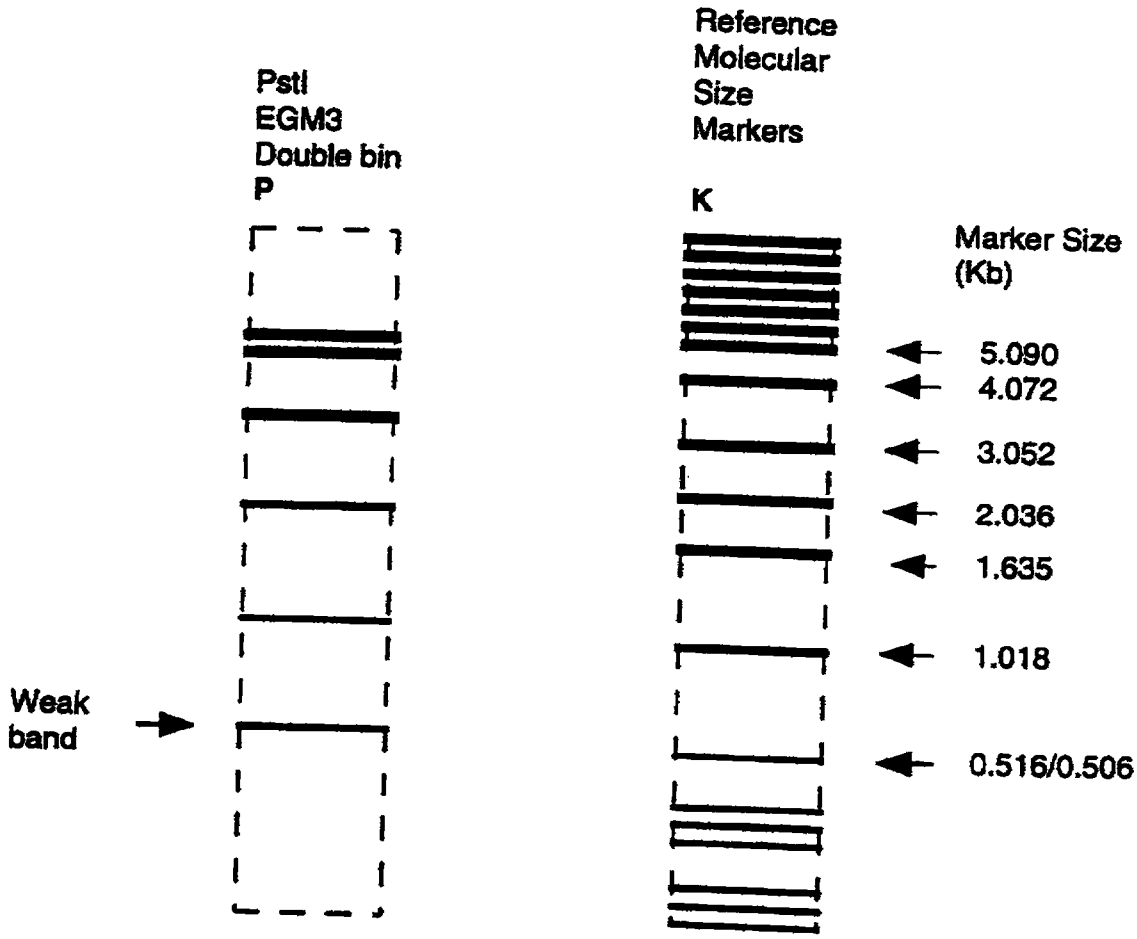


Figure 24

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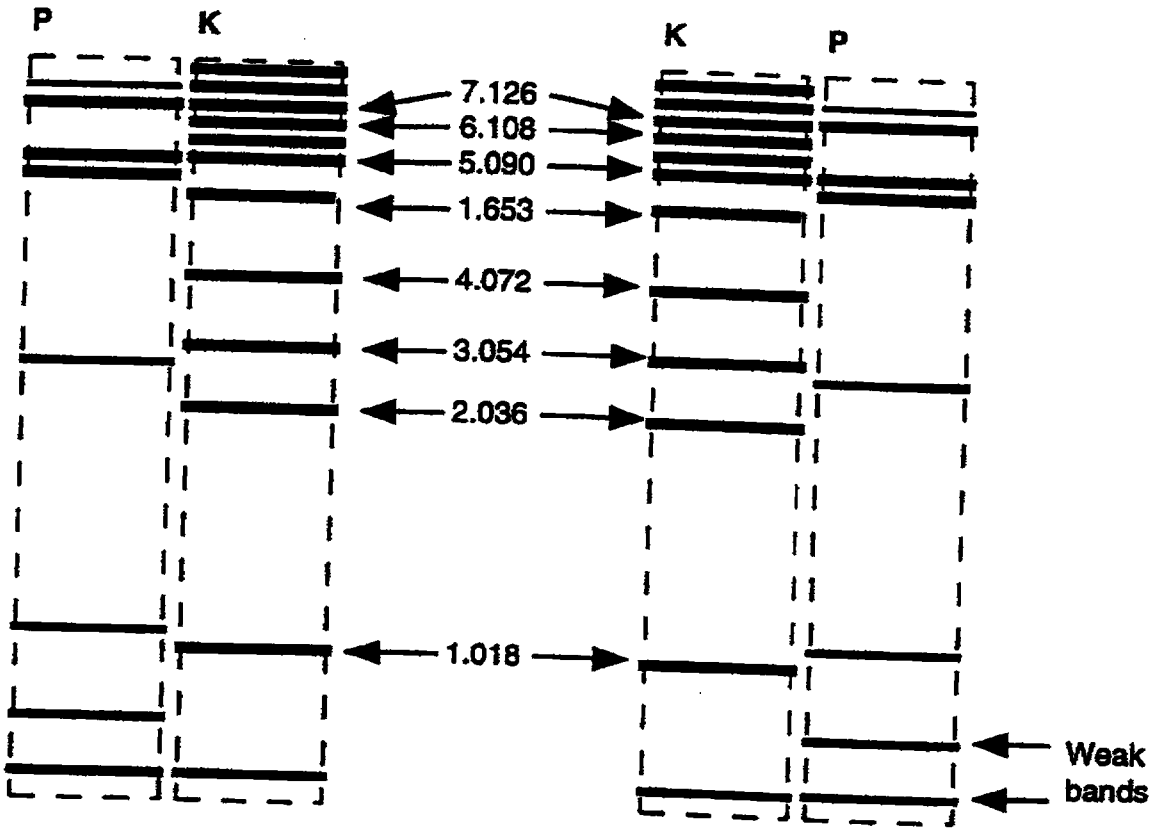


Figure 25

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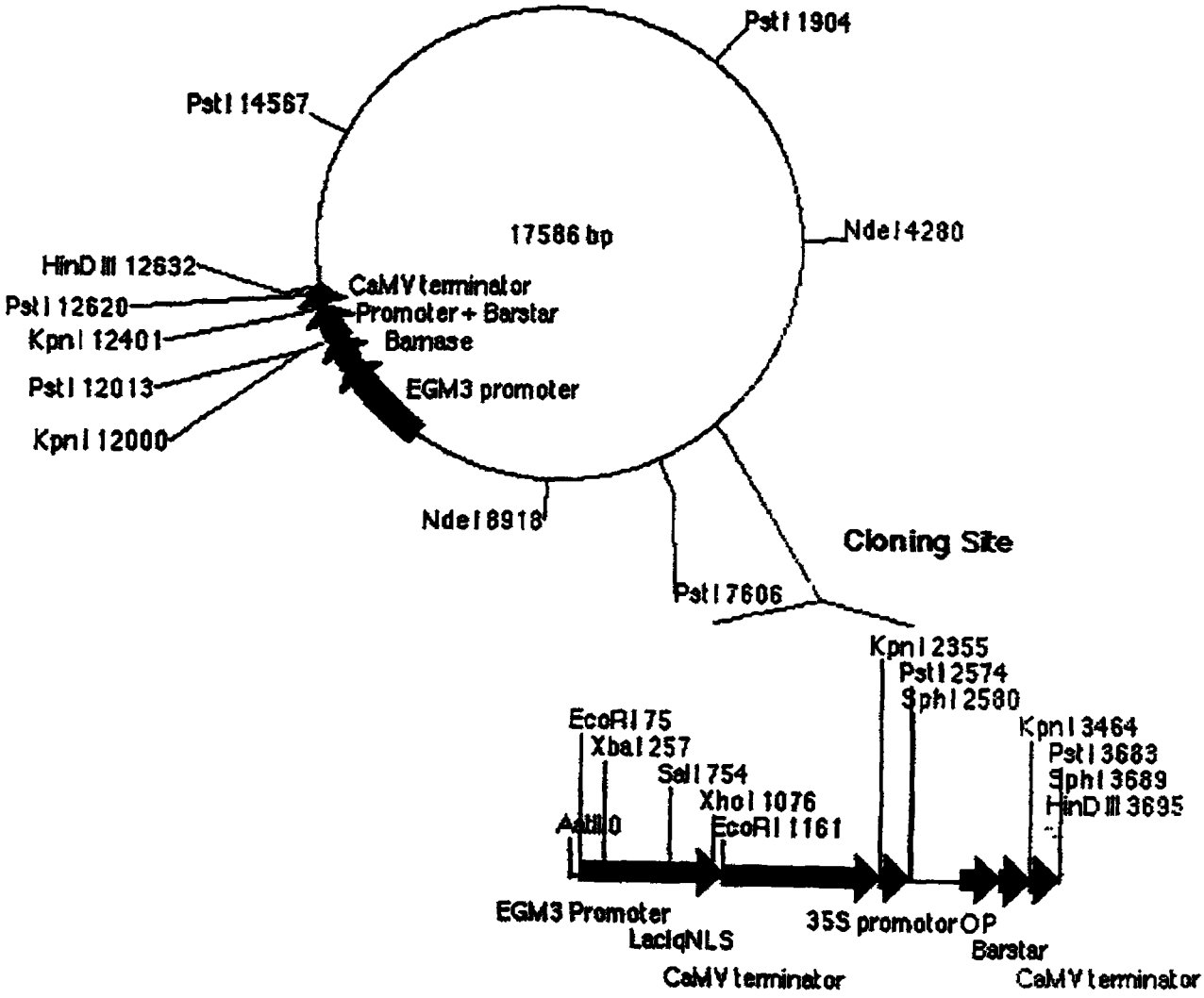
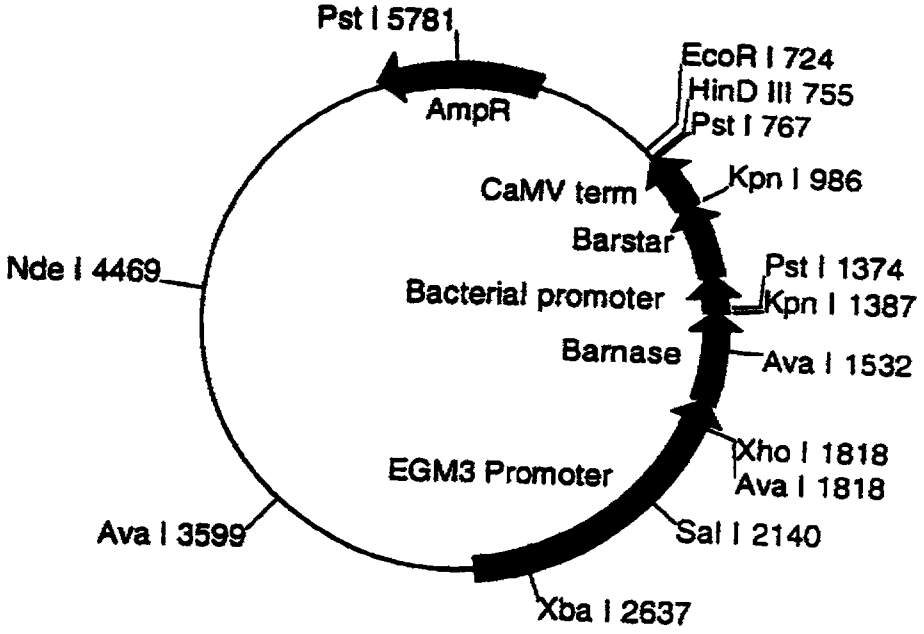


Figure 26

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5809 bp

Figure 27

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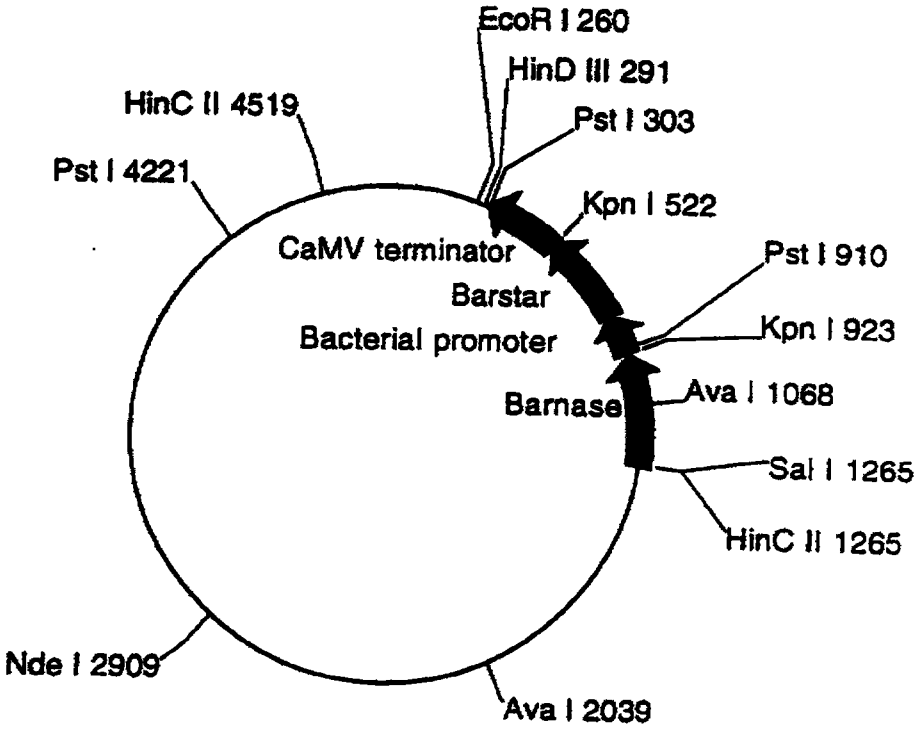


Figure 28

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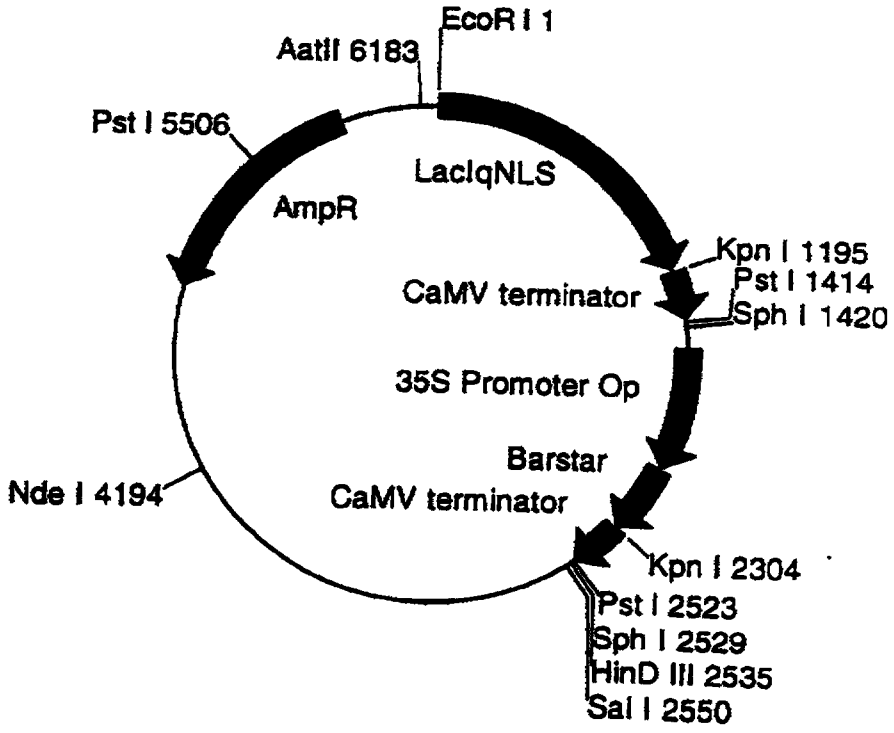


Figure 29

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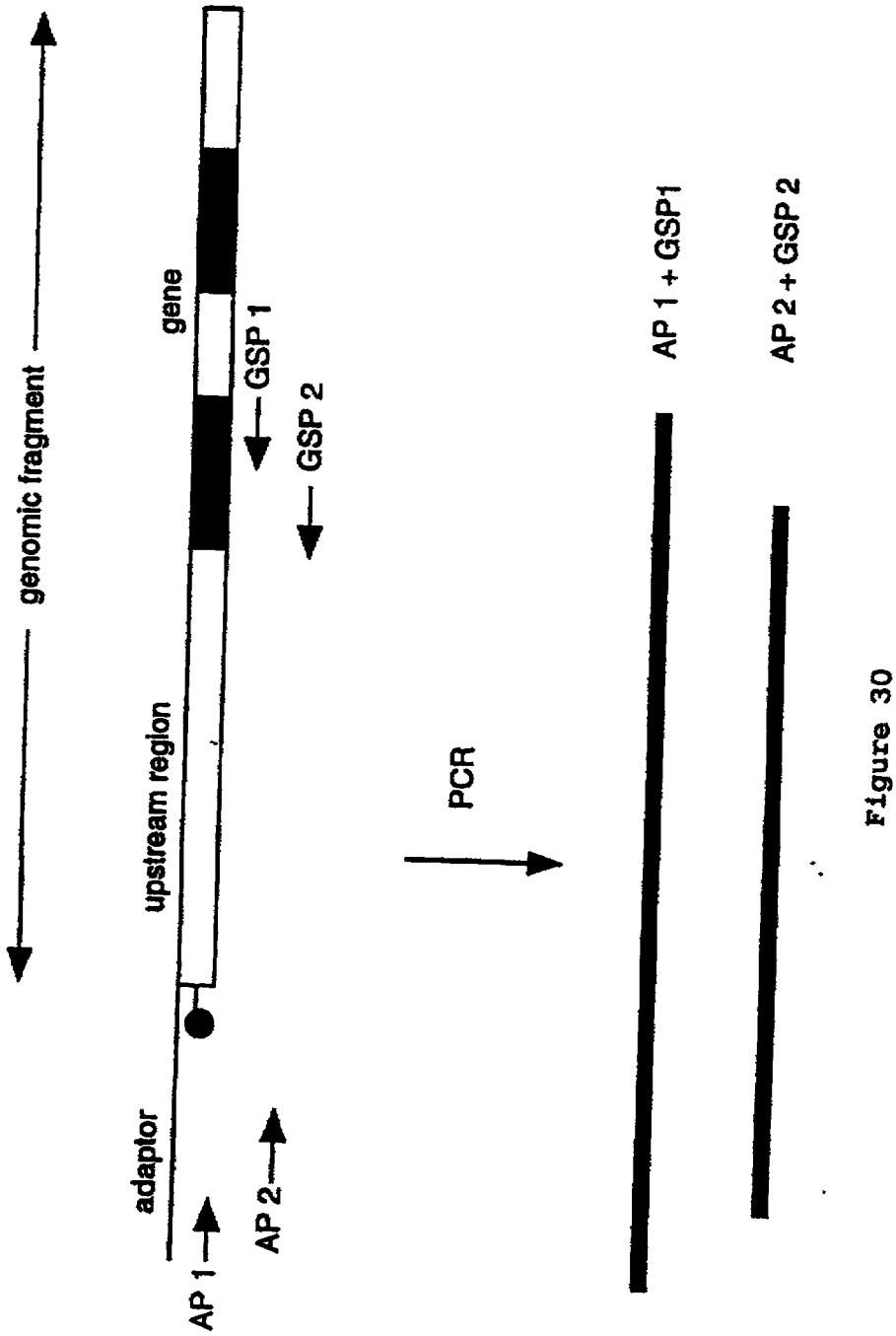


Figure 30

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CCCGGGCTGGTAAAAGACGTCCCTCCGATGAATTCGACAGTGGAGTCCACA

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CAAACATACTCTGTACGCAGATCGGCGGACCTGAAAATTTTCTTCGTATAAAT

CGTCTGTGTTAAGGATATTTGACCATCGGTCCGAAAGATGCGACTGTCCGAC

TGCATCTCGTCTCTGGTAATGTTTTTTTTCAAATTTATAAAGATTGTTTTGCATT

ATTATTATTTATCATGTTGCGTAAGATCTGGTGAGCAGTATTATTTGTCACTTTG

TGTGGAATCCCTTATGAAAATATATATTTATTCCAGAAAAGTATTTTAGCTTGTC

AAATTGTGTGGAATCTGGTATAAGTACCTGTATTTATCAGACGTATTATTTGTTA

AATTGTGTGGAATCTGGTATAAATTTATATACTTCGTGAGGAAATGTATTTATTA

GCAGTATTACTGGTCAAACAGAGTGGAATCCTGTATAAATTTATCATATTTAGG

AACAAATGTATTTATCAGCGTTATTATCTGTCAAAATGTGTGGAGACGCGATG

ACTTTAATTTTTTGATAATAGGCGGAGCGTATAGGTACGCTGTAGTATTGTAGA

CTGGCATGTAGAGTACAAAGTTTAGATATTAACCTAGATTATTTTTGCTATTCG

CAGGCTATCGCGTACTCATGGTGAAATGCGTACAGTTTCAAAGTAAATGGTTG

ATGTTGAGTGCGGTGGCGGGGGCAGACACACAAATAGGAATCGGAGAGCGA

GAGTACTGCAGCAATTGTTGTTTACTTTTTGAGCAAGACGAGGATCAAAGAAG

AATAAGGAAGAGGCCGAGAGAGGGAGCATTATCGTCGAGCAGGAGGGGGAA

AGAAAGAAAGAAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAGCTGAAGC

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Figure 31

This Sheet Has Been Replaced

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CCAAGAAGTTACAAAGTTGAAGGGTAAGGTTGAGCTCCTGCAGCGATCACAAAG

GCATTTGTTGGGGGAAGATCTGGGTCCGTTAAATGTTAAGGAGCTACAGCAGCTT

GAACGTCAGCTGGAGGTTGCTCTGACACATCTTAGGTCAAGGAAAACGCAGGTA

ATGCTGGACCAGATTGAGGAACTTCGCCAAAGGGAACGGTTGCTACATGAAGTA

AACAAGTCTCTGCAGAAAAAGCTTTCCGAAACAGAGGGAAGAGATGTAATAACTG

GCATAGAGCAAACTTCTAATACTAATACTGGTACTAACGGTCCTTGGGATTCTTCT

ATCACAAACACTGCGTATGCTCTCTCACACCCTCAACAAGATTCAAATTCAAGCC

TCCACCATGTGGACTGTGAACCCACGCTACAGATTGGTTATCAGCCTGTGGCTC

CTGAAAGCATCGTCCCTCCTCATCAGCCGCCGCACAACCAAACGCCGAACCAA

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CACAACAAAAGCCCAAAGCGTGGTAAATTACGAAATTAGAATTATATTATCATTAA

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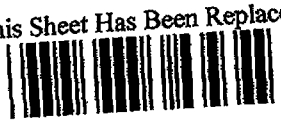
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AAATATTATTATACACTACGGTTTATCAAAAAAAAAAAAAAAAAA

Figure 32

This Sheet Has Been Replaced



MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSTRGKLYEF

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LQKKLSETEGRDVITGIEQTSNTNTGTNGPWDSSITNTAYALSHPQQDSNSSLHH

VDCEPTLQIGYQPVAPESIVPPHQPPHNQTPNQYMQGWWV

(261)

Figure 33

This Sheet Has Been Replaced



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GTACTTCGGTTTGTAAGTAAAACTAAAACAGAAAATTTCTCTTCTAGTATT

CTTAAACCGAGTGGAGTTGAGCTGAAGTGAGAGGAGGTATATATATATA

TGTATATTTATTTAGAGTCCAAAGAATCGAGGCGAAAGGCATGTGATGG

ACGAAAGCAACCTTAATCTCTCTGCAAATTGTCCCTCCAAATTCCAACT

AAAATCAATCCGGACCCGCATGAGCATCGATCGAATCATTTGGGTCATG

CATCCTATGCATGCTCA

Figure 34

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CATCTAAGGGAACTGAGAGCAAATGACCCACTGATTTTCCCAGAAAGCACAAGTGC
ACGATCATGCCCCAATGAATATAGCCAGCTGCAAAGACAGTACTCTCATTCTCCAG
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CAGTGAGAGCGATGAAAGGAAAGCTGATACGAATAAGCAGAAAAGGAGGCGGTCC
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CGGAACCAGGAGAACTGGCAAGAGGGAAGAAAAATGGTCTGGATTATCTCTTTGAT
CTCTATGAGCAGTGTGGGAAATTTTTATTAGAAGTACAAAGGATTGCTAAGGAAAAG
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CTTCAAGTGTAGCTCCGTGATTATTGGGCATTTGTGTTCTCATTGTGACCTTGCATAT
CAGATTTGATATGCATTGTGTCATGCCGTTCTTTCAAACATTGTATATGTATTGGCAA
CTGGGTATGGATCTGCTCCTTTCTCCGTCACTAAACATGCGGAAACCTTTGTTTCTC
CTTGTGATAAATTTCAGTACATTACTGTTCATAGTTATTTTCTTTCAAAAAAAAAAAAA
AAAAA

Figure 35

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DPLIFPESTSADHAPMNIASCKDSTLILQNSNQAQFCGSGLIGVPEHSSESDERK

ADTNKQKRRRSKEPGEDGEDRPREHPFIVTEPGELARGKKNGLDYLFDLYEQCGK

FLLEVQRIAKEKGEKCPTKVTNQVFRHAKHNGAVYINKPKMRHYVHCYALHCLDS

EQSNHLRRLYKERGVNVGAWRQACYYPLVAIARENNWDIEGIFNRNEKLKIWYVPT

KLRQLCHMERSKECQ

(404)

Figure 36

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TGGAACACCATTTTCTTCTAATCACATCACAGTAGTAGCTATCATCCAAACTTATCCA
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GTCTGATTGCTATGTTTTATCAAATGATTTCTCAAATAATAATTCAGTGTACCCATTTG
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GCCCTATAGTATCCCGCGGCCATGGCGGCCGG

Figure 37

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GGGTCAGACTGCAGTAAGATGAAGTCGGTGTGACTCACACTTTGAAGAA

CAGCCATACATACTATTAGTGTTTCAAGTCGATCGAAGTTTGTTTATTCAG

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AAAAAAAAAAAAAAAAAA

Figure 38

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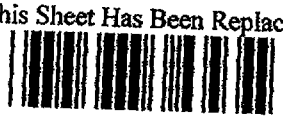




Figure 39

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REGULATION OF GENE EXPRESSION IN EUKARYOTES

This invention relates to a method for regulation of gene expression in eukaryotes.

This invention has particular but not exclusive application to regulation of highly specific expression in target organs or functions of plants, and for illustrative purposes reference will be made to such application. However, it is to be understood that this invention could be used in other applications, such as regulation of expression of other eukaryotic genes such as regulation of specific expression in target organs and functions of yeasts and animals.

Eukaryotic organisms of commercial benefit including plants, animals and lower organisms such as yeasts sometimes divert growth resources into non commercial structures, or express other genes of economic detriment. Other commercial species may benefit from the introduction of characteristics which would enhance commercial worth.

For example, the development of reproductive structures on forest trees represents a significant burden on the resources of the trees, the reproductive effort occurring at the expense of wood growth. Seeds from mature trees produce an undergrowth of saplings which must be removed periodically to prevent competition with the tree crop for soil resources and to minimize the risk of damaging fire.

Australian Patent Specification No. 86224/91 discloses a method of enhancing vegetative growth in a plant. This disclosure exemplifies a method wherein a substantially tissue-specific promoter for reproductive-structure specific genes was selected on the basis of the early appearance of the gene product and specificity of expression in both male and female reproductive buds. Several genes and promoters were isolated and characterised, each gene was modified by fusing a portion of the gene including the tissue-specific promoter of the gene with a structural gene for a ribonuclease.

Whilst the transformants exhibit a lack of reproductive structures, promoter leakage tends to result in a low level of accumulation of the lethal ribonuclease in non target tissues, resulting in reduced growth and thus reduced commercial potential.

Gene expression promoters are to be generally regarded as falling in one of

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three broad classes. Constitutive promoters continuously express the gene in all tissues. Developmental promoters express the gene in specific tissues or in tissues at specific stages. Inducible promoters respond by switching expression on or off in response to the presence of metabolites, foreign compounds or other stimuli such as light, heat or pressure.

However, the inserted promoters are often leaky and the preferred strong promoters may deleteriously express in other tissues with insufficient inhibition to prevent death of non target cells, which may adversely affect commercially important non-target tissues. Promoter leakage cannot be predicted and, for example, where the transformants such as trees have a long growth to first flowering, the resulting gene product activity in non target cells may negate or reduce the advantage of flower suppression. The generally preferred strong promoters limit the promoter choices.

It is known that the expression of a lethal gene product such as Barnase under the control of a substantially flower-tissue specific promoter may be rendered more tissue specific by constitutive coexpression of an inhibitor (B* or "Barstar") for the gene product. The action of the expressed gene product in the target cell will occur where the inhibition is insufficient to suppress gene product action, and non target cell Barnase activity arising out of a degree of non-specificity of the promoter or promoter leakage is substantially suppressed.

The present invention aims to substantially alleviate at least one of the above disadvantages and to provide a method for regulation of eukaryotic gene expression which will be reliable and efficient in use. Other objects and advantages of this invention will hereinafter become apparent.

With the foregoing and other objects in view, this invention in one aspect resides broadly in a method of regulating a eukaryotically active gene, comprising transforming a cell with construct expressing a modulator gene product regulating the eukaryotically active gene or its product and a further gene product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

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The eukaryotically active gene may comprise a native gene to the organism of interest or may comprise an introduced gene. The gene may be promoted by its natural promoter or comprise a heterologous construct with the gene. The eukaryotically active gene may code for an expression product of any selected cellular function. For example the gene may code for an active polypeptide including enzymes such as a ribonuclease having a lethal effect on a target tissue or a peroxidase conferring nematode resistance on root tissue. The gene may code for an enabling product for pathways for production of pigments and dyes such as anthocyanin, an insecticidal compound such as Bt toxin, or fragrances.

Expression of reporter genes under control of inducible promoters may display exact location of target cells in target tissues without any leakage to surrounded cells. This may be very useful for investigation of primary steps of interactions between pathogens and insects and plant cells. The GUS, luc, anthocyanin gene and GFP may comprise candidates as reporters. Colourimetric or fluorescent analysis may be made of micropropagated materials before commercialisation.

As used herein, the expression "lethal gene" is taken to mean a gene or genes which encode a peptide or antisense or ribozyme or other non peptide which significantly disrupts a target cell leading thereby to the death of the target cell. The invention will be described hereinafter with reference to embodiments comprising a eukaryotically effective lethal gene.

The lethal gene may be any gene or combination of genes which encode a peptide or antisense or ribozyme which significantly disrupt a target cell leading thereby to the death of the target cell. For example, the lethal gene may be selected from those that encode ribonucleases such as Barnase from B. amyloliquefaciens, RNase T1 from A. aryzae, bovine RNase A, RNase I and RNase H from E. coli or a set of plant RNases (family of S-proteins). Alternatively, the lethal gene may be selected from nucleases such as the family of restriction endonucleases, enzymes including glucanases such as 1-4-ß-glucanases or 1-3-ß-glucanases, ubiquitins, acid pyrophosphatases and inhibitors of plant cell wall synthesis.

Preferably, the lethal gene is an RNase. A chimaeric lethal gene may be

constructed including the coding region of Barnase from B. amyloliquefaciens under the control of upstream (promoter) sequences of genes expressed in the male and female parts of plant reproductive organs at the early stages of development.

Restriction of expression of a lethal gene in target cells during first events of pathogen-host plant interaction in target cells may be achieved. For example, expression of Barnase or other toxins like ß-glucanase or chitinase in attacked cells will lead to necrosis of these cells which will prevent spreading of infection to surrounding cells. The critical stage in this artificial hypersensitive reaction project is blocking of possible promoter leakage in surrounding cells.

Expression of bacterial salicylate-hydroxylate gene may reduce level of salicylic acid which is responsible for systemic expression of cassettes under control of pathogen-inducible promoter in all plants. For example, a pathogen-inducible promoter may be isolated from barley which is strongly expressed after inoculation with powdery mildew Eresify graminis (Mouradov et al.,1993). The genomic clone of this gene was isolated and characterised (Mouradov et al.,1994). This promoter region may be fused to cassettes including a lethal or other functional gene.

Three eucalypt genes designated EGM1, 2 & 3 with homology to other plant MADS-box genes have been cloned and sequenced. Phylogenetic analysis has been performed to examine the relatedness of the eucalypt genes to other MADS-box genes. This revealed that EGM2 is likely to be homologous to the GLOBOSA MADS-box gene group which are involved in development of petals and stamens, EGM3 and EGM1 both fall in the AGL2 group of MADS-box genes. These genes are most commonly expressed in the inner three whorls of the flower (petals, stamens and carpels).

The pattern of expression of all three EGM genes has been characterised. Expression of these genes was not detected in any vegetative tissue. The EGM1 gene is expressed in the petals, stamens and the carpel of eucalypt flowers. EGM3 is expressed in the floral meristem and in sepals. EGM2 is expressed in petals and stamens. The EGM3 and EGM2 genes have been shown by Southern analysis to be single genes. EGM1 is also likely to be a single gene.

The promoter regions of all three of these genes may be used in sterility gene

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constructs and may be isolated from a eucalypt genomic library and engineered into lethal gene constructs.

The eukaryotically active gene, modulator gene and further gene and their respective promoters may comprise a transformation cassette with which the target organism may be transformed by any known means. The promoters of the eukaryotically active gene, modulator gene and further gene or genes are selected in combination such that the specificity of expression of the eukaryotically active gene is enhanced. For example, the eukaryotically active gene may be promoted by a promoter which is inducible or developmental whereas the modulator gene may be constitutive. Specificity and the usefulness of even weak promoters may then be enhanced by promoting the further gene or genes with a promoter with the same specificity as that used to promote the eukaryotically active gene.

Alternatively, a tissue specific promoter for the eukaryotically active gene may be inhibited by a constitutively-promoted inhibitor gene product, which in turn is controlled by an inducibly-promoted further gene product.

In a yet further alternative, the eukaryotically active gene product may be constitutively expressed subject to the modulating effect of a modulator gene expressing under the control of a tissue specific promoter, the modulator gene or product being itself controlled by the expression of a further gene under the control of a promoter specific for a different or complementary tissue. By this means for example, a constitutively-promoted gene conferring insect resistance may be localized in its effect to exclude the seeds by expressing a gene inhibitor under the control of a seed specific promoter, the gene inhibitor itself being controlled by a further gene product expressed under the control of a promoter specific for non-seed tissues.

In a further aspect, this invention resides broadly in a method of regulating expression of a eukaryotically active gene, comprising transforming a cell with a transformation cassette comprising said eukaryotically active gene, a modulator gene expressing a product regulating the eukaryotically active gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible

promoters and developmental promoters for the same or complementary tissues.

In a further aspect this invention resides broadly in a transformation cassette comprising a eukaryotically active gene, a modulator gene expressing a product regulating the eukaryotically active gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues.

In a yet further aspect this invention resides broadly in a method of transformation of a propagatable eukaryotic cell with an expression cassette including:

a lethal gene expressing a cytotoxic peptide in a target tissue under the control of a promoter substantially specific to said target tissue;

an modulator gene constitutively expressing an inhibitor of the production or action of said cytotoxic peptide, and

a further gene under the control of said substantially specific promoter and functioning to block said inhibitor or modulator gene.

In a further aspect, this invention resides broadly in an expression cassette including:

a gene expressing a peptide cytotoxic to a target tissue under the control of an inducible promoter substantially specific to said target tissue;

an inhibitor gene expressing an inhibitor of the production or action of said cytotoxic peptide, and

a repressor gene under the control of said inducible promoter and functioning to block said inhibitor or inhibitor gene.

In a further aspect, this invention relates to primers for PCR isolation of the Barnase and Barstar genes from an extract of B. amyloliquefaciens, designated P1 and P2 (for Barstar) and P3 and P4 (for Barnase) and comprising:

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
*Eco*RI

P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'
HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

The cytotoxic effect of the lethal gene expression product may be increased by coupling a localisation signal sequence to the construct. For example, the cytotoxic effect of the preferred RNase may be increased by targeting into the nucleus where most of the pre-RNAs and mature RNAs exist in non-protected form. For this purpose the coding region of, for example, the Barnase gene may be modified by fusion of a nuclear localisation signal (NLS) sequence to the RNase gene.

Accordingly in a further aspect this invention provides a set of PCR primers for fusing the NLS sequence from VirD2 gene of A. tumefaciens to the Barstar gene and comprising:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

The tissue-specific or developmental promoter may be selected from those tissue specific promoters that are controlling genes in plant reproductive organs during the early stages of flower development. These genes are maintained in the repressed state in all plant organs during the non-flowering period and are induced substantially in reproductive organs during the flowering period.

The developmental promoter can be isolated by any known means. For example,

mRNA only present during the development of the target tissue may be identified and isolated, cDNA from these specific mRNAs prepared and used to probe the genomic library, followed by identifying the portion of the plant genome which contains promoter region of these genes. The tissue-specific promoters may be homologous (native) or heterologous (foreign) in origin. For plant reproductive organ tissues, the promoters may be selected from those that are expressed in cells of different floral tissues in both male and female organs, or only in specific tissues such as sepals, petals, ovary, style, stigma, ovule, corola and others. For the generation of the sterile plants, promoters which express in tissues at early stages of both male and female organ development are preferable.

For example, the gene family encoding homeotic MADS-box genes which play an important role in flower development express at the early stages of the developing floral primordia. We have isolated MADS genes from P. radiata which displays strong homology to the Arabidopsis homeotic MADS-box genes known as the AGAMOUS AGL-2 and AGL-4 genes. These genes express in young floral primordia and are not expressed in the inflorescence meristem.

The gene encoding an inhibitor of the lethal gene product may comprise a gene repressing expression of the lethal gene, a gene transcribing an antisense RNA, or may comprise a gene expressing a protein capable of deactivating the lethal protein. The inhibitor gene will generally be under the expression control of a constitutive promoter which expresses the inhibitor in non target tissues, although it is envisaged that a packaged operator-repressor system may be used.

For example, a Barnase gene/reproductive organ-tissue specific promoter system may be associated with an anti-Barnase (B* or Barstar) gene/promoter system.

In the alternative, negative regulation in expression of bacteriophage lambda N gene and SOS system in E. coli could be used in expression cassettes. Expression of the bacteriophage lambda N gene is tightly regulated via the P^L promoter and controls the level of expression from this promoter. This system may be used to provide the requisite negative control of the expression of an inhibitor gene in the target tissue.

The SOS system is regulated by the interplay of two proteins. A transcriptional

repressor (LexA) inhibits the initiation of transcription through specific binding to its operator targets. A RecA protein blocks LexA protein and thus prevents inhibition at its specific site. This would be a one component lethal gene system and would not require a toxin blocking gene.

The repressor gene under the control of the inducible promoter may be selected to repress transcription of the inhibitor gene, translation of the RNA transcript or action of a peptide product thereof. Preferably, the repressor gene codes for a peptide product which directly represses expression of the inhibitor gene. Accordingly, it is preferred that the repressor gene and inhibitor gene be adapted to form a specific repressor/operator couple.

For example, the E. coli *lacIq* gene under control of the same tissue specific promoter as the lethal gene construct may be used to repress the action of the repressor gene promoter modified by insertion of a lac operator sequence.

The repressor gene promoter may be a modified 35S-RNA-CaMV promoter having at least one lac operator between the promoter and coding region of the repressor. In the selection of the modified 35S-RNA-CaMV promoter exemplified hereinafter, the present inventors have developed primers for amplification of the modified 35S-RNA-CaMV sequences.

Accordingly, in a further aspect this invention provides primers for PCR amplification of modified 35S-RNA-CaMV sequences, designated 35S1 and 35S2 and comprising:

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

For the introduction of lac operators into both the 3' and 5' region of the modified 35S-RNA-CaMV promoter, the sequence may be amplified by PCR primers:

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

binding protein (repressor, *lacIq*) and the target promoter sequence (operator) is understood in great detail. Expression of the lac operon is under the strong and tightly negative control from the *lacIq* repressor. Purified repressor is a tetramer, formed from four identical subunits, each with 360 amino acids (MW 38,350 kDa). The operator sequence constitutes 35 base pairs, including 28 base pairs of symmetrical sequence.

For the maximum repression of the expression of the inhibitor gene, the repressor protein is preferably targeted into the nucleus of the target cells. This may be achieved by fusion of the nuclear localisation signal sequence to the C-end of the repressor.

In a further aspect this invention relates to a set of primers suitable for isolation of the *lacIq* gene and having suitable restriction enzyme cleavage sites for cloning into the plant vectors, the two primers corresponding to the 5' and 3' ends of the gene and designated AM 1 and AM 2:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
KpnI

In a yet further aspect of the present invention, there is provided a set of PCR primers for use in fusing the NLS sequence to the 3' end of the *lacIq* gene and comprising:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

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LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of *lacIq* 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of *lacIq*

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

If desired, the method of the present invention may be a reversible method. For example, the gene cascade may use molecular elements which will allow the reversal of the engineered sterility to fertility thus permitting seed production. The reversal mechanism may consists of placing an anti-repressor gene under the control of a chemically switchable promoter. When, for example, restoration of fertility is required plants may be sprayed or otherwise administered a chemical which induces expression of anti-repressor RNA which will block inhibition of expression of the modified inhibitor gene. Accumulation of the inhibitor product in floral organs will inhibit lethal gene function, thus allowing normal flower formation.

For example, for the lac/*lacIq* system described, a chemical may be sprayed on the plant which induces expression of anti-*lacIq* RNA which will block inhibition of expression of a modified Barstar gene. Accumulation of the Barstar product in floral organs will inhibit lethal gene function, thus allowing normal flower formation. The promoter region of the maize glutathione-S-transferase (GST II) gene could be used for chemically-induced expression. Expression of the GST II promoter can be switched on by chemicals such as dichloramin (N,N-diallyl-2,2-dichloracetamide), or fluorazole (benzyl-2-chloro-4-trifluoromethyl-5-thiazole-carboxylate).

In the case of Eucalyptus, the EGM3 promoter, comprising the upstream sequences of the EGM3 gene, may be used to express the lethal gene specifically in eucalypt floral tissue. Using this promoter, the cytotoxic Barnase gene may be specifically expressed in the floral meristem at a very early stage of development. The inhibitor Barstar may be expressed under the control of 35S-RNA-CaMV promoter

carrying the lac-operator sequences.

The E. coli *lacIq* gene under the control of the EGM3 promoter could be used as repressor gene to prevent expression of Barstar in the floral meristem tissue during flowering initiation. During flowering, the EGM3 promoter would express Barnase and *lacIq* in the floral meristem while the modified 35S-RNA-CaMV promoter would express Barstar in all tissue except the floral meristem, giving protection from promoter leakage. During non-flowering period, the 35S-RNA-CaMV promoter would express Barstar generally, giving protection from leakage.

Nematodes cause annual losses well in excess of US$100 billion to world agriculture. The most important pathogens by far are the nematodes Meloidogyne incognita and Meloidogyne javanica which attack a wide range of plants. Control measures include use of hazardous chemicals, cultural practices such as rotation and the use of resistant varieties in a limited range of crops. The advantages of engineering resistance to nematodes include no requirement for specific cultural practices and reduced environmental risks.

The lethal gene/inhibitor/repressor approach can be used to engineer resistance to nematodes. The ideal site to activate a plant defence against nematodes is in the feeding cells in the vascular region of the plant root. In several plant-nematode interactions, gene promoters are being identified by differential screening which could be used to express the Barnase gene specifically at the site of nematode feeding. Recently two genes (Lemmi9 and LemmilO) have been identified that are expressed at high levels in giant cell feeding sites in tomato roots infected with Meloidogyne incognita. (Van der Eycken et al., (1996) Plant Journal 9, 45-54).

Expression of a lethal gene in feeding cells would rapidly kill the cell and disrupt the feeding process which is very critical particularly for the development of the females. Expression of the inhibitor gene in non-target tissue would be required to prevent damage to other parts of the root due to promoter leakage. This is particularly important since the promoter which shows high level expression in the giant feeding cell is also expressed at lower levels in non target tissue. Expression of the repressor gene under the feeding site promoter should block the expression of the inhibitor gene sufficiently

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in the target tissue to allow the lethal gene to destroy the cell.

In order that this invention may be more readily understood and put into practical effect, reference will now be made to the following Examples and the accompanying drawings which illustrate preferred embodiments of the invention and wherein:

FIG. 1 represents the construction of the Barnase/NLS gene localised lethal gene system;

FIG. 2 represents constructs including lac operon- modified 35S-RNA-CaMV operator/Barstar inhibitor gene system;

FIG. 3 represents the construction of *lacIq*/NLS localised repressor gene system;

FIG. 4 represents the mode of action of Barnase/Barstar:lac/*lacIq* cascade in accordance with the present invention;

FIG. 5 represents an alternative cascade Barnase:op/LexA/RecA in accordance with the present invention;

FIG. 6 represents the mode of action of a reversible Barnase/Barstar:lac/*lacIq*/antisense-*lacIq* cascade in accordance with the present invention;

FIG. 7 is a diagrammatic illustration of the control cassette and modes of action of the lethal gene/NLS system of FIG. 1;

FIG. 8 is expression of PrMADS1, PrMADS2, PrMADS3, PrFL1 and PrCON1 genes in reproductive and vegetative tissues of P. radiata.

FIG. 9 is expression of PrMADS3 gene in male and female cones.

Fig. 10 is the gel mobility shift assay of PrMADS1, PrMADS2, and PrMADS3.

FIG 11 is the nucleotide sequence of the EGM1 promoter;

FIG. 12 is the nucleotide sequence of the EGM2 promoter;

FIG. 13 is the nucleotide sequence of the promoter of the eucalypt floral-specific MADS gene EGM3;

Fig. 14 is a graphical representation of Northern blot of total RNA extracted from Eucalypt tissue probed with EGM1, EGM2 and EGM3 cDNA;

Fig. 15 is a graphical representation of Northern blots of total RNA extracted from Eucalypt tissue probed with EGM2 cDNA (6 hour exposure) and EGM3 cDNA;

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Fig. 16 is an alignment of the predicted protein sequence of the MADS-box region of several plant MADS-box genes;

FIG. 17 is a phylogenetic tree showing relatedness of several plant MADS-box genes;

FIG. 18 is a Southern blot of Eucalyptus grandis DNA digested with *Eco*RI and HinDIII and then probed with the EGM3 and EGM2 genes;

FIG. 19 is a Northern blot of total RNA extracted from eucalypt tissue probed with the EGM3 cDNA (6 hour exposure).;

FIG. 20 is a diagrammatic illustration of plasmid 7 prom Barstar;

FIG. 21 is a diagrammatic illustration of plasmid pBR Barnase prom Barstar containing V1-promoters;

FIG. 22 is a diagrammatic illustration of a plasmid containing V2-promoters;

FIG. 23 is a diagrammatic representation of an agarose separation of a *Pst1* digest of an EGM3 double bin. Given that the *Eco*RI cloning site is at base 6772 in EGM3 bin, the predicted fragment sizes from the map for this digestion are 4.9, 4.8, 4.4, 3.3, 1.9, 1.1, and 0.6 kb.

FIG. 24 is a diagrammatic representation of two gel exposures of a *Pst1* digest of an EGM3 bin showing the other possible orientations of the cloned insert. The sizes of fragments expected from this digest are 7.4, 4.9, 4.4, 1.1, 0.7, and 0.6 kb.

FIG. 25 is a plasmid diagram of EGM3 double bin. The fragment containing EGM3 promoter driving *lacIq*Nls and the fagment containing 35S pomoterOP driving Barstar can be cloned in either orientation.

FIG 26 is a plasmid diagram of EGM3 V1 sense.

FIG 27 is a plasmid diagram of plasmid V1 (Barnase-Barstar).

FIG. 28 is a plasmid V2 (*lacIq*NLS-35s promoterOp-Barstar).

FIG. 29 is a representation of a promoter finder strategy;

FIG. 30 is the nucleotide sequence of PrMADS2 promoter;

FIG. 31 is the nucleotide sequence of PrMADS3 cDNA;

FIG. 32 is the amino acid sequence of PrMADS3 protein;

FIG. 33 is the nucleotide sequence of PrMADS promoter;

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FIG. 34 is the nucleotide sequence of PrFL1 cDNA clone;

FIG. 35 is the amino acid sequence of PrFL1 protein;

FIG. 36 is the nucleotide sequence of PrFL1 promoter; and

FIG. 37 is the nucleotide sequence of PrCon1 gene (partial).

EXAMPLE 1

A chimaeric reproductive organ-specific expression cassette was constructed including a reproductive organ-specific promoter and Barnase gene from B. amyloliquefaciens, under the positive control of a gene encoding an inhibitor of Barnase, Barstar (B*), from B. amyloliquefaciens under control of the modified constitutive promoter (35S RNA CaMV) with introduced lac-operator sequence, and under the negative control of the E. coli *lacIq* gene under control of the same reproductive organ-specific promoter as used to express the lethal gene as per FIG. 4.

The reproductive organ specific promoter comprises the upstream sequences of genes expressed in reproductive organs at the early stages of flower development as described hereinafter. The cytotoxic effect of the Barnase was increased by targeting it into the nucleus where most of the pre-RNAs and mature RNAs exist in non-protected form. For this purpose the coding region of the B gene was modified by fusion of the nuclear localisation signal (NLS) sequence from VirD2 gene of A. tumefaciens strain C58 to the 3'-region of the B gene, as illustrated in FIG. 1.

The inhibitor Barstar (B*) gene portion vectors carrying lac-operator sequences in 5'-, 3'- and 5'- plus 3'- regions of the 35S-RNA-CaMV promoter region were constructed as per FIG.2.

The E.coli *lacIq* gene under control of the same reproductive organ-specific promoter as used to express the lethal gene has its *lacIq* protein targeted into the nucleus by fusion of the NLS from VirD2 gene of A. tumefaciens to the C-end of the *lacIq*

protein as per FIG. 3.

EXAMPLE 2

An alternative chimaeric reproductive organ-specific expression cassette was constructed as per FIG. 5, wherein the reproductive organ-specific promoter and Barnase gene is under the positive transcriptional control of the LexA product under control of 35S-RNA-CaMV promoter, and under the negative control of the RecA gene product under control of the same reproductive organ-specific promoter as used to express the lethal gene, as per FIG. 5.

EXAMPLE 3

A reversible system was constructed substantially in accordance with Example 1. In addition a chemically switchable promoter from the maize glutathione-S-transferase (GST II) gene regulating an antisense *lacIq* RNA gene was included. This allowed seed production as per FIG. 6. Restoration of fertility may be thus achieved by spraying with an inducer such as dichloramin (N,N-diallyl-2,2-dichloracetamide) or fluorazole(benzyl-2-chloro-4-trifluoromethyl-5-thiazole-carboxylate). This chemical induced expression of anti-*lacIq* RNA blocks inhibition of expression of modified 35S-RNA-CaMV-B* gene. Accumulation of the B* in floral organs will inhibit lethal gene function, thus allowing normal flower formation.

EXAMPLE 4

Modification and insertion of the *lacIq* repressor gene into plant expression vectors.

The pRT99GUS vector was digested with XbaI and KpnI to release the coding region of the GUS gene and become a suitable vector for expression in plants.

The *lacIq* repressor is available on almost all commercial E. coli strains. Some commercial plasmids also contain the repressor gene. In order to express this gene in plants, the prokaryotic translational initiation codon (GTG) was changed into ATG. In order to isolate the *lacIq* gene two primers corresponding to the 5' and 3' ends of the gene were designed (AM 1 and AM 2). Both primers have suitable restriction enzyme cleavage sites for cloning into the plant vectors.

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
Kpn I

After amplification the PCR fragment was digested with XbaI and KpnI and ligated into the pBR-35SGUS vector digested with same enzymes in order to replace the GUS gene (pBR-35SlacIq).

In order to fuse the NLS sequence to the 3' end of the *lacIq* gene several PCR were performed.

Primers:

AM 1 as mentioned above.

LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of *lacIq* 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of *lacIq*

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

Final PCR fragment containing NLS from VirD2 gene from A. tumefaciens fused to the coding region of the *lacIq* gene was digested with Xba I and KpnI and ligated into the plant expression vector pBR322-35SGUS digested with the same enzymes (pBR-35S*lacIq*-NLS). This vector was transformed from the XL1 Blue into the JC 8697(recA-) E. coli strain in order to eliminate possible homologous recombination.

EXAMPLE 5

Barnase and Barstar genes.

These genes were isolated by PCR from the crude extract of the B. amyloliquefaciens using P1 and P2 (for Barstar) and P3 and P4 (for Barnase) primers.

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
*Eco*RI

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P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'
HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

The P1-P2 PCR fragment (Barstar) was digested with *Eco*RI and HindIII and ligated into the Bluescript SK vector digested with same enzymes (BS-B*). The BamHI-HindIII fragment of the BS-B* vector was introduced into the protein expression vector pQE32 (pQE-B*).

The P3-P4 PCR fragment (Barnase) was ligated into the PCR cloning vector p/GEM-T (pGEM-T-B). In order to fuse NLS sequence to the 3' end of the B gene several PCR were performed.

Primers:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

Final PCR fragment containing NLS from VirD2 gene from A. tumefaciens fused to the coding region of the Barnase gene was ligated into the PCR cloning vector pGEM-T (pGEM-T-B-NLS).

EXAMPLE 6

Introduction of lac-operator sequence into the 35S-RNA-CaMV promoter.

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The commercial plasmid pQE32 contains the ideal combination of two lac-operators (operators I and II). As a first step the HindIII fragment from the pRT99GUS vector was introduced into the Bluescript SK vector (A 1). The *Eco*RI-*Sal*I fragment of the A 1 vector carrying the GUS gene fused into the 35S-RNA-CaMV promoter and terminator regions was introduced into the pQE32 vector digested with the same enzymes.

The resulting vector contains 35S-RNA-CaMV promoter with two lac operons in the upstream region (Op+ 35S-GUS).

In order to introduce the lac operator between the 35S-RNA-CaMV promoter and coding region of the GUS gene initially a BamHI-SalI fragment of the A1 vector carrying promoterless GUS gene was ligated into the pUC19 vector digested with same enzymes (pUC19-promoterGUS). In the next step the pUC19-promoterGUS vector was digested with *Eco*RI and *Sal*I enzymes and ligated into the pQE32 vector digested with the same enzymes (pQE-promoterGUS). The 35S-RNA-CaMV promoter region was amplified by PCR using the 35S1 and 35S2 primers, corresponding to the 5' and 3'-region of the 35S-RNA-CaMV promoter.

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
*Sal*I

The PCR fragment was digested with XhoI and *Sal*I and introduced in to the pQE-promoterGUS digested with XhoI. Clones containing the promoter in the correct orientation were selected and resulting plasmid (35S- GUS) was transformed from the XL1 Blue into the JC 8697 (recA-) E. coli strains in order to eliminate possible homologous recombination.

In order to introduce lac-operators into both the 5'- and 3'-region of the 35S-RNA-CaMV promoter the operator-35S promoter region of the -35S-GUS vector was amplified by PCR using the OP1 and 35S2 primers, corresponding to the 5'- and 3'-region of the -35S promoter.

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

The PCR fragment was digested with XhoI and *SalI* and introduced into the pQE-promoterGUS digested with XhoI. Clones containing the promoter in the correct orientation were selected and resulting plasmid (-35S-promoterGUS) was transformed from the XL1 Blue into the JC 8697 (recA-) E. coli strains in order to eliminate possible homologous recombination.

Barstar gene was cloned into the pQE-promoterGUS vector after digestion of both with *Eco*RI+KpnI.

One difficulty with the tissue ablation approach is the effect of promoter leakage or expression in other tissues. To overcome this we have developed a control system (gene cascade) whereby expression of a lethal gene(s) is countered in non-target tissues (FIG. 7). Two different vectors carrying Barnase (V1) and Barstar plus repressor (*lacIq*) (V2) parts of these cascade were designed.

In order to clone promoter regions into the vectors V1 and V2 they have been amplified using sets of primers with *SalI* restriction sites on the ends for V1 (*SalI* primers) and *EcoR*I restriction site for V2 (*Eco*RI primers). In order to check the orientation of the promoters in V1 and V2 vectors an *EcoR*I restriction site was designed in the forward *SalI* primer downstream from *SalI* site and a *SalI* site was designed in the *EcoR*I primer downstream from *EcoR*I:

***SalI* primers:**

PrMADS2 promoter:

Forward primer:

5' CCGGTCGACGAATTCCGACAGTGGAGTCCACAAAGAAAGATGCG3'
SalI *EcoR*I

Reverse primer: 5' CCGGTCGACTTCTTTCCTTCTTTTCTTTCTGC 3'
SalI

PrMADS3 promoter:

Reverse primer: 5' GCGAATTCTTCATCTTACGTCACGCGAGG 3'
*Eco*RI

PCR fragments of *Sal*I primers were digested with *Sal*I restriction enzyme and introduced into the V1 vector digested with *Sal*I. The orientation of promoters was checked using digestion with *Eco*RI enzyme.

PCR fragments of *Eco*RI primers were digested with *Eco*RI restriction enzyme and introduced into the V1 vector digested with *Eco*RI. The orientation of promoters was checked using digestion with *Sal*I enzyme. Maps of the resulting plasmids are shown in FIGS. 20 and 21.

These vectors were linearised with HindIII enzyme and introduced into the Bin19 binary vector digested with HindIII. Colonies carrying modified Bin 19 vectors were selected on plates with kanamycin and ampicillin antibiotics. As a next step Bin19-V1-promoter and Bin19 V2-promoter vectors were transformed into several agrobacterium strains. Arabidopsis thaliana, Eucalyptus grandis and Pinus radiata embryos and explants were co-transformed with *Agrobacterium* strains.

The HindIII fragment from the plasmid pRT99GUS (Töpfer *et al.* Nucleic Acids Research (1988) 16 (17): 8725) was cloned into the HindIII site of pBR 322. This insertion resulted in two plasmids corresponding to the insert being cloned in both orientations. The inserted region contains the Cauliflower Mosaic Virus (CaMV) 35S RNA promoter, beta glucuronidase (GUS) gene, and CaMV 35S terminator region. These plasmids were called pBrGUS1 and pBrGUS2 and provided the basis for the lethal gene constructs. The orientation of the insertion event was checked with a BamHI digestion: pBRGUS 2 results in BamHI fragments of 2.5kb and 4.4kb, pBRGUS 1 in fragments of 6.1kb and 0.8kb.

The DNA encoding the *lacIq* nuclear localisation signal peptide was amplified from the plasmid pGEM *lacIq*NLS by PCR using the 5' primer *lacI* (this has an *Eco*R1 site) and the 3' primer D23 which has a KpnI site. The amplified DNA fragment was restricted with *Eco*RI and KpnI and cloned into pBRGUS 2 cut with the same enzymes. The resulting plasmid was called pBRLac. The pBRLac plasmid was then cut with SphI and run on an agarose gel to allow the purification of the plasmid fragment containing

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the *lacIq* gene, ampicillin resistance gene, and origin of replication away from the small SphI fragment, which contained restriction sites that we wished to remove. The resulting plasmid was called pBRLac BH-.

The second stage in the plasmid construction was the preparation for cloning of the Barstar gene under the regulation of the modified CaMV plus lac operator promoter. This was amplified from the plasmid p35S-op-Barstar *Eco*RI- which contained the promoter/gene sequence which had been modified to remove the *Eco*RI site between the promoter and coding sequence. This modification was accomplished by cutting the 35S-op-Barstar plasmid with *Eco*RI, carrying out a blunting reaction with T4 DNA polymerase, and then religating the blunted plasmid. The PCR was carried out using the 5' primer pQE-F and the 3' primer Bar-3'. The PCR fragment was cut with XhoI and KpnI. This was cloned into the 35S-op-Barstar *Eco*RI- plasmid also cut with XhoI and KpnI, and from which the unwanted gene had been purified away by gel electrophoresis. This enabled the removal of restriction sites at the three prime end of the Barstar coding region. This plasmid was called p35S-op-Barstar *Eco*RI-2.

The Barstar gene was then cut out of the p35S-op-Barstar *Eco*RI-2 plasmid with XhoI and *SalI* and cloned into pBRLac BH- cut with *SalI*. The Barstar gene could go into the *SalI* site in either orientation but only one orientation was found. The orientation of the insert was ascertained by a KpnI digest. Only a 1kb band was seen which was indicative of the orientation of the insert seen in plasmid pBRLac Op Barstar 1. The resulting plasmid was called V2.

The plasmid was then ready for the cloning of, in this case, a flower specific promoter, into the unique *Eco*RI cloning site 5 prime of the *lacIq* gene. The EGM3 promoter from the Eucalyptus MADS gene EGM3 was used for this purpose. This was cut from plasmid pEGM3 with *Eco*RI. This promoter was cloned in both orientations, and the resulting plasmids were called V2 EGM3 sense and V2 EGM3 antisense. The plasmid V2 EGM3 sense was used as a source of the EGM3 regulated *lacIq* and CaMVop Barstar genes for the plant transformation vector. The orientation of the EGM3 promoter was determined by an XbaI *Pst1* digest. The presence of 2.3 and 0.9 kb bands was indicative of the correct orientation.

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The second construct V1, containing the promoterless Barnase gene, was constructed using pBrGUS 2 as the starting point. A Barnase gene was amplified from genomic Bacillus amyloliquefaciens using the primers Barnase 5 prime Sal, and Barnase 3 prime Kpn. The resulting PCR product was restricted with KpnI and *SalI* as was pBrGus 1 and a ligation reaction performed. The plasmids from the resulting colonies were used as templates for sequencing using the amplification primers. Plasmid SB4 was found to contain a Barnase fragment of the same sequence as the B. amyloliquefaciens Barnase gene and this called pBRBarnase and used for further constructions.

Previous work using Barnase as a lethal gene in plants had shown that the anti-toxin gene for Barstar was required to be present and expressed from the same plasmid as the Barnase before a promoter region could be cloned 5 prime of the Barnase gene (Paul *et al.* 1992 Plant Molecular Biology 19: 611-622). This was achieved by using a plasmid that contained the Barstar gene and promoter from B. amyloliquefaciens in cis with the Barnase sequence. To this end the promoter plus Barstar DNA region was amplified from the B. amyloliquefaciens genomic DNA using primers 5 prime Barstar promoter and Bar 3 prime. The PCR fragment was restricted with KpnI as was pGEM 3f and a ligation reaction performed. The resulting white colonies were screened for inserts. The DNA from colony 7 was used for seqencing and found to contain the Barstar promoter plus Barstar sequence as in the genomic B. amyloliquefaciens DNA. This plasmid was called 7 prom Barstar, as shown in Figure 20.

The 7 prom Barstar plasmid was resticted with KpnI as was pBR Barnase and the promoter Barnase fragment was cloned into the KpnI site of the PBR Barnase DNA. This resulted in a plasmid known as V1. The EGM3 promoter was cut out of pEGM3 using *Eco*RI, blunted using DNA polymerase I (Klenow fragment) and cloned into the unique *SalI* cloning site of the V1 DNA which had also been restricted and blunted. The resulting plasmid with the promoter inserted in the correct orientation was called EGM3 VI sense (FIG. 26).

The EM3 V1 sense DNA was linearised with HindIII and cloned into the plant

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transformation vector Bin19 also linearised with HindIII. This resulted in two plasmids, EGM3 V1 Bin 1 and 2, corresponding to the two orientations of insertion. In orientation 2, the two *Eco*RI site present in EGM3 V1 Bin are approximately 80 bp appart. The EGM3 V1 Bin 2 was cut with *Eco*RI and blunted using DNA polymerase I (Klenow fragment). The EGM3 promoter lacIq gene, and the CaMV 35S op Barstar genes were cut out of EGM3 V2 sense using AatII and HindIII. This fragment was also blunted and ligated into the *Eco*RI cut and blunted EGM3 V1 Bin 2 to create the plasmids EGM3 Double Bin I and II (FIG. 25), again corresponding to the two possible orientations. These plasmids were used for transformation of plants via A. Tumefaciens.

The procedure was then repeated with further Eucalyptus MADS promoters. These were EGM2 long and short, and a shorter version of EGM3.

Primers.

Barnase 5 prime Sal

5' CCGTCGACATGGCACAGGTTATCAA 3'
SalI

Barnase 3 prime Kpn

5' CGGGTACCTTATCTGATTTTTGTAAAGG 3'
KpnI

Five prime Barstar promoter

5' CGGGTACCGTCCAATCTGCAGCCGTCCGA 3'
KpnI

Bar 3'

5' GCGGTACCTTAAGAAAGTATGATGGTG 3'
KpnI

D23

5' CGGGTACCTATCTCCTATTTCCCCCACG 3'
KpnI

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pQE-F

5' GCGTATCACGAGGCCCTTTC 3'

lacI

5' GCGAATTCAACATGGAACCAGTAACGTTATA 3'
*Eco*RI

EXAMPLE 7

Isolation of reproductive organ-specific promoters

Five cone-specific genes displaying strong homology to Arabidopsis thaliana and Anthirrhinum majus floral meristem and organ identity genes were isolated from P. radiata cDNA library prepared from immature female and male cones. Three of them, PrMADS1, 2 and 3, belong to the family of MADS-box genes showing homology to Arabidopsis AGL-2, AGL-4 and AGL-6 genes and *dal1* gene from another non-angiosperm, Picea abies (Norway spruce), respectively. The PrFL1 gene is the pine ortholog of Arabidopsis Leafy (*Lfy*) and Floricaula (*Flo*) gene from Anthirrhinum. The PrCon1 shows strong homology to Arabidopsis CONSTANS (co) gene. To elucidate the function of these genes we took the approach of characterising their expression pattern in male and female cones during different stages of cone development.

A significantly lower level of expression was detected in vegetative tissues: vegetative buds, needles, stem and roots. *In situ* hybridisation showed that expression of these genes is detectable only in reproductive tissue cells.

Expression analysis revealed that all five genes show different patterns of expression in different stages of development of male and female cones (FIGS. 8 and 9). PrMADS1, 2 and 3 genes are cone-specific: expression of both genes was restricted to reproductive organ primordium tissues. No detectable expression of these genes was observed in vegetative tissues: vegetative buds, needles, stems, roots. For PrFL1 and PrCon1 low detectable expression was observed in vegetative buds.

In reproductive organs low level of expression of both genes was detected at early stages of cone development (5 mg cones) which was increased during cone development (50 mg cones). In male cones expression of both genes was restricted

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to microsporangium containing primary sporogenous cells. In female cones expression of both genes was restricted to premature ovules.

To characterise MADS proteins as DNA-binding proteins *in vitro*, we expressed both proteins in E. coli and characterised their DNA-binding properties. PrMADS1,2,3 proteins are sequence-specific DNA-binding proteins. Their DNA-binding consensus sequence is similar to that of the AGAMOUS protein. All three proteins bind a DNA sequence matching the consensus sequence of CArG box TT(A/T)CC(A/T)$(A/t)_2$(T/A)NNGG(-G)$(A/T)_2$ (oligo A) for PrMADS1 and PrMADS2) (FIG. 31). Mutation of these consensus sequences (oligo B) significantly decreases their binding of PrMADS 1,2 or 3 proteins. Competition with non-radioactive oligos did not decrease binding of any of the proteins to the CArG consensus. This indicates that we are dealing with specific DNA-protein interactions.

Upstream sequences were isolated using a 'Promoter finder' strategy (FIG. 29). A special adaptor was ligated to the ends of DNA fragments generated by digestion of genomic DNA from P. radiata with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I separately. The enzymes used were selected because they have six-base recognition sites and generate blunt ends. Following adaptor ligation, these DNA fragments were used as a template for PCR using first adaptor primers AP1, AP2 and gene-specific primers GSP-1,2.

The sequences of adaptor (first sequence), adaptor-primers and polymerase blocking primer shown below. It is noted that adaptor primer AP1 corresponds to bases 1 to 22 of 22 of the adaptor, adaptor primer AP2 corresponds to bases 13 to 31, and the polymerase block corresponds to bases 41 to 48.

Adaptor:

5'GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGG-CTGGT-3'

Polymerase block:

AP1 _____ 3'-NH_2-CCCGACCA-$P0_4$-5'

AP2

Adaptor primer 1. (AP1): 5'-GTAATACGACTCACTATAGGGC-3'

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PrMADS2: 5' CGCCTTTTTCAGCAGACCATTCCGGC 3'

PRMADS3: 5' CAGCAGTCCGTTTCCGGCGCTTCG 3'

PrFL1: 5' CGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGG 3'

The 1-2 kb PCR fragments were cloned into the TA-type cloning vector and sequenced. Sequences of cDNAs, proteins and promoter regions for PrMADS2, PrMADS3 and PrFL1 are shown in FIGS 30, 33 & 36.

EXAMPLE 8

Introduction of the chimaeric DNA sequences into the plants

Chimaeric DNA sequences were co-transferred into the plant tissue using the A. tumefaciens system. Transformants were retained by growing on selective medium. Presence of two plasmids was proved using standard molecular biology techniques (Southern, northern hybridizations, PCR). Plasmid A has Barnase gene and plasmid B has Barstar gene under control of plant promoters. This creates an additional selection for co-transformation.

The vector system designed involves a cascade of genes expressing under the positive control of the reproductive organ-specific promoters and negative control of the E. coli lactose (lac) repressor-operator system. During the non-flowering period, in the absence of the repressor protein, the operator sequence will permit constitutive expression of the Barstar gene or antisense RNA in all organs. Because of possible leakage of the reproductive organ-specific promoter, low level of the lethal gene product will exist in different plant organs and cells.

Accumulation of the Barstar protein in cytoplasm or antisense RNA in nuclei of these cells will block the cytotoxic effect of the lethal gene product. At the early stages of flowering, expression of the B (or other RNases) and *lacIq* genes in the appropriate cells of the reproductive organ will dramatically increase. Accumulation of the *lacIq* protein in the nucleus of these reproductive organ cells will block expression of the Barstar gene (or antisense RNA) via the lac repressor-operator interaction which will

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increase the level of lethal gene products in the target reproductive organ cells.

EXAMPLE 9

Three Eucalypt genes (EGM1, 2 & 3) with homology to other plant MADS-box genes have been cloned and sequenced (FIGS. IO-13 and 16). Phylogenetic analysis has been performed to examine the relatedness of the eucalypt genes to other MADS-box genes (FIG. 17). This revealed that EGM2 is likely to be homologous to the GLOBOSA MADS-box gene group which are involved in development of petals and stamens. EGM3 and EGM1 both fall in the AGL2 group of MADS-box genes. These genes are most commonly expressed in the inner three whorls of the flower (petals, stamens and carpels) but their function in flower development is yet to be determined.

The pattern of expression of all three EGM genes has been characterised (FIGS. 14 and 15). Expression of these genes was not detected in any vegetative tissue. The EGM1 gene is expressed in the petals, stamens and the carpel of eucalypt flowers. EGM3 is expressed in the floral meristem and in sepals. EGM2 is expressed in petals and stamens. The EGM3 and EGM2 genes have been shown by Southern analysis (FIG. 18) to be single genes. EGM1 is also likely to be a single gene.

The promoter regions of all three of these genes may be used in sterility gene constructs and have been isolated from a eucalypt genomic library. The EGM3 promoter has been engineered into lethal gene constructs.

Northern blots were performed on a range of tissues in order to determine the tissue specificity of expression of the three EGM MADS-box genes. Tissue used for isolation of RNA from roots, seedlings stems shoots leaves and mature flowers was obtained from Eucalyptus grandis plants. Tissue for isolation of RNA from floral tissues including receptacles, petals, stamens, carpels, and styles was collected from E. globulus flowers. This species was used because it produces very large flowers, making collection of sufficient amounts of RNA much easier. Ten micrograms of total RNA was used in the blots which included the different floral tissues and 20 micrograms was used in blots of E. grandis RNA which included the vegetative tissue. Blots were probed with the three EGM genes which had been digested with appropriate restriction enzymes to remove the MADS-box.

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The northern blots indicated that all three EGM genes are expressed at a high level in eucalypt flowers. When northerns were exposed to film for long periods, weak expression of the EGM2 and EGM1 genes was detected in vegetative tissue. The EGM3 gene was specifically expressed in floral tissue. Within the flower, the EGM2 gene was observed to be expressed in stamens and petals. The EGM3 gene is expressed in receptacles, petals, stamens, carpels and styles.

Example 10

The first part (component 1) of the cascade contains the reporter GUS gene under control of floral meristem identity gene promoter (ie. LEAFY from Arabidopsis thaliana) with lac-operator (*LEAFY-op*). The second construct (component 2) contains the repressor *lacIq* gene under control of maize *GST-27* gene promoter. This promoter is up-regulated by treatment of plants with the safeners, dichlormid and R-29148. The Arabidopsis plants were co-transformed with two components.

An alternative strategy is for two transgenic Arabidopsis lines carrying components 1 and 2 separately to be crossed. Expression from component 1 alone produces blue flowers after staining with X-gal. Chemically-inducible induction of expression from component two, in plants expressing component one, eliminated the blue colour seen in flowers that were not chemically induced.

Example 11

The first component of the cascade contains the Barnase gene under control of floral meristem identity gene promoter (ie. LEAFY from Arabidopsis thaliana) with lac-operator. The second component contains the repressor *lacIq* gene under control of maize *GST-27* gene promoter. Arabidopsis plants were co-transformed with the two components. In non induced plants expression of only component one produces sterile Arabidopsis plants. Induction of expression from component two, in plants expressing component one, stopped expression from *LEAFY-op* promoter and restored the fertility in initially sterile Arabidopsis plants.

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Example 12

A number of abbreviations are used in the following text. These are in common use in the field of plant tissue culture.

BA: benzyladenine also known as 6-benzylaminopurine.

IBA: indole-3-butyric acid.

NAA: 1-naphthaleneacetic acid

TDZ: thidiazuron also known as 1-phenyl-3-[1,2,3-thiadiazol-5-yl] urea.

The following is a detailed description of the preferred steps for producing transgenic Eucalyptus from shoot and seedling explant material.

Shoot explants

1. Subculture shoots monthly on solid KG medium containing 0.2mM BA. Keep in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) and 22.5°C.

2. Use whole shoots 3-4 weeks after subculture.

3. Remove lower leaves leaving the top 4-6 leaves.

4. Wound leaves 5 to 6 times with a needle (e.g. 25G) and place whole shoots in an Agrobacterium suspension (approximately $1x10^8$ cfu ml^{-1}) containing 10-100mM acetosyringone for 10 minutes to 2 hours. An Agrobacterium suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Best results when wound near base of leaf. Alternatively, instead of leaving the wounded shoots in an Agrobacterium suspension for 1 hour, the shoots can be vacuum-infiltrated with the Agrobacterium for 10-30 mins at 40-100 Kpa. The shoots do not need to be wounded for the vacuum infiltration procedure. However, callus formation is greater when wounding is effected with a needle rather than by vacuum-infiltration.

5. Blot shoots between sterile filter papers and insert shoots vertically into KG medium containing 0.2mM BA. Co-culture for 2 days in the dark.

6. Transfer shoots (still upright) to KG medium containing 0.2mM BA and 200 mgL^{-1}

Seedling explants

1. Disinfest seeds and germinate on KG containing 0.2 mM BA.

2. Using 10-12 day old seedlings, remove roots and place them in an overnight-grown Agrobacterium suspension (approximately $1x10^8$ cfu ml^{-1}) containing 50mM acetosyringone. An Agrobacterium suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately $1X10^8$ cfu mL^{-1}. Wound the cotyledons and hypocotyl by gently stabbing with a 30 gauge syringe needle under a dissecting microscope. Incubate for 1 hour then remove the seedlings from the suspension and blot them between sterile filter papers to remove excess liquid. Instead of leaving the wounded cotyledons in an Agrobacterium suspension for 1 hour, the cotyledons can be vacuum-infiltrated with the Agrobacterium for 20 mins at 95 KPa (28mm Hg). The cotyledons do not need to be wounded for the vacuum infiltration procedure. However, the hypocotyls require wounding even when using vacuum infiltration.

3. Co-cultivate on KG medium (containing 0.2 mM BA) for 2 days in the dark making sure that the seedlings are standing upright in the medium.

4. Transfer the seedlings (still upright) to KG medium (containing 0.2mM BA) and containing 200 mgL^{-1} cefotaxime. Continue incubation in low light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$ PAR) for 5 days.

5. Excise the hypocotyls and cotyledons. Transfer the hypocotyls to callus induction medium G22 containing 0.5mM BA, 1.0mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Transfer the cotyledons to G22 medium containing 1.0mM BA, 1.0mM NAA, 0.3mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Continue incubation in the dark for 2 weeks.

6. Transfer explants to the same medium containing 15 $mg.L^{-1}$ geneticin and 200 $mg.L^{-1}$ cefotaxime. Continue dark incubation. Subculture every two weeks until about 6 weeks have elapsed.

7. After 6 weeks, transfer to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 15 mgL^{-1} geneticin. Leave the cultures in the dark for 5-7 days then transfer them to the light (16 hour photoperiod, 100-350 Lux or 1-8 $mmolm^{-2}s^{-1}$

PAR).

8. After about 10 weeks on the GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime, a number of explants will have produced shoots. These shoots are excised and transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime. Meanwhile, the callus that has also formed on the original explants is subcultured back to GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime and left for a further month, by which time more shoots may develop which can then be transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime.

9. All shoots and callus are now transferred to fresh KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime for another month or so.

10. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks.

11. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 $mmolm^{-2}s^{-1}$ PAR). Subculture every 2 weeks.

12. Assay putative transformed shoots for marker gene(s) activity.

13. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

Seedlings of 12 days old or younger are best for transformation but seedlings up to 20 days old can be used for regeneration.

Shoot regeneration frequencies for non-transformed cotyledon and hypocotyl explants without selection are approximately 80%.

Experience has shown that removal of cefotaxime from the medium, even after 3 months, will result in rapid overgrowth by Agrobacterium. The concentration of cefotaxime varies between liquid and solid media.

Shoot material grew faster in liquid medium and formed more shoots when

compared to shoots grown on solid medium. This liquid selection step has the advantages of reducing false positives by increasing selection pressure, reducing residual Agrobacterium and increasing the amount of shoot tissue compared to solid grown cultures.

The regenerated rooted transgenic plants are then moved to a soil based medium and grown into trees of a size and form suitable for planting. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

The media used in this study were G22, GBA and KG as described by Laine and David (1994). However, various basal media, including MS, B5 and P24, have been tested and found to support shoot regeneration. The plant growth regulator regimes were generally different from those of Laine and David (1994), which were in the combination of 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for callus induction from leaves; 1-3 mM BA, 0.05-1 mM TDZ and 0.5-2.5 mM NAA for cotyledons; and 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for hypocotyls. The differentiation medium was generally a GBA medium, which was a G22 but supplemented with 2.5 - 5 mM BA and 0.5 mM NAA (Laine and David, 1994). A KG medium containing 0.2 mM BA is generally used as subculture medium for clone materials. All media were solidified with 0.25% Gelrite or Phytagel. pH was adjusted to 5.7 - 5.8 using potassium hydroxide before autoclaving for 15 minutes at 121°C.

A preferred method for the preparation of Agrobacterium inoculum is described below.

1. Streak out the Agrobacterium strain containing the construct onto plates with selection, eg YEP + rifampicin (50 mg/L) + kanamycin (100 mg/L) for LBA4404, EHA105 and AGL1 containing the pBin GUSINT construct.

2. Incubate plate at 280°C for 2 days.

3. Pick a single colony into YEP broth with selection and grow on shaker overnight at 280°C.

4. Use the overnight culture to inoculate (1% inoculum) fresh medium with selection

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and grow on shaker overnight at 280°C. This additional step will produce a more consistent inoculum for plant transformation.

5. Harvest Agrobacterium, wash and resuspend in tissue culture medium. Dilute to an appropriate concentration ready for transformation.

6. Streak out the resuspended Agrobacterium on to lactose yeast medium plate and incubate plate at 280°C for 2 days. Do a Benedict's test (Bernaerts and Deley, 1963) on the colonies to confirm they are Agrobacterium.

This strategy will give good thick cultures of Agrobacterium on the day of transformation. A culture grown overnight which was inoculated from a plate or glycerol stock will give variable results.

An AGL1 culture grown as described above and diluted to an optical density (600nm) of 0.98 had a viable count of 2.37×10^9 cfu/ml.

It will of course be realised that while the above has been given by way of illustrative example of this invention, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as claimed in the claims appended hereto.

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CLAIMS

1. A method of regulating a eukaryotically active gene, comprising transforming a cell with a construct constitutively expressing a modulator gene product regulating the eukaryotically active gene or its product and a further gene product regulating said modulator gene or its product, the promoter of said gene being developmental for a target tissue and the promoter of said further gene being selected from inducible promoters and developmental promoters for the same said tissue or complementary tissues.

2. A method of regulating a eukaryotically active gene according to claim 1, wherein said eukaryotically active gene is selected from a native gene to the organism of interest or an introduced gene.

3. A method of regulating a eukaryotically active gene according to claim 2, wherein said eukaryotically active gene codes for an expression product selected from an active polypeptide, an enzyme or an enabling gene product for a synthetic pathway.

4. A method of regulating a eukaryotically active gene according to claim 3, wherein said synthetic pathway is selected for production of pigments and dyes.

5. A method of regulating a eukaryotically active gene according to claim 4, wherein said dye is such as anthocyanin.

6. A method of regulating a eukaryotically active gene according to claim 4, wherein said synthetic pathway is selected for production of an insecticidal compound.

7. A method of regulating a eukaryotically active gene according to claim 6, wherein said insecticidal compound is Bt toxin.

8. A method of regulating a eukaryotically active gene according to claim 3,

wherein said enzyme is selected from a ribonuclease having a lethal effect on a target tissue and a peroxidase conferring nematode resistance on root tissue.

9. A method of regulating a eukaryotically active gene according to claim 8, wherein said ribonuclease gene is that of Barnase from B. amyloliquefaciens.

10. A method of regulating a eukaryotically active gene according to claim 9, wherein said gene is constructed including the coding region of Barnase from B. amyloliquefaciens under the control of promoter sequences of genes expressed in the male and female parts of plant reproductive organs at the early stages of development.

11. A method of regulating a eukaryotically active gene according to any one of the preceding claims, wherein said eukaryotically active gene is constructed with a promoter selected from those promoting eucalypt genes designated EGM1, 3 & 2 in Eucalyptus grandis.

12. A method of regulating a eukaryotically active gene according to any one of claims 1 to 10, wherein said eukaryotically active gene is constructed with a promoter selected from those promoting pine genes designated PrMADS1, 2 and 3 in Pinus radiata.

13. A method of regulating a eukaryotically active gene according to any one of the preceding claims, wherein said eukaryotically active gene, modulator gene and further gene and their respective promoters may comprise a transformation cassette with which the target organism may be transformed.

14. A method of regulating a eukaryotically active gene according to claim 13, wherein said promoter for said eukaryotically active gene is tissue specific and is inhibited by a constitutively-promoted inhibitor gene product, which in turn is controlled by an inducibly-promoted further gene product.

15. A method of regulating expression of a eukaryotically active gene, comprising transforming a cell with a transformation cassette comprising said eukaryotically active gene, a modulator gene constitutively expressing a product regulating the eukaryotically gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoter of said gene being developmental for a target tissue and the promoter of said further gene being selected from inducible promoters and developmental promoters for the same said tissue or complementary tissues.

16. A method of transformation of a propagatable eukaryotic cell with an expression cassette including:

a lethal gene expressing a cytotoxic peptide in a target tissue under the control of a promoter substantially specific to said target tissue;

an modulator gene constitutively expressing an inhibitor of the production or action of said cytotoxic peptide, and

a further gene under the control of said substantially specific promoter and functioning to block said inhibitor or modulator gene.

17. A method of regulating a eukaryotically active gene according to any one of the preceding claims 1 to 14, wherein said eukaryotically active gene is selected from Barnase and Barstar genes from an extract of B. amyloliquefaciens, isolated by PCR by the primers designated P1 and P2 (for Barstar) and P3 and P4 (for Barnase) and comprising:

P1 5' GCG AAT TCC GCA CAT GAA AAA AGC 3'
*Eco*RI

P2 5' GCA AGC TTA AGA AAG TAT GAT GGT G3'
HindIII

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

P4 5' CGG GTA CCT TAT CTG ATT TTT GTA A3'
KpnI

18. A method of regulating a eukaryotically active gene according to any one of the preceding claims 1 to 14, wherein said eukaryotically active gene is a lethal gene wherein the cytotoxic effect of the lethal gene expression product is increased by coupling a localisation signal sequence to the construct.

19. A method of regulating a eukaryotically active gene according to claim 18, wherein said eukaryotically active gene codes for an RNase targeted into the nucleus by fusion of a nuclear localisation signal (NLS) sequence to the RNase gene.

20. A method of regulating a eukaryotically active gene according to claim 19, wherein the gene is the Barnase gene and said fusion is by PCR, wherein a set of PCR primers for fusing the NLS sequence from VirD2 gene of A. tumefaciens to the B gene are used and comprising:

P3 5' GCC CAT GGC ACA GGT TAT CAA CAC G3'
NcoI

BD1 5' CCT TAC AAA AAT CAG A GT CCT TTC AAA GCG TCC G3'
3' end of Barnase 5' end of NLS of VirD2

BD2 5' CGG ACG CTT TGA AAG GAC TCT GAT TTT TGT AAA G3'
5' end of NLS of VirD2 3' end of Barnase

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

21. A method of regulating a eukaryotically active gene according to any one of the preceding claims 1 to 20, wherein said repressor gene and inhibitor gene are adapted to form a specific repressor/operator couple.

22. A method of regulating a eukaryotically active gene according to claim 21, wherein said repressor/operator couple comprises the E.coli *lacIq* gene under control of the same promoter as the eukaryotically active gene construct and used to repress the action of the repressor gene promoter modified by insertion of a lac operator sequence.

23. A method of regulating a eukaryotically active gene according to claim 22, wherein said repressor gene promoter is a modified 35S RNA CaMV promoter having at least one lac operator between the promoter and coding region of the repressor.

24. A method of regulating a eukaryotically active gene according to claim 23, wherein said modified 35S-RNA-CaMV sequences are amplified by PCR using primers designated 35S1 and 35S2 and comprising:

35S1 5' GCC TCG AGC ATG GTG GAG CAC GAC AC3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

25. A method of regulating a eukaryotically active gene according to claim 23, wherein said modified 35S-RNA-CaMV sequences are amplified by PCR using primers designated OP1 and 35S2 for the introduction of lac operators into both the 3' and 5' region of the modified 35S promoter:

OP1 5' GCC TCG AGC TTT GTG AGC GGA TAA C3'
XhoI

35S2 5' GCG TCG ACT CTC CAA ATG AAA TGA AC3'
SalI

26. A method of regulating a eukaryotically active gene according to claim 22, wherein said *lacIq* gene is isolated with enzyme cleavage sites for cloning into the

plant vectors, using two primers corresponding to the 5' and 3' ends of the gene and designated AM 1 and AM 2:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

AM 2 5'GCG GTA CCT CAC TGC CCG CTT TC3'
KpnI

27. A method of regulating a eukaryotically active gene according to claim 26, wherein the 3' end of the *lacIq* gene is fused to the NLS sequence by a set of PCR primers:

AM 1 5'GCT CTA GAC CAT GGA ACC AGT AAC GTT ATA 3'
Xba I

LD 1 5' CTG GAA AGC GGG CAG GTC CTT TCA AAG CGT CCG 3'
3' end of *lacIq* 5' end of NLS in VirD2 gene

LD2 5' CGG ACG CTT TGA AAG GAC CTG CCC GCT TTC CAG 3'
5' end of NLS in VirD2 3' end of *lacIq*

D 23 5' CGG GTA CCT ATC TCC TAT TTC CCC CAG G3'
KpnI

28. A transformation cassette comprising a eukaryotically active gene, a modulator gene constitutively expressing a product regulating the eukaryotic gene or its product, and a further gene expressing a product regulating said modulator gene or its product, the promoter of said gene being developmental for a target tissue and the promoter of said further gene being selected from inducible promoters and developmental promoters for the same said tissue or complementary tissues.

29. An expression cassette including:

a gene expressing a peptide cytotoxic to a target tissue under the control of an inducible promoter substantially specific to said target tissue;

an inhibitor gene constitutively expressing an inhibitor of the production or

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action of said cytotoxic peptide, and

a repressor gene under the control of said inducible promoter and functioning to block said inhibitor or inhibitor gene.

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ABSTRACT

There is provided a method of regulating a eukaryotically active gene, comprising transforming a cell with a transformation cassette expressing a modulator gene product regulating the eukaryotically gene or its product and a further gene product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues. The lethal gene expressing Barnase, a ribonuclease of B. amyloliquefaciens, is placed under the control of a tissue specific promoter, such as those derived from PrMADS 1, 2 or 3 of Pinus radiata or EGM1, 2 or 3 of Eucalyptus grandis. The same tissue specific promoter is used to express *lacIq* gene, a repressor for Barnase (Barstar) being promoted by a modified 35s RNA CaMV promoter including the lac operon. The cassette is used to transform plant cells for regeneration into plants expressing the Barnase in the target tissues with improved specificity and reduced promoter leakage.

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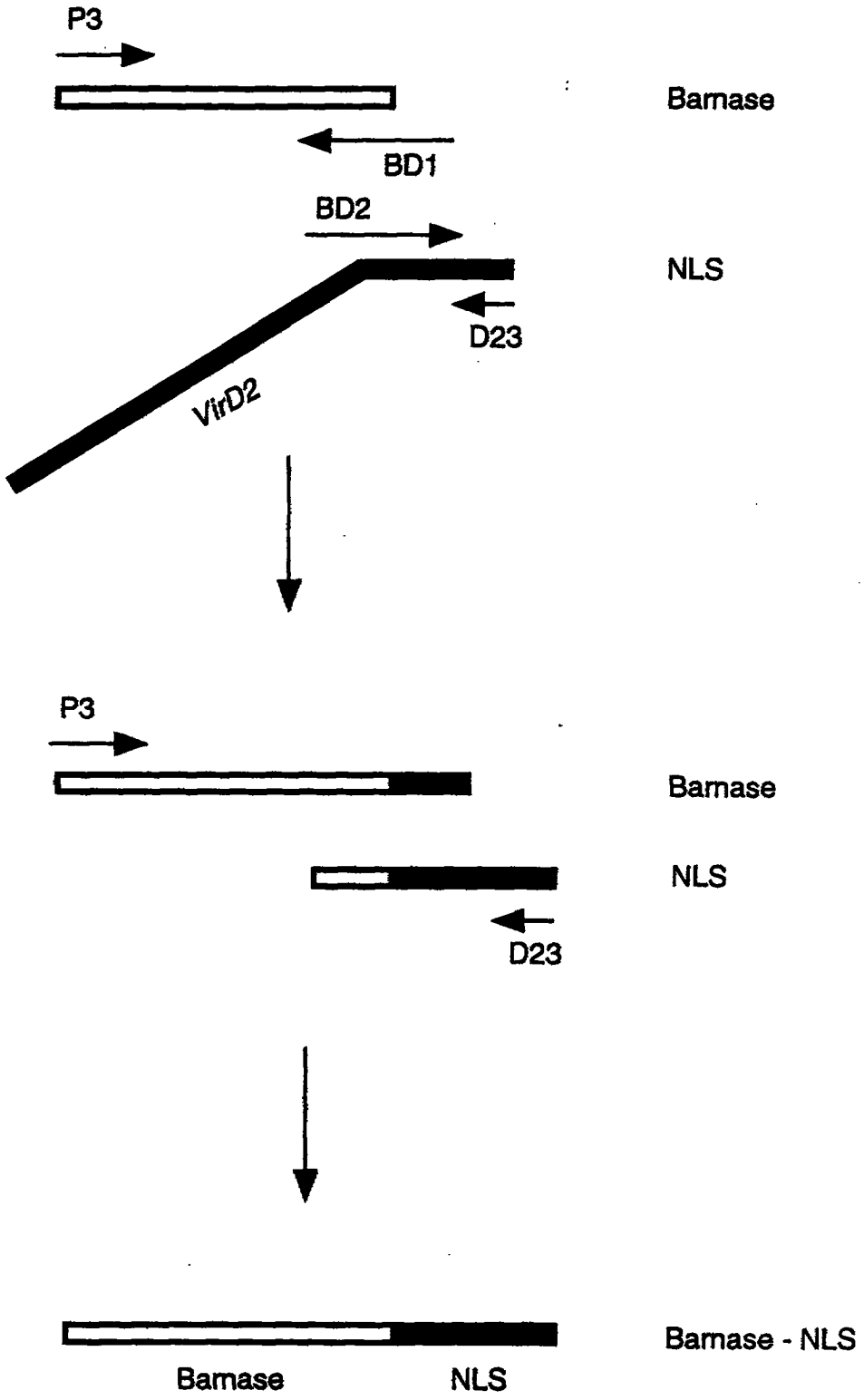


Figure 1

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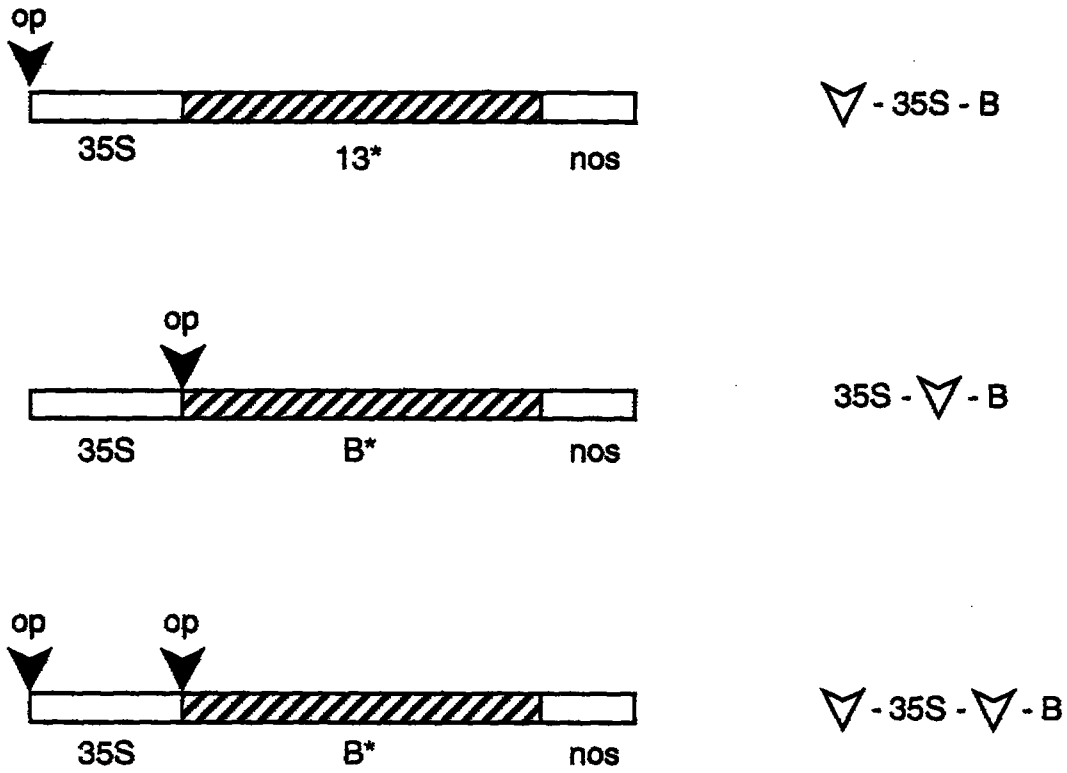


Figure 2

PCT/AU 97/00089
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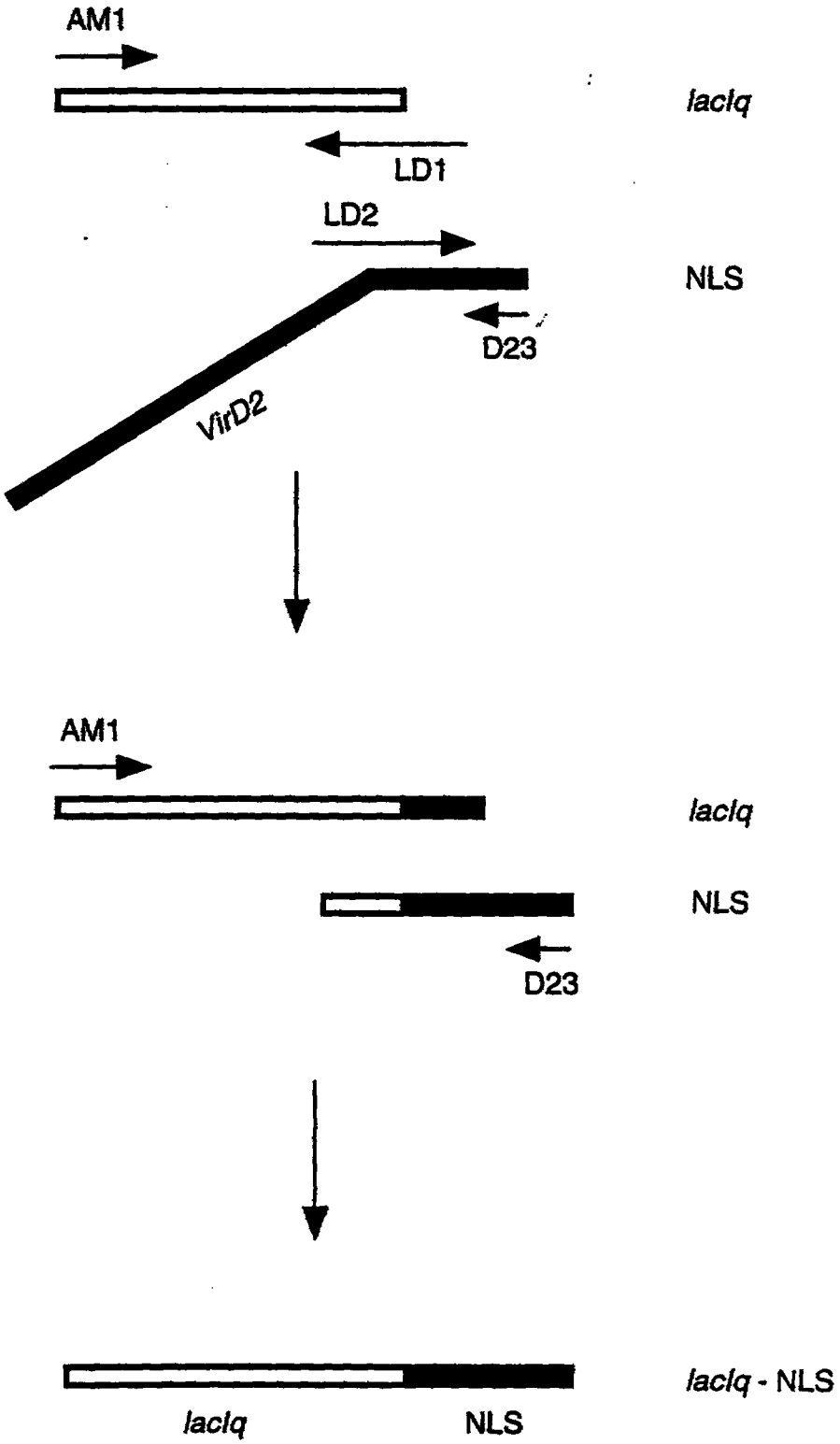
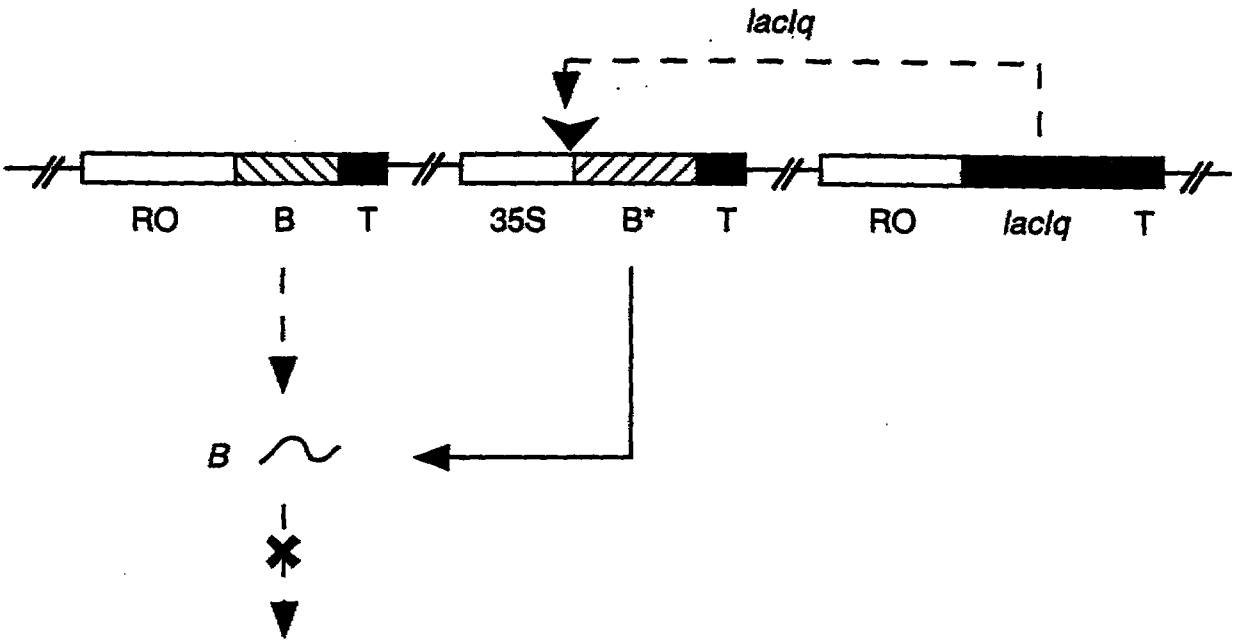


Figure 3

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IPEA/AU

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A. non - flowering period



B. flowering period

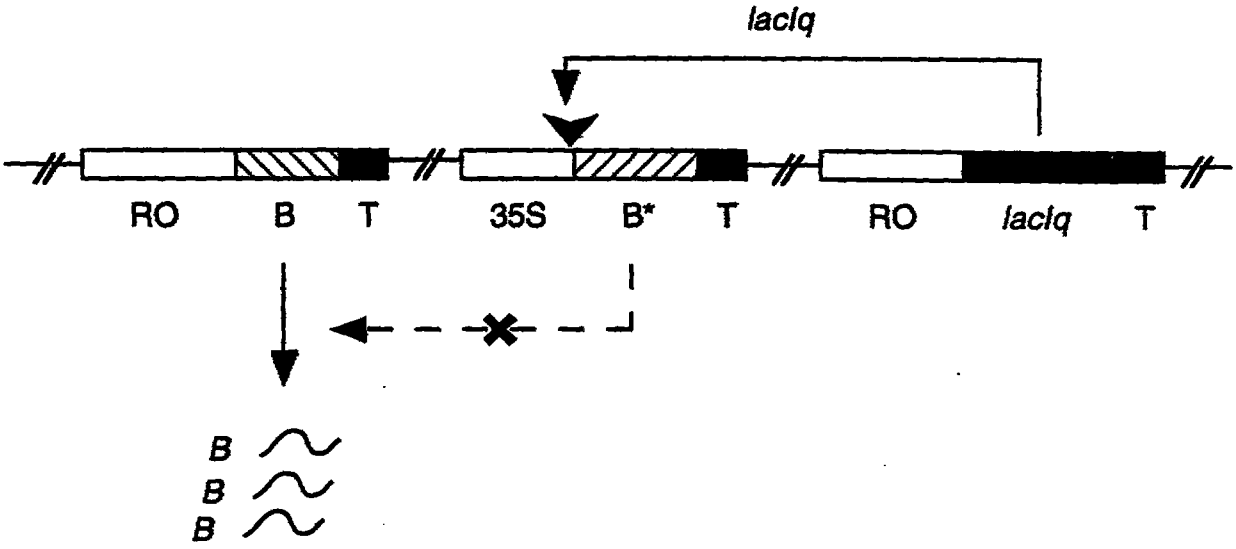
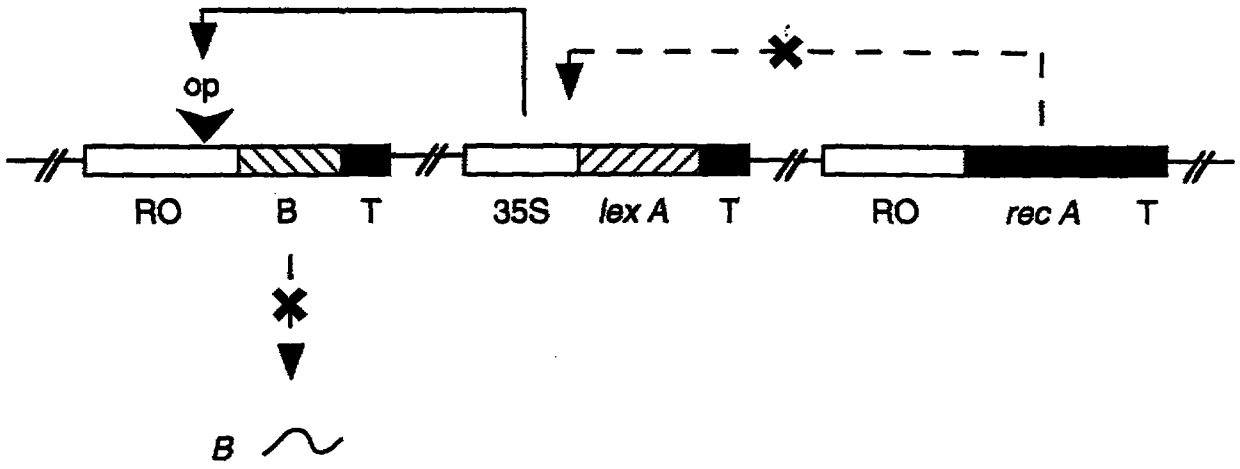


Figure 4

AMENDED SHEET
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PCT/ - 97/00089
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A. non - flowering period



B. flowering period

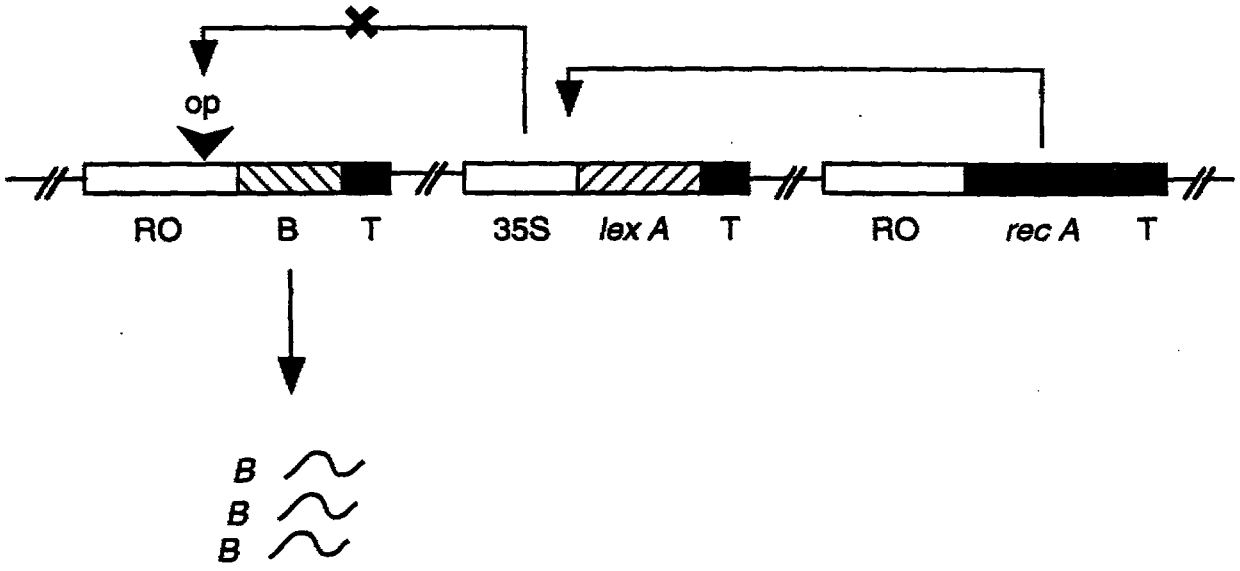


Figure 5

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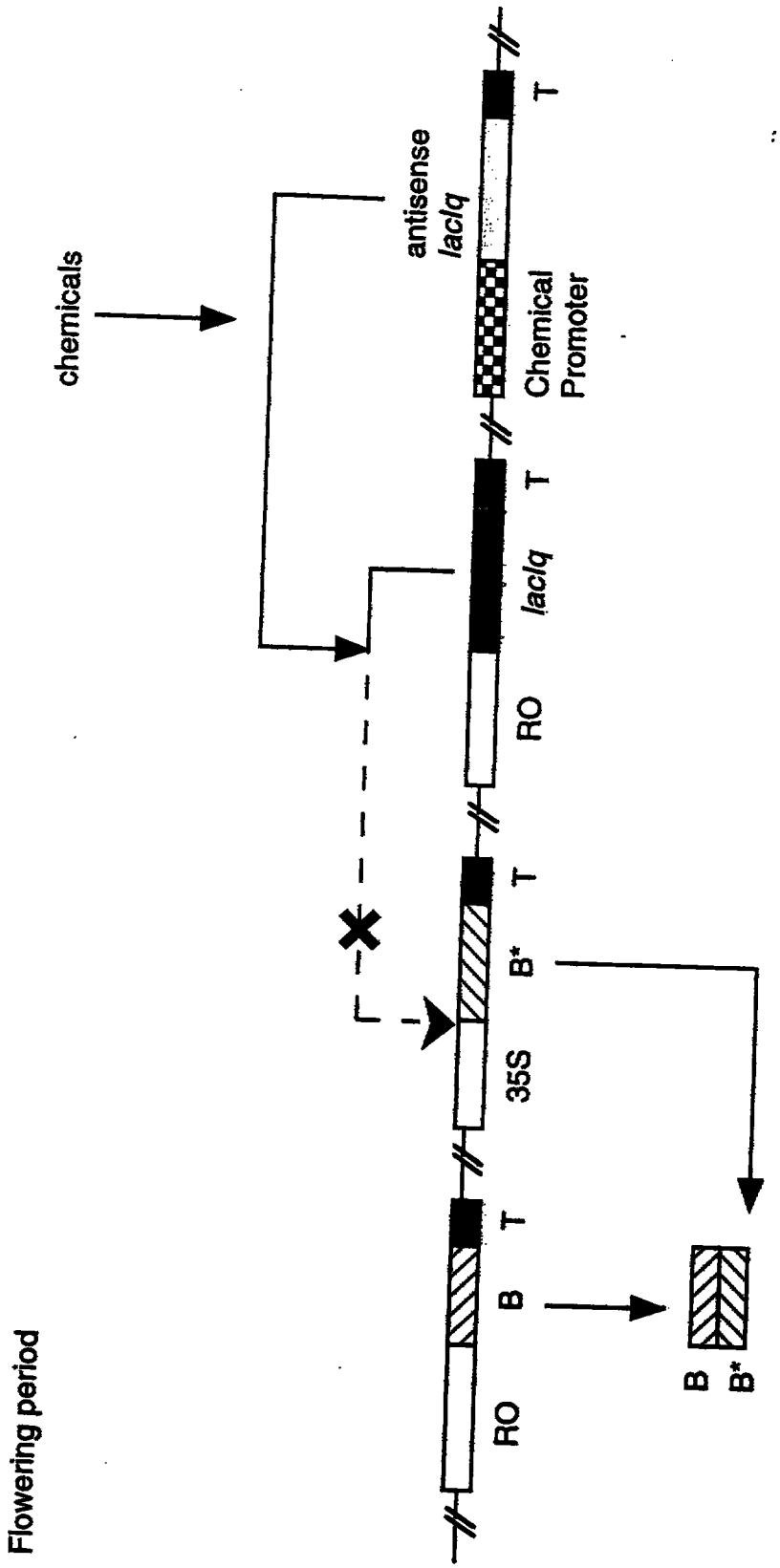
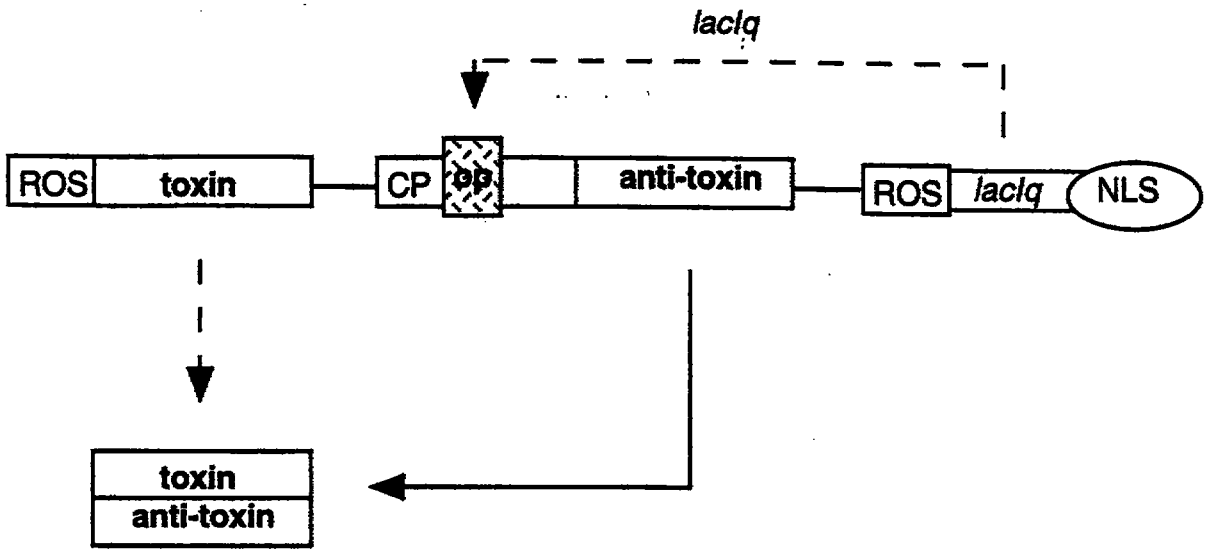


Figure 6

PCT/A 97/00089
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A. non - flowering period
(in all tissues)



B. flowering period
(in reproductive tissues)

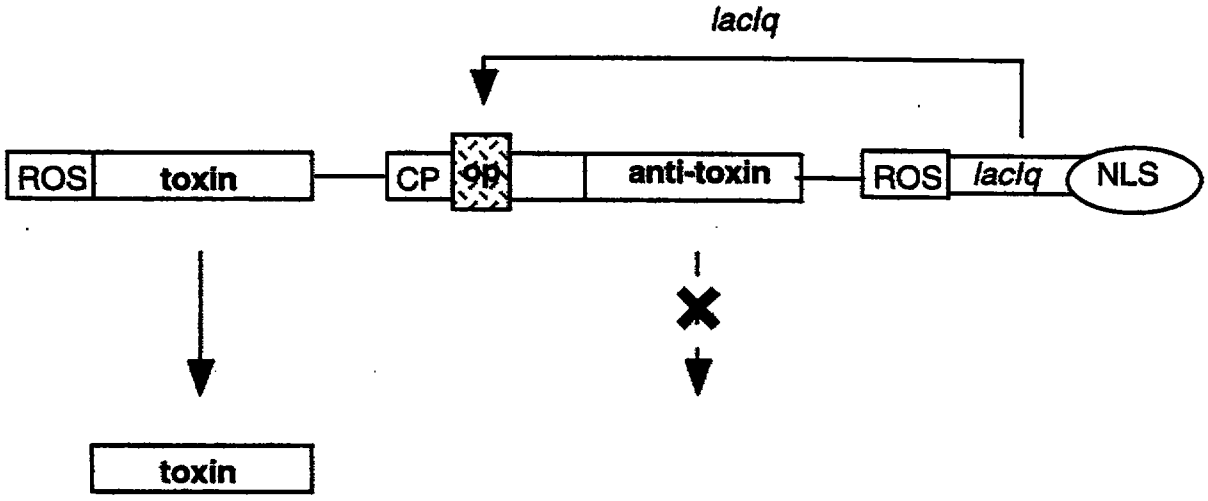
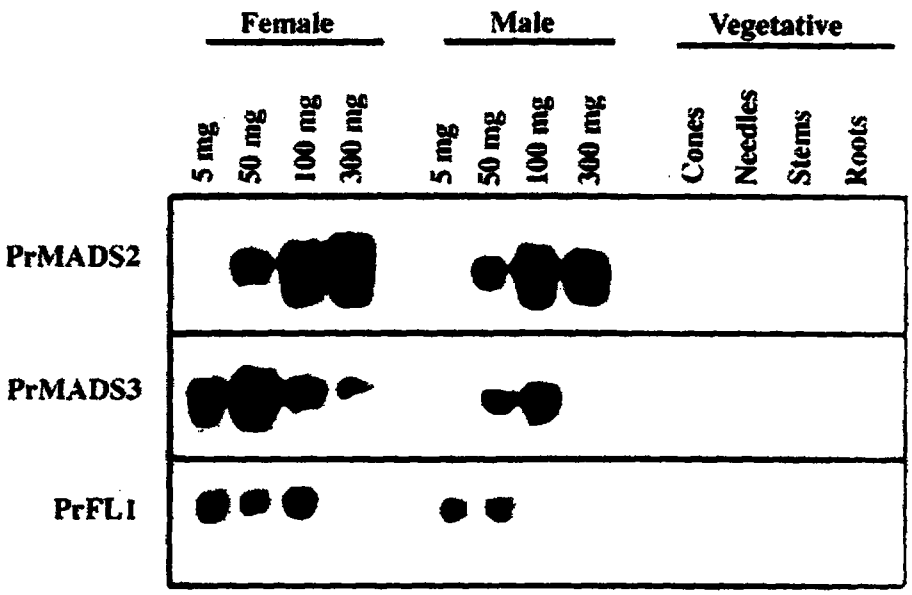


Figure 7

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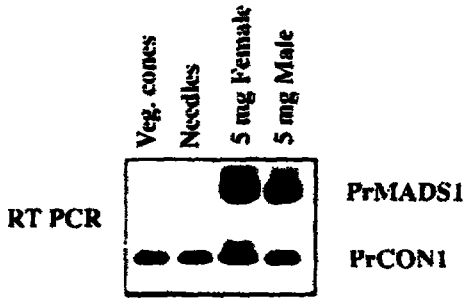


Figure 8

AMENDED SHEET
IPEA/AU

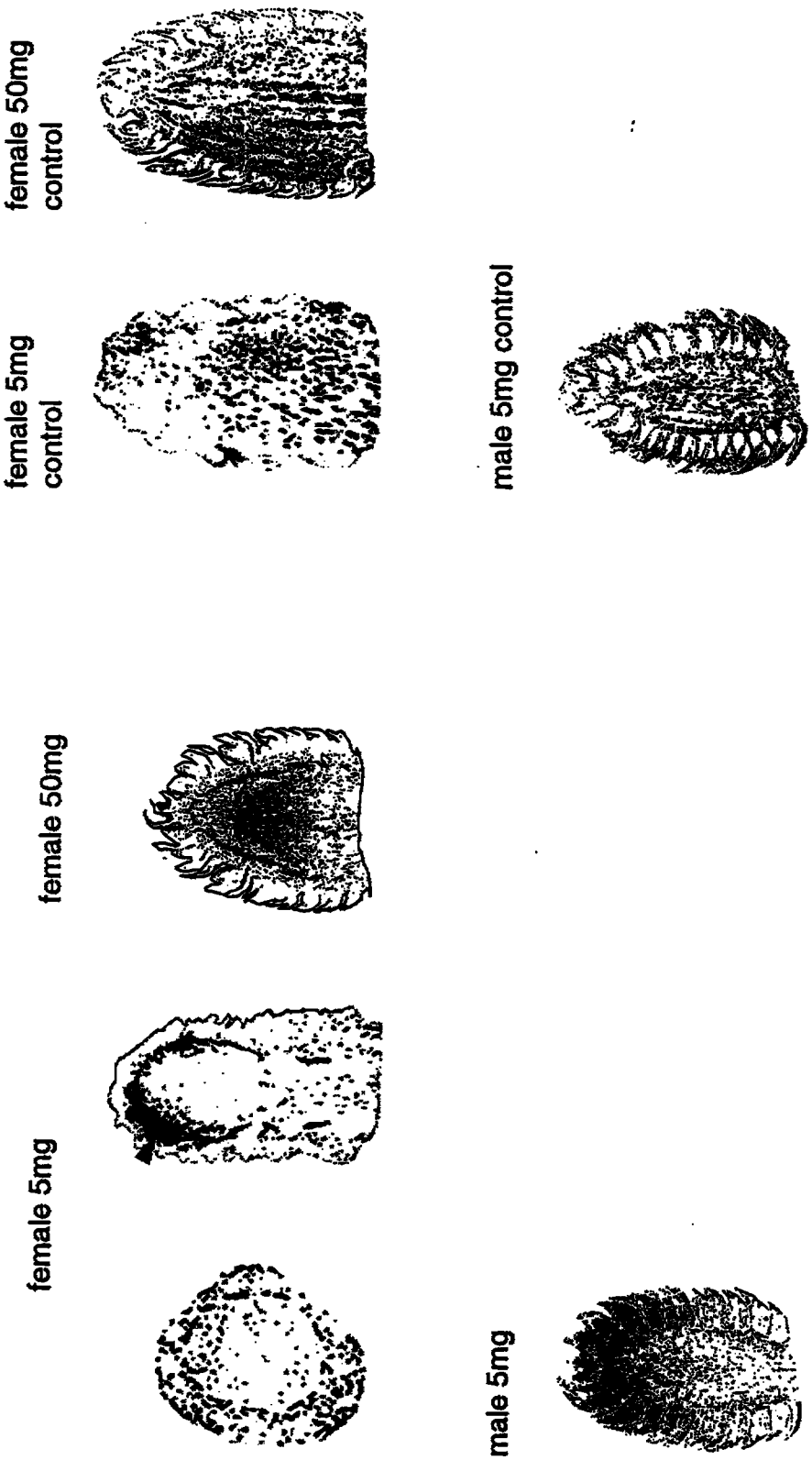
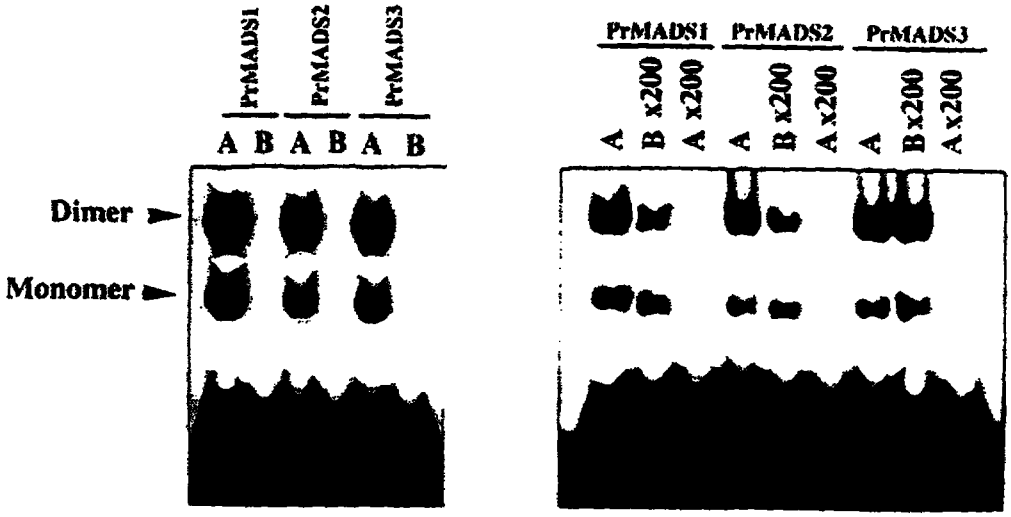

female 50mg control
female 5mg control
male 5mg control
female 50mg
female 5mg
male 5mg

Figure 9

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AG consensus CArG-box
NTT(A/T)CC(A/T)NNGG(-G)(A/t)2N

A: TTACCAAAAAAGGAAA

B: TTAGGAAAAAACCAAA

Figure 10

-373 TTTTACTTGGCATAATACATGCACGAACCACTATGCAATCAACCAATTGGGAAGGTTTTTTTTTTTTGTTTTTTTTTTTTTT

-293 TGGGGGGGGGGTGTTCTGGTTTTATTGGGTGGTTCTCTTGACATTGTAATTTCCCCTTAAAATTCCGCTTCATGACA

-213 AAAAGTACATAGAGGGGCTACTATCTTTGTTGACTTTTACCGTGTAGAGGGTTCCTAAGAGAGAGGGGGAGGGAAGAGA

-133 GAGACCATGCATAAGTATGGCTAGACACAAGAAAGGTTGCAGAACCGAGAAGAGATCTCTTTTGAAGAAGTGAACAAATT

-53 TGTGGGATTGTGGGTTGGTGACGAAAACAGCAAGACAAGAGAAAGGGGAAAGATG

Figure 11

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-940 ATAGAGCAGTACACTTTAAGGGAGCACGCACCCCAATTTGATATAAGGTACAAAGTTTTGTTCCAAGATGTGGGTGACAT

-860 CATTAAATGAGATTTACCATCTTTCAACAGGTTCAAAGGGTACCCTGTACTATAAAGCCCACGAAGCTATTTGTATGTTA

-780 AAAAAAAATTCACATAGACTAGGTACGATCGAAAAGCTCATTAAGGAGCTGCCAACGAAAAGTTCACTTTGTTAACCTCT

-700 CCTCTTATCTTATATTCAATAAGAATATGCACATCGGTTGTGATTTGATGCTCAATTGAACTTGACATTTGGTTATTGGT

-620 TCTACCCACTTAAACCCTAAGCAAAAAGGCCAGAAATGTGCACACATTATTCATCAAGCCGGGTGCCACGTGTCCAGTAA

-540 GACAAACTAAATAAGGAGAAAATACTGGTAAGGAAGCCTATGTTGTGTATGTAAGATACCCAATGATAGTCCGTGGTTTT

-460 GATAAGTTCAAACTTAACATTTGAAAAAGGGACAATATGACGAAGCTGCAGTTAGAACAGGAGAACACCCCAACCCGAGA

-380 AAATATCCGGTCGTTTAATATCAATAGCTCACGTCAGTGCAGGATGTCTGAAAATGACCGATGGTCACACGCGCGTGACA

-300 TGGAAGAGAAGAACCAATAACCAGAAAGCCCTAATAATGAACAGTAGACTTTAGCATGAAACCTCTCACCTTTTCTTTTG

-220 TGCCAAGAAAGTCGCAGTTCCCCTTAACCCTAAATGGAATGTTGTGTCTTTACTTCTGTACCTCTCCTTTTTTTCAGCCT

-140 CTCTCTCTCTCTCTCTCTCTCTCTCTCAGTCTCAGCTATTCAGCTATTCTGCTCCTCAGCTTTCATTTGCAAACAAGAGC

-60 AGATAAGGAAGATAATACGAGAGAGAACAAGAGAGAAAGAGAAGGCCCGGAAAAAGAAAG**ATC**GGTCGAC
(mutated ATG)

Figure 12

GATTGTTACGATCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGG

CCGCCATGGCCGCGGGATTTAATACGACTCACTATAGGGAGCTCGATCTCTT

GAAAGGGAAAATCCGTGTCGTTTTTCTTCGTAAACAAAAGTCGATGAAATAATA

TGTATTGTTACTATATATAAAACAAAAGTGCTCGTGAACATTGTCAAATACTATA

AGGTATAGACAGGTTTATGACTCACAATTACGAAGGGGCCTAAAAGCCGAGAG

GGAAAAGGTAATGCTAGGTATAGTACATCTAGACTATTAATTGATATAATAAGTA

AGTGGATCATGTTCAATTTCAGATAAAAATTTCTATGTCACGTTGTTTAATATTTG

AATTTAATTATTGATTTTATTATATTATTCTTTGTCATATTAATTAATTAGATTGTG

GATATTTGCCGATTCTCATGAGAGAAAATTGATCTTTAATTAATTTCAAAATACC

ATGGCAAGAAATCATAAGAAAACTCACTCTTCCCCACACGCACGCCACATCG

TGCTCTAGGTTAAATAGAGATGTGATCTCCAAATTTTTTGTCTGGAGCAATGCT

AAAGATGCTAAAAAAGTTTAGCAAGTAGGATGACTAACTTGAAATAGGGCAGTC

AATAAACCAAGTCTAACGATCAATTACTACATTACACGTAATGAGAAACATTTC

GAGAAAATCTCTCTATTTATTGTGGTTACATAAGTAGCCTACCTATTTGTAATTA

TGCAAATTAACCTAGGACGAGCCATATATGCAGTCGACATTCATAAGGCTTTAT

AGTTTGTGCTTCCGGAAATAGCTTAGAAAACCACATACACTTTTCATCAAGACT

AATCTGACAGTAAAAAAAGGAAAAAGAAAAGAACACCATCAAGACTAATGTAAT

GAAAAGAAAACCCTATTACTAATTTTGAGAGTTTTCTTGTAGACCCATCACGTC

ATCCATCTAAAACGATGCAATGCACACAACTAATGACAAGGGACATGTTAATAT

ACAGCAAACCTACCAGGTTTCCCCTTCCTTCATTAGAGTATAATAACTGTTCCC

TTTTCTACCTCCTTTTTGTACCCTAGCTCTCGAGCTCCTTAAGAAAGTTTGCTC

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CAGAGATATCGGAATTC

Figure 13

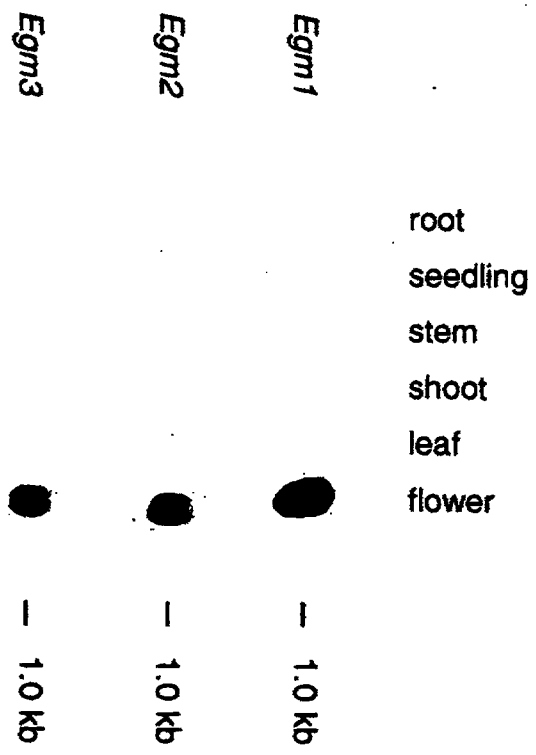

Egm3
Egm2
Egm1
root
seedling
stem
shoot
leaf
flower
1.0 kb
1.0 kb
1.0 kb

Figure 14

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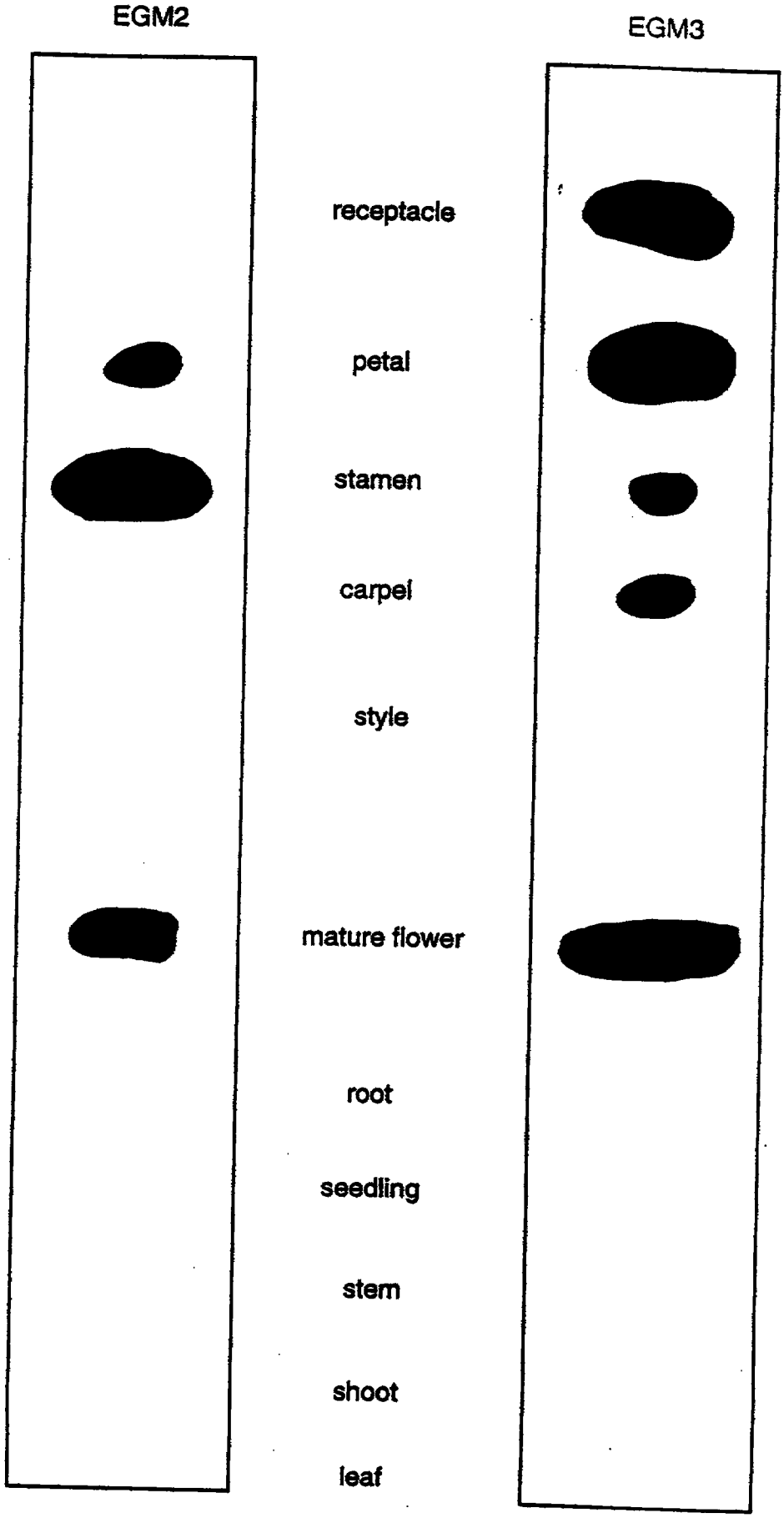


Figure 15

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```
MADS
CONSENSUS MGRGKIEI KRIENKTNRQ VTFSKRRNGL LKKAYELSVL CDAEVALIIF SSRGKLYEY

SLM5      ----RVQL ----------I--- ----------T-- ----H-I--- ---D-G--V- -TK---F--
TDR4      ----RVQL ----------I--- ----------S-- ----H-I--- ----G--V- -TK---F--
AP1       ----RVQL ----------I--- ----------A-- ----H-I--- ---------V- -HK---F--
CAL       ----RV-L ----------I--- ----------T-- ----Q-I--- ----S--V- -HK---F--
SQUA      ----VQL  ----------I--- ----------G-- ----H----- ---------V- -NK---F--
FBP2      ----V-L  ----------I--- ----------A-- ---------- ---------- -N-------F
TDR5      ----RV-L ----------G-I- ----------A-- ---------- ---------- -N-------F
EGM1      ----RV-L ----------I--- ----------A-- ---------- ----G----- -N-------F
AGL2      ----RV-L ----------I--- ----------A-- ---------- ----S--V- -N-------F
AGL4      ----RV-L ----------I--- ----------A-- ---------- ---------V- -N-------F
CMB1      ----RV-L ----------I--- ----------A-- ---------- ---------- -N-------F
EGM3      ----V-L  ----------I--- ----------A-- ---------- ---------- -T-------F
DAL1      ----RVQL R---------I--- ---------- -- ---------- ----G----- -P-------F
TDR3      -V---TQM R----A-S--- ---------- -- ----F----- ---------V- ----R-----
PLE       N------- -----I----- ----C----- -- ---------- ---------- ----R-----
AG        S------- -----T----- ----C----- -- ---------- ----------I- -PS--A-HF
TDR8      -----V-L -----Q----- ---------- -- ---------- ---R-SI-M- --SN--H--
AP3       -A----Q- -----Q----- --Y------- -- F---H--T-- ---------- --TQ--H--
DEF       -A----Q- -----Q----- --Y------- -- F---H----- ---K-SI-MI ---------
FBP1      --------- -----SS--- --Y------I -- ---K-I--- ---R-SV--- A-S--MH-F
GLOBOSA   --------- -----SS--- --Y------I -- M---K-I--- ---H-SV--- A-S--MH-F
EGM2      --------- -----SN--- --Y------- -- I---K-I--- ---Q-SV--- G-S--MH--
```

Figure 16

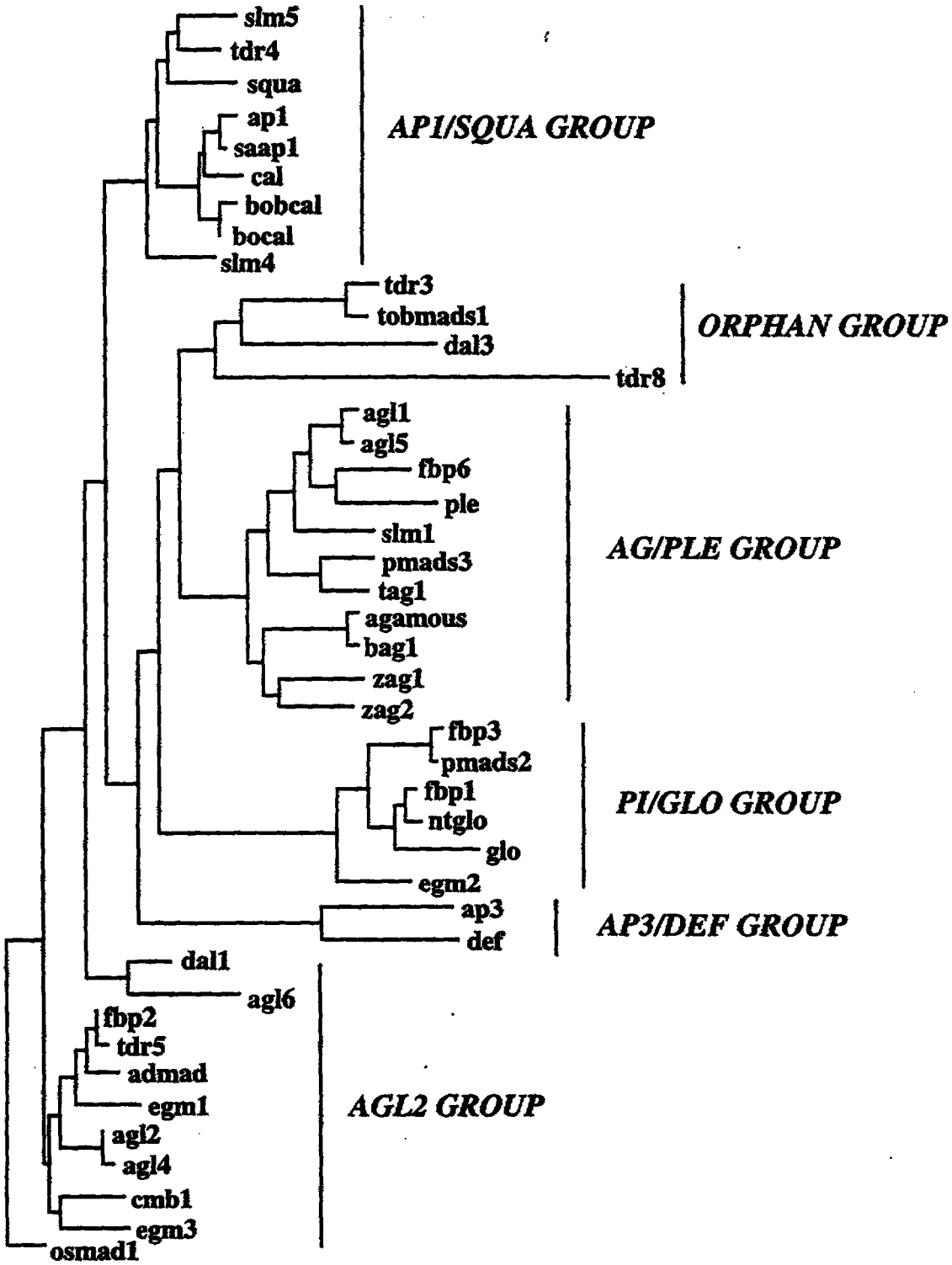

slm5
tdr4
squa
ap1
saap1
cal
bobcal
bocal
slm4
AP1/SQUA GROUP
tdr3
tobmads1
dal3
tdr8
ORPHAN GROUP
agl1
agl5
fbp6
ple
slm1
pmads3
tag1
agamous
bag1
zag1
zag2
AG/PLE GROUP
fbp3
pmads2
fbp1
ntglo
glo
egm2
PI/GLO GROUP
ap3
def
AP3/DEF GROUP
dal1
agl6
fbp2
tdr5
admad
egm1
agl2
agl4
cmb1
egm3
osmad1
AGL2 GROUP

Figure 17

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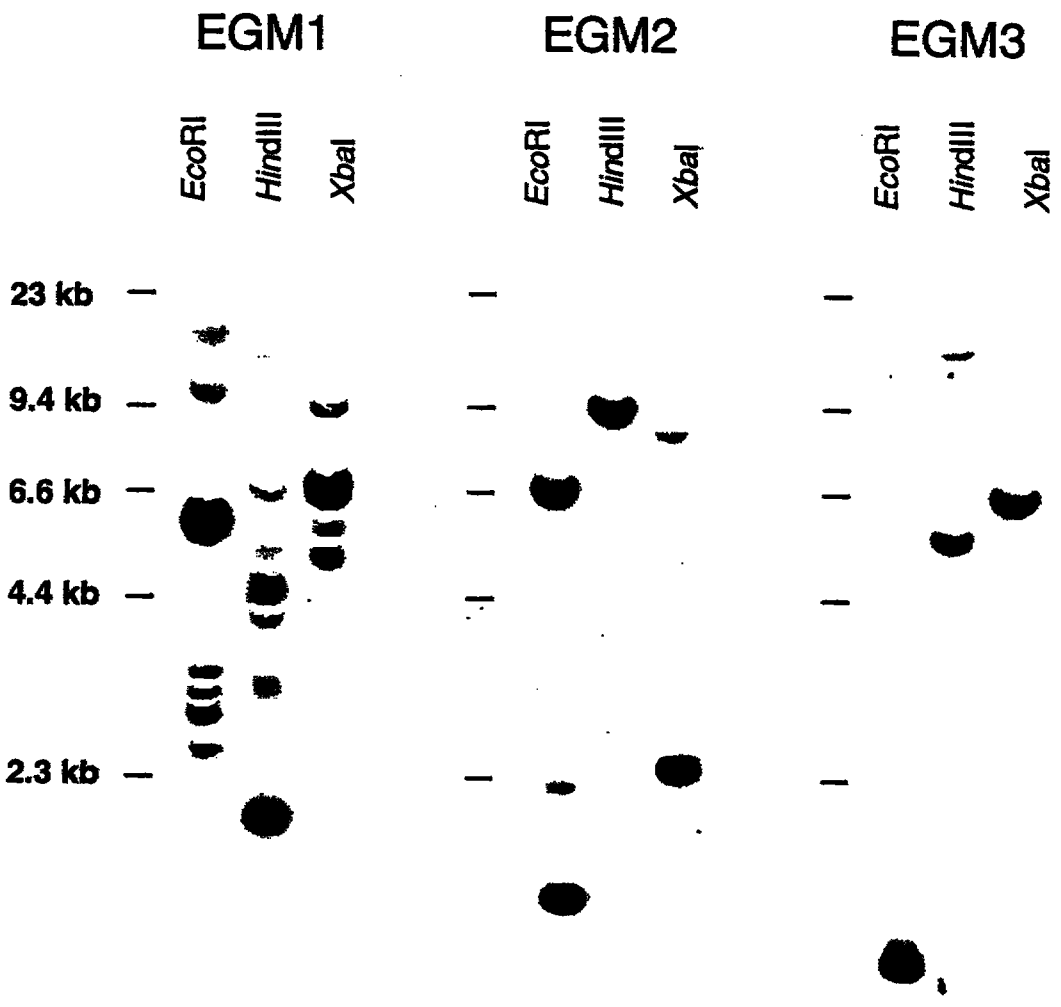


Figure 18

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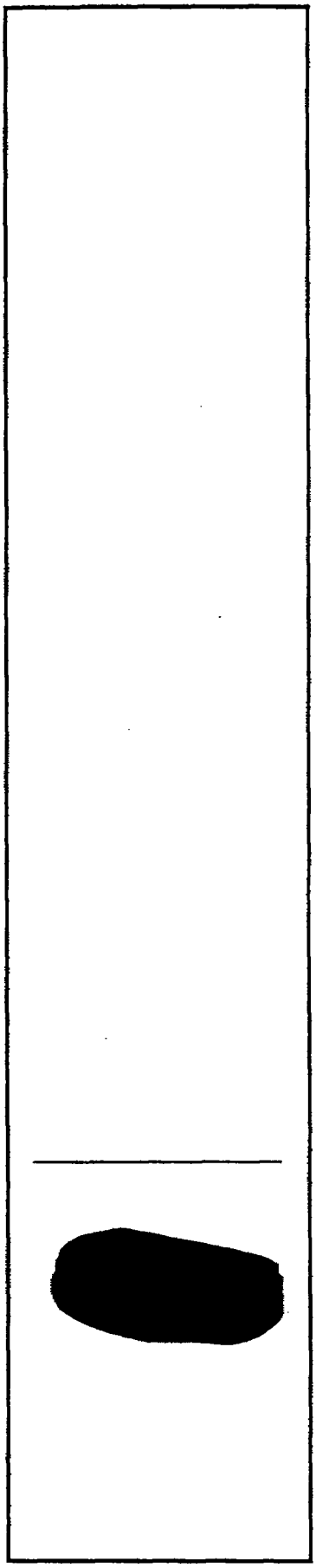


Figure 19

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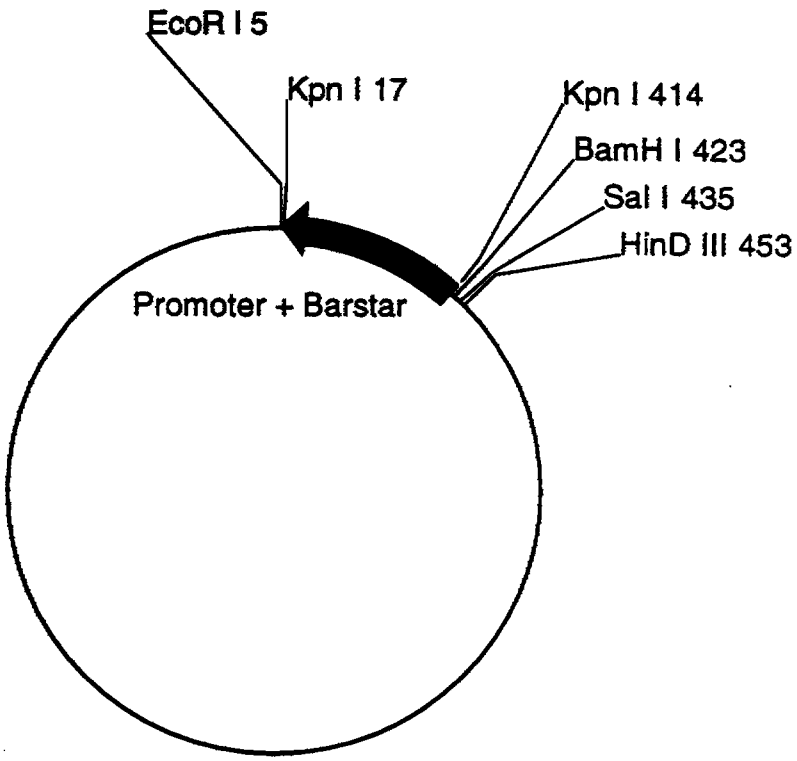


Fig.20

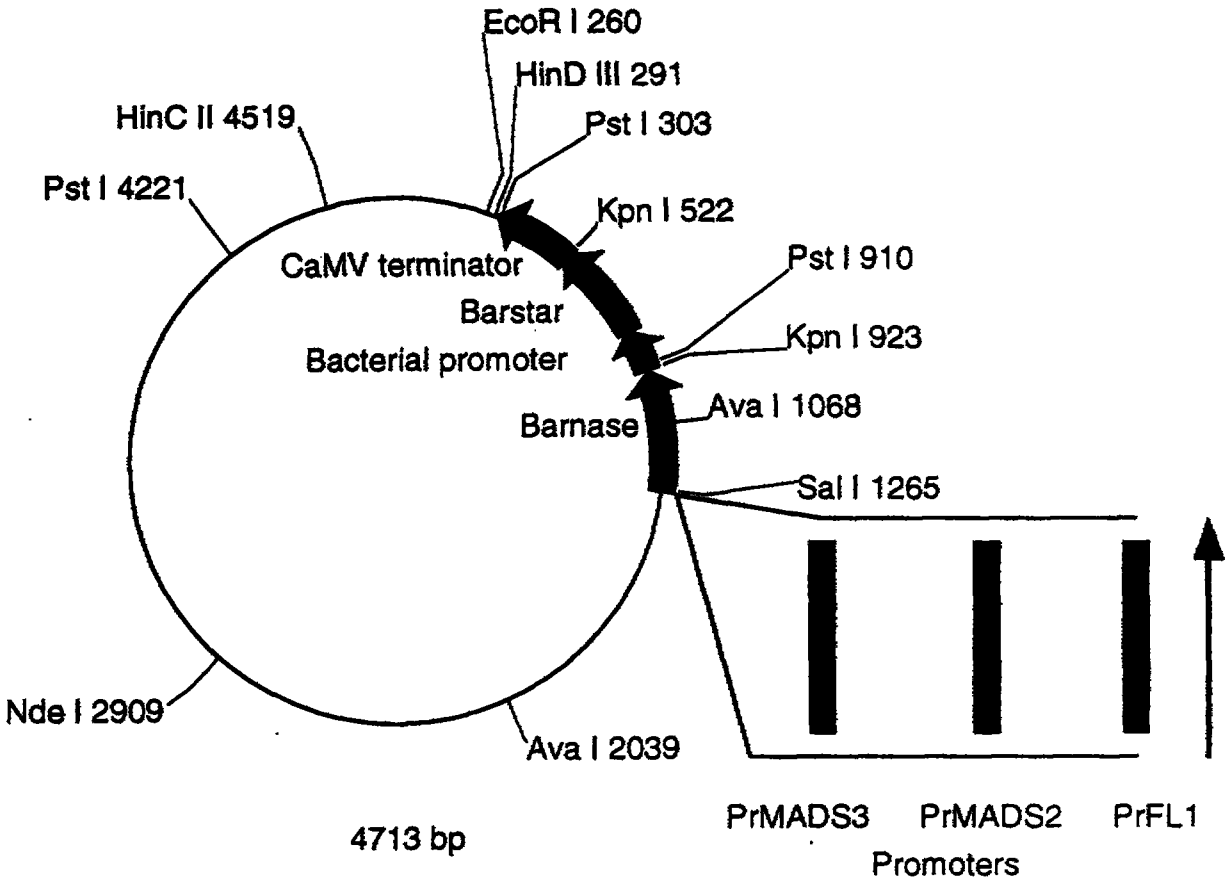

EcoR I 260
HinD III 291
Pst I 303
HinC II 4519
Pst I 4221
Kpn I 522
CaMV terminator
Barstar
Bacterial promoter
Pst I 910
Kpn I 923
Ava I 1068
Barnase
Sal I 1265
Nde I 2909
Ava I 2039
4713 bp
PrMADS3
PrMADS2
PrFL1
Promoters

Fig.21

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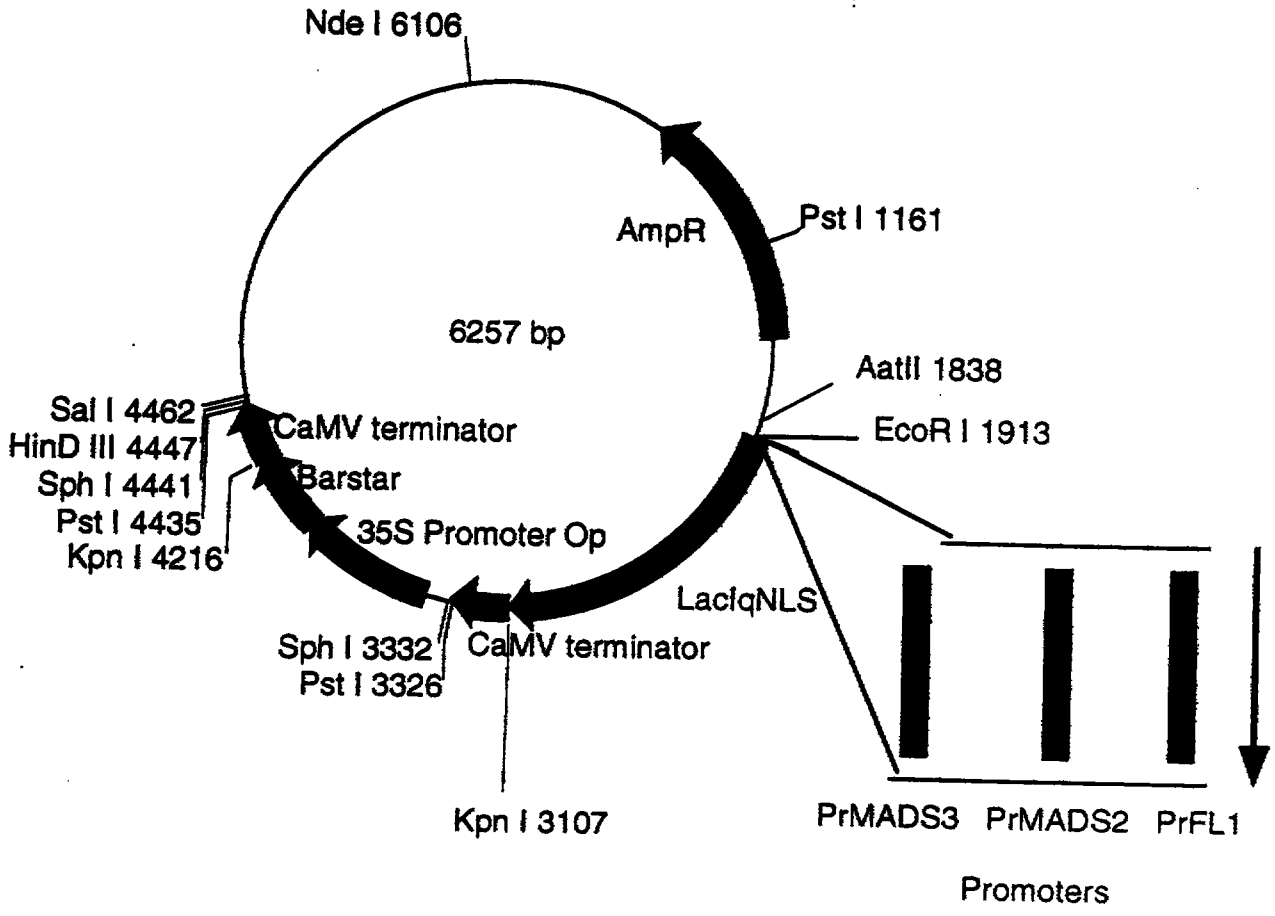


Fig.22

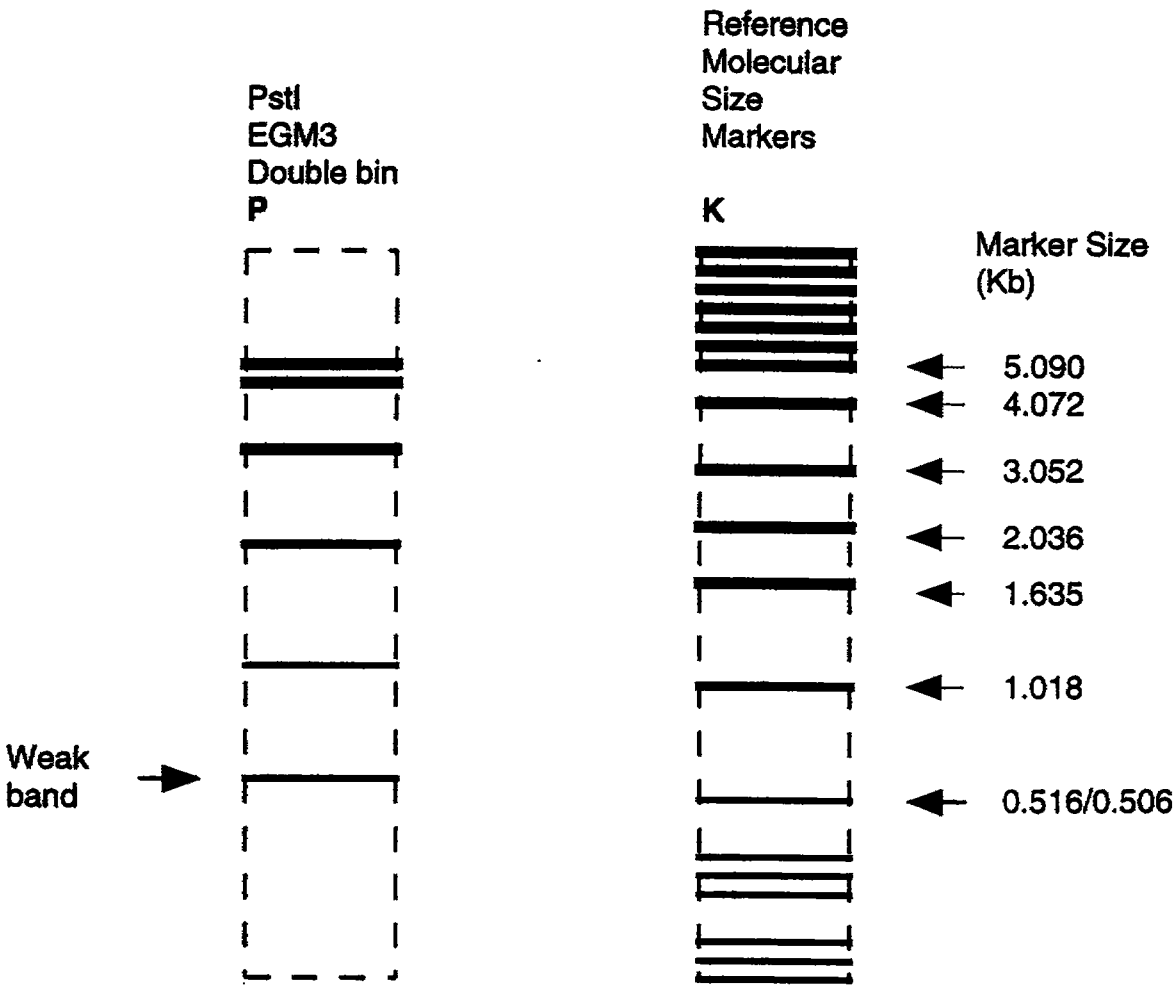

PstI
EGM3
Double bin
P
Reference
Molecular
Size
Markers
K
Marker Size
(Kb)
5.090
4.072
3.052
2.036
1.635
1.018
0.516/0.506
Weak
band

Figure 23

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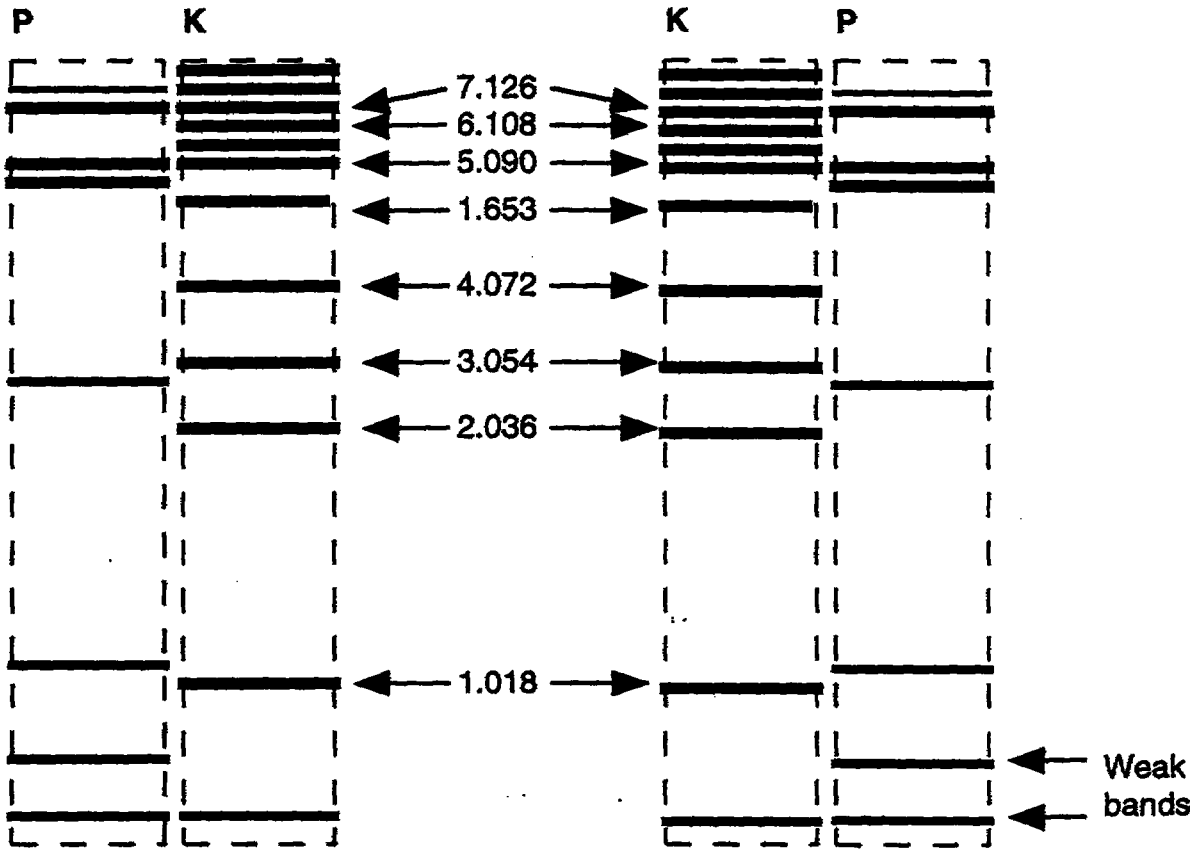


Figure 24

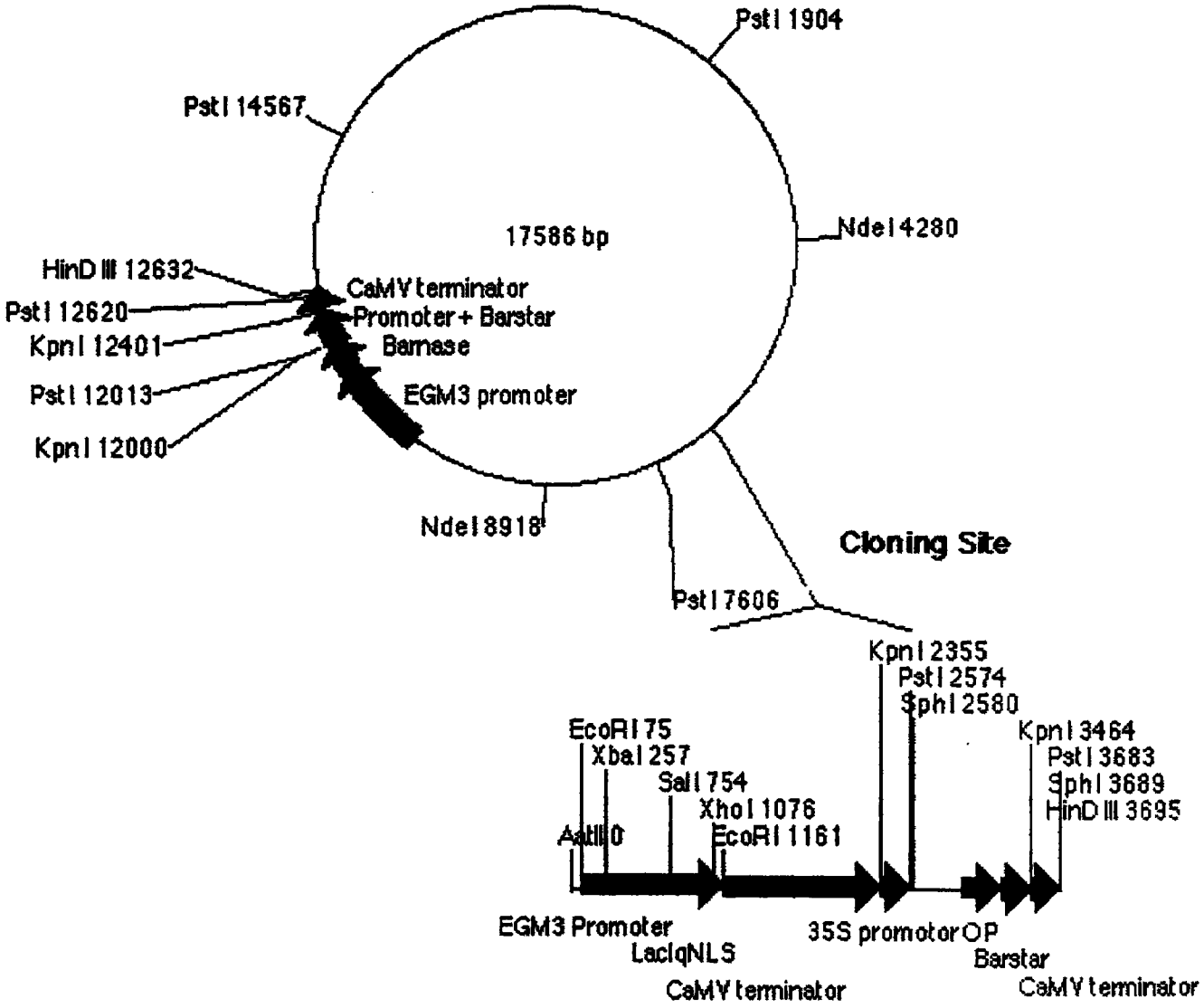
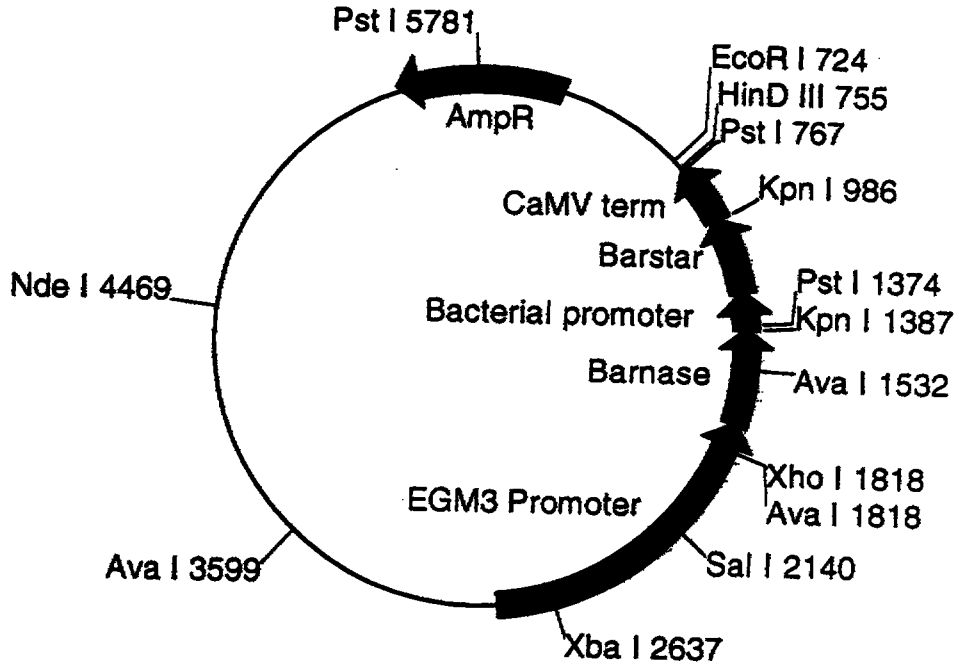

PstI 1904
PstI 14567
NdeI 4280
17586 bp
HinD III 12632
PstI 12620
KpnI 12401
PstI 12013
KpnI 12000
CaMV terminator
Promoter + Barstar
Barnase
EGM3 promoter
NdeI 8918
Cloning Site
PstI 7606
KpnI 2355
PstI 2574
SphI 2580
KpnI 3464
PstI 3683
SphI 3689
HinD III 3695
EcoRI 75
XbaI 257
SalI 754
XhoI 1076
AatII 0
EcoRI 1161
EGM3 Promoter
LacIqNLS
CaMV terminator
35S promotor OP
Barstar
CaMV terminator

Fig.25

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5809 bp

Fig. 26

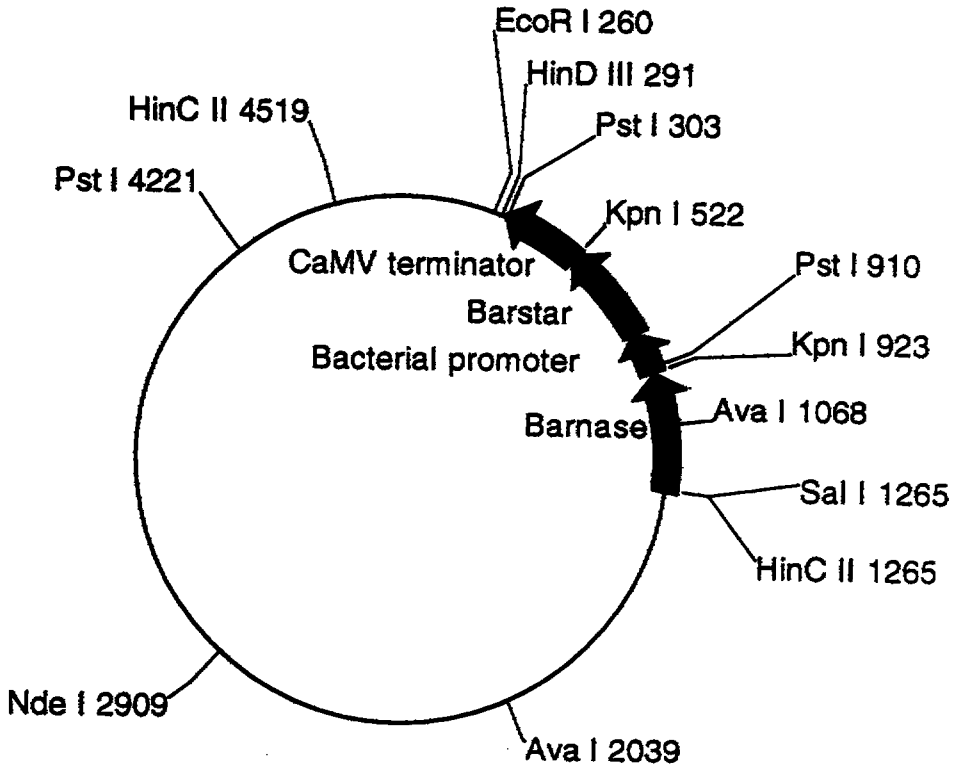

EcoR I 260
HinD III 291
Pst I 303
HinC II 4519
Pst I 4221
Kpn I 522
CaMV terminator
Barstar
Bacterial promoter
Pst I 910
Kpn I 923
Barnase
Ava I 1068
Sal I 1265
HinC II 1265
Nde I 2909
Ava I 2039
4713 bp

Fig.27

PCT/AU 97/00009
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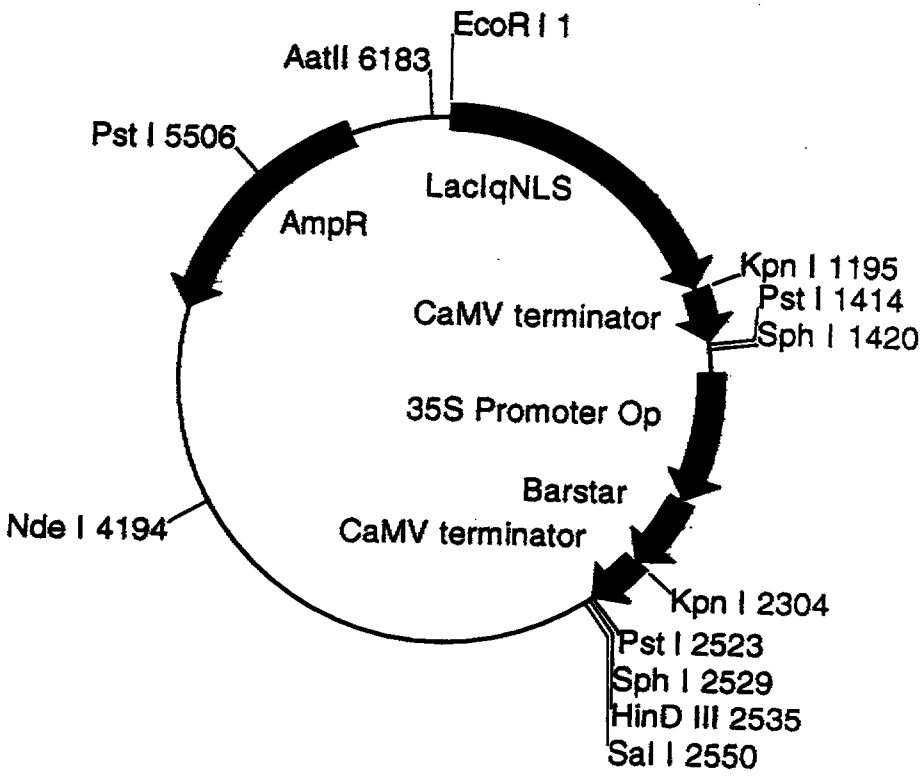


Fig.28

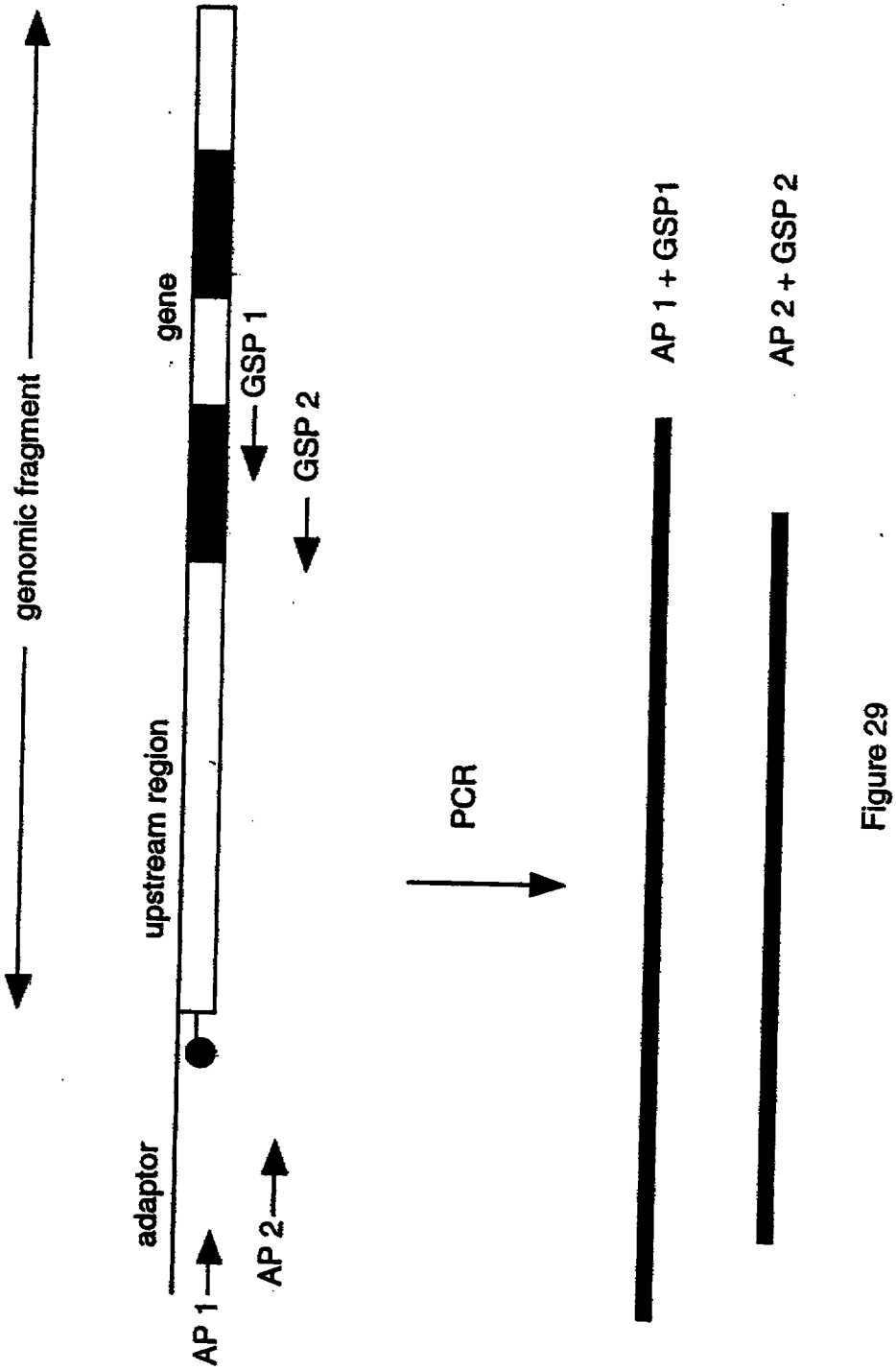

genomic fragment
gene
GSP 1
GSP 2
upstream region
adaptor
AP 1
AP 2
PCR
AP 1 + GSP1
AP 2 + GSP 2

Figure 29

PCT/AU 97/00086
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CTCCCGGCCGCCATGGCCGCGGGATACTATAGGGCACGCGTGGTCGACGG

CCCGGGCTGGTAAAAGACGTCCCTCCGATGAATTCGACAGTGGAGTCCACA

AAGAAAGATGCGTACGCTGAATGCCACGTGTCTATAGTTTGCCCACACCTTT

CAAACATACTCTGTACGCAGATCGGCGGACCTGAAAATTTTCTTCGTATAAAT

CGTCTGTGTTAAGGATATTTGACCATCGGTCCGAAAGATGCGACTGTCCGAC

TGCATCTCGTCTCTGGTAATGTTTTTTTTCAAATTTATAAAGATTGTTTTGCATT

ATTATTATTTATCATGTTGCGTAAGATCTGGTGAGCAGTATTATTTGTCACTTTG

TGTGGAATCCCTTATGAAAATATATATTTATTCCAGAAAAGTATTTTAGCTTGTC

AAATTGTGTGGAATCTGGTATAAGTACCTGTATTTATCAGACGTATTATTTGTTA

AATTGTGTGGAATCTGGTATAAATTTATATACTTCGTGAGGAAATGTATTTATTA

GCAGTATTACTGGTCAAACAGAGTGGAATCCTGTATAAATTTATCATATTTAGG

AACAAATGTATTTATCAGCGTTATTATCTGTCAAAATGTGTGGAGACGCGATG

ACTTTAATTTTTTGATAATAGGCGGAGCGTATAGGTACGCTGTAGTATTGTAGA

CTGGCATGTAGAGTACAAAGTTTAGATATTAACCTAGATTATTTTTGCTATTCG

CAGGCTATCGCGTACTCATGGTGAAATGCGTACAGTTTCAAAGTAAATGGTTG

ATGTTGAGTGCGGTGGCGGGGGCAGACACACAAATAGGAATCGGAGAGCGA

GAGTACTGCAGCAATTGTTGTTTACTTTTTGAGCAAGACGAGGATCAAAGAAG

AATAAGGAAGAGGCCGAGAGAGGGAGCATTATCGTCGAGCAGGAGGGGGAA

AGAAAGAAAGAAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAGCTGAAGC

GGATCGAGAATAAGATTAACCGTCAGGTCACGTTTTCGAAACGCCGGAATGG

TCTGCTGAAAAAGGCG

Figure 30

ATGGGGCGGGGTCGGGTTCAGCTGAGGCGAATAGAAAACAAAATAAATCGACAA

GTCACGTTTTCGAAGCGCCGGAACGGACTGCTGAAGAAGGCGTACGAGCTATCA

GTGCTGTGCGATGCCGAAGTGGCGCTAATAATTTTCTCTACCAGAGGAAAGCTTT

ACGAGTTTGCCAGTTCCAGCATGAACAAGACGTTGGAAAGATACGAAAAATGTTC

ATATGCAATGCAAGATACCACAGGCGTTTCGGACCGGGAAGCACAGAATTGGCA

CCAAGAAGTTACAAAGTTGAAGGGTAAGGTTGAGCTCCTGCAGCGATCACAAAG

GCATTTGTTGGGGGAAGATCTGGGTCCGTTAAATGTTAAGGAGCTACAGCAGCTT

GAACGTCAGCTGGAGGTTGCTCTGACACATCTTAGGTCAAGGAAAACGCAGGTA

ATGCTGGACCAGATTGAGGAACTTCGCCAAAGGGAACGGTTGCTACATGAAGTA

AACAAGTCTCTGCAGAAAAAGCTTTCCGAAACAGAGGGAAGAGATGTAATAACTG

GCATAGAGCAAACTTCTAATACTAATACTGGTACTAACGGTCCTTGGGATTCTTCT

ATCACAAACACTGCGTATGCTCTCTCACACCCTCAACAAGATTCAAATTCAAGCC

TCCACCATGTGGACTGTGAACCCACGCTACAGATTGGTTATCAGCCTGTGGCTC

CTGAAAGCATCGTCCCTCCTCATCAGCCGCCGCACAACCAAACGCCGAACCAA

TACATGCAAGGATGGTGGGTTTGATATTTAACATTTATCATTATCAGTTACTTCAAT

CACAACAAAAGCCCAAAGCGTGGTAAATTACGAAATTAGAATTATATTATCATTAA

AAAAAAACCCTATTTTCATTGTATAGCAGTAGGCTTGATTTACTGCTATGATAGCG

GAGGTTTTATTGGGCAAACAAACCCTACTGGTATATTAGACCTTCTTGTCGACAAA

GTTTAATTGCATAAATCTTGTATGCTAATCTGGCCGCTAAAAGAGCGATGGAAAAA

TAGTTGTCCCATTCACAACACATGATATGTTTAAATCCAACGTGTATGTGTCTGCA

AAATATTATTATACACTACGGTTTATCAAAAAAAAAAAAAAAAA

Figure 31

PCT US 97/00089
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          *         *         *         *         *
MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSTRGKLYEF

  *         *         *         *         *         *
ASSSMNKTLERYEKCSYAMQDTTGVSDREAQNWHQEVTKLKGKVELLQRSQRH

          *         *         *         *         *
LLGEDLGPLNVKELQQLERQLEVALTHLRSRKTQVMLDQIEELRQRERLLHEVNKS

  *         *         *         *         *         *
LQKKLSETEGRDVITGIEQTSNTNTGTNGPWDSSITNTAYALSHPQQDSNSSLHH

        *         *         *         *
VDCEPTLQIGYQPVAPESIVPPHQPPHNQTPNQYMQGWWV
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(261)

Figure 32

PC 97/00
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GGCCCGGGCTGGTATTTGGTGTAAGTTTTAAAGGAAGCTGGAAGATTTC

AAATCAGTCCTAATCCTTTAAAAACTCAGCTTTTGCAAACATGTAACAAT

CTATGAGTATTATTATGCGTTATGGATGTGGCATTTCCACTGCCTTTTAA

GTACTTCGGTTTGTAAGTAAAACTAAAACAGAAAATTTCTCTTCTAGTATT

CTTAAACCGAGTGGAGTTGAGCTGAAGTGAGAGGAGGTATATATATATA

TGTATATTTATTTAGAGTCCAAAGAATCGAGGCGAAAGGCATGTGATGG

ACGAAAGCAACCTTAATCTCTCTGCAAATTGTCCCTCCAAATTCCAACT

AAAATCAATCCGGACCCGCATGAGCATCGATCGAATCATTTGGGTCATG

CATCCTATGCATGCTCA

Figure 33

PCT/AU 97/00089
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GAAATTCGGCACGAGGGTAGAGAGATCCCTGGGCCTAGAAGTGTTTTCGGAGGAGC
GCACTGCATTCTACTTCGGAAAAAAATATGGATGCAGAGCACTTTCCTGTAGGTTTC
TTTAGGTGGGATCAGAGACCAGCACCAGTTGTAGCGGCAGCAGCAGCACCAACAA
CAACTGTCTTTAACAAGGACCATGGACGACCGTTGGAAGTCATTCTTCCCATGAAAT
GGGAGAAAGGATTTGAAGTCCCTTGAAGATCTGTTTAAAGAGTATGGAGTTCGATAC
GTAACTCTTGCCAAGATGACCGAGATGGGCTTCACTGCCAACACCCTTGTCAATAT
GACAGAGGAAGAGATTGAAGATTTGATGAAGACCCTGGTAGAACTCTATCATATGGA
TCTTCTTATAGGGGAGAGATATGGGATTAAATCTGCCATAAGAGCAGAGAAGAAAAG
GTTGCAGGATAGCTTGGAGATGCAAAGGTTGGAAATCTTGTCTGAGGCAGAGAGAA
AGAGGATATTACATGATGATCAGAATACTTTTGCAGCTGCTATGGCATCCGAAGGAA
CATCTAAGGGAACTGAGAGCAAATGACCCACTGATTTTCCCAGAAAGCACAAGTGC
ACGATCATGCCCCAATGAATATAGCCAGCTGCAAAGACAGTACTCTCATTCTCCAG
AACAGTAACCAGGCACAGTTTTGTGGCTCGGGATTGATTGGAGTGCCTGAGCACAG
CAGTGAGAGCGATGAAAGGAAAGCTGATACGAATAAGCAGAAAAGGAGGCGGTCC
AAGGAGCCTGGAGAGGATGGGGAGGACAGGCCTAGAGAGCATCCTTTCATTGTCA
CGGAACCAGGAGAACTGGCAAGAGGGAAGAAAAATGGTCTGGATTATCTCTTTGAT
CTCTATGAGCAGTGTGGGAAATTTTTATTAGAAGTACAAAGGATTGCTAAGGAAAAG
GGAGAAAAATGCCCAACAAAGGTTACAAATCAAGTGTTCCGTCATGCCAAGCACAA
TGGTGCTGTCTACATAAACAAACCTAAAATGCGACATTATGTTCATTGCTATGCTCTG
CATTGCTTGGACAGTGAGCAATCCAATCACCTCAGAAGACTATACAAGGAGAGGGG
AGTAAATGTTGGGGCCTGGCGCCAGGCCTGTTACTATCCCCTGGTAGCCATAGCCA
GAGAGAATAATTGGGATATTGAGGGCATTTTTAATAGGAACGAAAAGCTTAAGATTTG
GTATGTTCCCACAAAACTTAGACAACTGTGTCATATGGAGAGAAGCAAAGAGTGTCA
ATAGTTTCATTGAGATTAATGTGTGTAATTTAACTTAGGCAGCTGTGTCACATAGAGA
GAAGCAAAAAGTGCCAATAGTTTCATTGAGATTAATGTGTGTAATTTGAGCTGCTCAG
CTTCAAGTGTAGCTCCGTGATTATTGGGCATTTGTGTTCTCATTGTGACCTTGCATAT
CAGATTTGATATGCATTGTGTCATGCCGTTCTTTCAAACATTGTATATGTATTGGCAA
CTGGGTATGGATCTGCTCCTTTCTCCGTCACTAAACATGCGGAAACCTTTGTTTCTC
CTTGTGATAAATTTCAGTACATTACTGTTCATAGTTATTTTCTTTCAAAAAAAAAAAA
AAAAA

Figure 34

AMENDED SHEET
IPEA/AU

```
          *          *          *          *          *
MDAEHFPVGFFRWDQRPAPVVAAAAAPTTTVFNKDHGRPLEVILPMNGRKDLKSL
     *          *          *          *          *          *
EDLFKEYGVRYVTLAKMTEMGFTANTLVNMTEEEIEDLMKTLVELYHMDLLIGERY
          *          *          *          *          *
GIKSAIRAEKKRLQDSLEMQRLEILSEAERKRILHDDQNTFAAAMASEGTSKELRAN
  *          *          *          *          *          *
DPLIFPESTSADHAPMNIASCKDSTLILQNSNQAQFCGSGLIGVPEHSSESDERK
          *          *          *          *          *
ADTNKQKRRRSKEPGEDGEDRPREHPFIVTEPGELARGKKNGLDYLFDLYEQCGK
  *          *          *          *          *          *
FLLEVQRIAKEKGEKCPTKVTNQVFRHAKHNGAVYINKPKMRHYVHCYALHCLDS
          *          *          *          *          *
EQSNHLRRLYKERGVNVGAWRQACYYPLVAIARENNWDIEGIFNRNEKLKIWYVPT
*          *
KLRQLCHMERSKECQ
```

(404)

Figure 35

PCT/AT 97/00089
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GCCGCACTAGTGATTCGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGGTGCTGCT
GCTGCCGCTACAACTGGTGCTGGTCTCTGATCCCACCTAAAGAAACCTACAGGAAA
GTGCTCTGCATCCATATTTTTTTCCGAAGTAGAATGCAGTGCGCTCCTACACCAAAC
AAAAACCACTCATAGAATTAGCACATGGGTGATAAATTCTGAGATGCTTTTCATTGAT
TACCGATTGAACTGCAAGATTTGAAGTTGATGGGTTACAAATTCCTCCAGATATTTAA
CCAGACCAAGTGGGTTCCAAGCGGTACTTGAAGAAGCAGAATGTGTACCTCCGAAA
ACACTTCTAGGCCCAGGGACCTCTCTACCATCCAGTGACTTTACATTAGAAAATGTA
AGGCTGCTCCCATATTTAAGGCTGTCTTGTGAGTTTAATGCTTCTTAATCTAGTTTAC
TGGAACACCATTTTCTTCTAATCACATCACAGTAGTAGCTATCATCCAAACTTATCCA
AGACAAAATATCTGAAACCATTCCTGAACTAATGATTTGACTATACTTTGAGTTAGCG
TTGTACTCAAATTATTCATTGCGGGGCCATGTTTGCAGGTTAAACAAAAATCTCCACA
GTCTGATTGCTATGTTTTATCAAATGATTTCTCAAATAATAATTCAGTGTACCCATTTG
TATTTTTACTTTAATACTTTTCATTAATGTCCTCGAGAGCAATGGCTCTAGGGTCAAA
GGAACCTTATCCCAGTACCGTACGCTGTAAATACTGTAATGTTGCTTTTATGGTGTC
CTATAGATTACAGCGAGACTTGCTAGTCATGTGACTTTTAAAAATTTCATGTCAGGCA
CTTGAAAATTGTTTCAAGTACAATGCAATTTTCCCATGGACTACTGGAACTGCCTGCT
AAAACTGTCTTTTCATGGTCTAATGAGAAGTATATGACCAGGCCTCTTCTCCCTCCA
TTTTAAGAAAAACAAATTAAATTATTTAAAAAATAAAATTACAATAATAATCTTGACAGA
TTGATTTTGAGTTGAATGTTAGAATAGATGGAGTGCTGCCTAAGATTTGTTGGCCAAG
AAATCCGCAGCTTATTCCAGAAGTTCACTCATTACAATGTTTTATCTAAGAAATCCTC
AACTTATTCCAAAATGTAACTTATTACAATACCAGCCCGGGCCGTCGACCACGCGT
GCCCTATAGTATCCCGCGGCCATGGCGGCCGG

Figure 36

AMENDED SHEET
IPEA/AT

GAACAACTACTATTACAACGCCAATAAACCCCACTGCCAATATGGGGGC

AGATAGTATGGTTCCTGTTCACACTCCTGAAGTTATTGAGCATTCTTCTAC

AAAAGTTTCTATTGATACAGCTGGGTCAATGGATGTGGATGCAGCATCCA

AGTGCAATCACGTTTACAGAACTACATCTCTCAATCACTGTGTCTCTTCCT

CCCCCATAGATGTTGGAATTGTACCTGACAGCAACATTACATCTGATATTT

CAACACCTTACCATGACCCAAGAGGAGTATTCGAGATTCCTCCTCGGGT

TGTTCATCCTGGAGGCCAAGGTGAGGTCATGGGAAGAGAAGCAAGAGTT

CTCAGATACAGAGAAAAAAGAAAGAACAGAAGGTTTGAGAAGACAATACG

ATATGCTTCTAGAAAAGCCTATGCAGAGACTCGGCCCAGGATAAAAGGC

CGATTTGCCAAGAGAACAGAGGTANAAGTGGAACAGATATACTCATCTTC

TTTGCTTCCTGATCAAGGATATGGAGTTGTTCCATCTTATTGAACCAGTCA

ATATAAATAATTAACTCTTCTCTAGTATCTATCCTTGTTATTCTAATTGTAAC

TGCCATCACAATCATAATGCCTTATAACTTCAATTTATTCCATGCCTATGA

GATTGGGCTGGTATGTAAAACTCATGGCAGGTCTATTCACATTTGGGTTGT

TCGCATACTCAAAGCCTCCTCCTTACTCATTCTGCCTTGCTCATTGATGT

GGGTCAGACTGCAGTAAGATGAAGTCGGTGTGACTCACACTTTGAAGAA

CAGCCATACATACTATTAGTGTTTCAAGTCGATCGAAGTTTGTTTATTCAG

CTTCCTGCAATCTTCTCTGCTCATGAATAAATCTTTTCCCAGTCTCCTACT

AAAAAAAAAAAAAAAAA

Figure 37

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IPEA/AU

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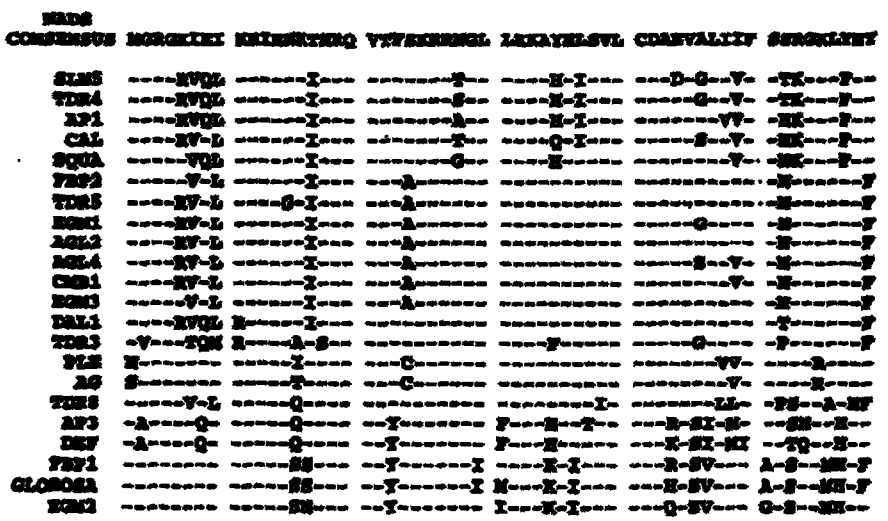
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☑ Description : paged 1-20, 22-27, 29-33, 34-37
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(54) Title: REGULATION OF GENE EXPRESSION IN EUKARYOTES



This Sheet Has Been Replaced

(57) Abstract

There is provided a method of regulating a eukaryotically active gene, comprising transforming a cell with a transformation cassette expressing a modulator gene product regulating the eukaryotically gene or its product and a further gene product regulating said modulator gene or its product, the promoters of two of said gene, modulator gene and further genes being selected from inducible promoters and developmental promoters for the same or complementary tissues. The lethal gene expressing barnase, a ribonuclease of *B. amiloliquefaciens*, is placed under the control of a tissue specific promoter, such as those derived from PrMADS1, 2 or 3 of *Pinus radiata* or EGM1, 2 or 3 of *Eucalyptus grandis*. The same tissue specific promoter is used to express LacIq gene, a repressor for barnase (barstar) being promoted by a modified 35s RNA CaMV promoter including the lac operon. The cassette is used to transform plant cells for regeneration into plants expressing the barnase in the target tissues with improved specificity and reduced promoter leakage.