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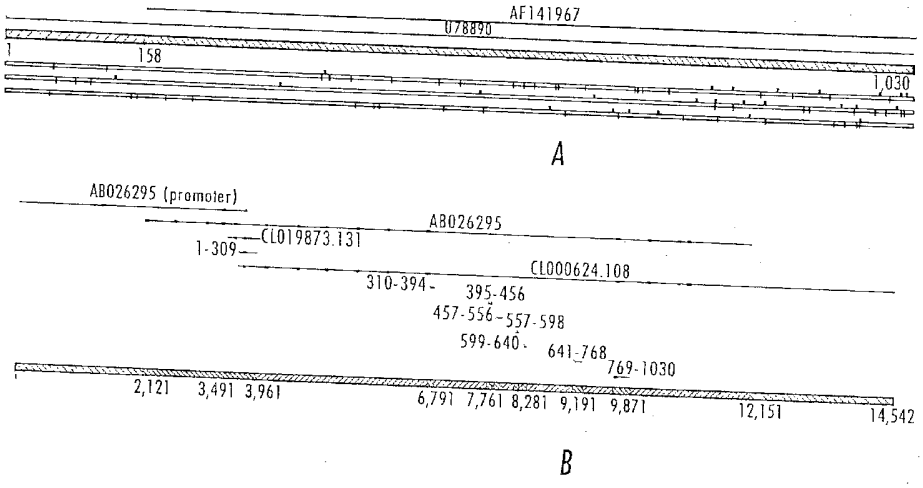
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(54) Title: REGULATORY SEQUENCES FOR EXPRESSING GENE PRODUCTS IN PLANT REPRODUCTIVE TISSUE



(57) Abstract: Expression cassettes causing specific regulatory control of transgene expression in plants, wherein the expression cassettes include regulatory sequences from the MARS gene family for expression of recombinant gene products in the reproductive tissue of plants for the purpose of generating abiotic stress tolerant plants.

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REGULATORY SEQUENCES FOR EXPRESSING GENE PRODUCTS IN PLANT REPRODUCTIVE TISSUE

FIELD OF THE INVENTION

The present invention includes expression cassettes that contain regulatory sequences derived from a target gene, for example, regulatory sequences from the MADS gene family, for tissue specific expression of recombinant gene products in plants.

BACKGROUND OF THE INVENTION

In agricultural biotechnology, plants can be modified according to one's needs. One way to accomplish this is by using modern genetic engineering techniques. For example, by introducing a gene of interest into a plant, the plant can be specifically modified to express a desirable phenotypic trait. For this, plants are transformed most commonly with a heterologous gene comprising a promoter region, a coding region and a termination region. When genetically engineering a heterologous gene for expression in plants, the selection of a promoter is often a critical factor. While it may be desirable to express certain genes constitutively, i.e. throughout the plant at all times and in most tissues and organs, other genes are more desirably expressed only in response to particular stimuli or confined to specific cells or tissues.

Promoters consist of several regions that are necessary for full function of the promoter. Some of these regions are modular, in other words they can be used in isolation to confer promoter activity or they may be assembled with other elements to construct new promoters. The first of these promoter regions lies immediately upstream of the coding sequence and forms the "core promoter region" containing consensus sequences, normally 20-70 base pairs immediately upstream of the coding sequence. The core promoter region contains a TATA box and often an initiator element as well as the initiation site. The precise length of the core promoter region is not fixed but is usually well recognizable. Such a region is normally present, with some variation, in most promoters. The base sequences lying between the various well-characterized elements appear to be of lesser importance. The core promoter region is often referred to as a minimal promoter region because it is functional on its own to promote a basal level of transcription.

The presence of the core promoter region defines a sequence as being a promoter: if the region is absent, the promoter is non-functional. The core region acts to attract the

general transcription machinery to the promoter for transcription initiation. However, the core promoter region is insufficient to provide full promoter activity. A series of regulatory sequences, often upstream of the core, constitute the remainder of the promoter. The regulatory sequences determine expression level, the spatial and temporal pattern of expression and, for a subset of promoters, expression under inductive conditions (regulation by external factors such as light, temperature, chemicals and hormones). Regulatory sequences may be short regions of DNA sequence 6-100 base pairs that define the binding sites for trans-acting factors, such as transcription factors. Regulatory sequences may also be enhancers, longer regions of DNA sequence that can act from a distance from the core promoter region, sometimes over several kilobases from the core region. Regulatory sequence activity may be influenced by trans-acting factors including general transcription machinery, transcription factors and chromatin assembly factors.

Frequently, it is desirable to have tissue-specific expression of a gene of interest in a plant. Tissue-specific promoters promote expression exclusively in one set of tissues without expression throughout the plant; tissue-preferred promoters promote expression at a higher level in a subset of tissues with significantly less expression in the other tissues of the plant. For example, one may desire to express a value-added product only in corn seed but not in the remainder of the plant. Another example is the production of male sterility by tissue-specific ablation.

Tissue specific promoters may be expressed in specific tissue at a specific time or times during the plant growth cycle. However, sufficient expression levels of gene products, especially those gene products directed to expression in specific tissues, is difficult to obtain. Iyer M., et al. (2001). It is known that the 5' untranslated leader sequence of mRNA, introns, and the 3' untranslated region of mRNA effect expression for particular genes. For example, Sieburth, L.E. and Meyerowitz, E.M. (1997) show that intragenic sequences appear to be necessary for the expression of the *AGAMOUS* (AG) gene, an Arabidopsis MADS box gene, in the distinct expression patterns of normal early and later flower development. Larkin J.C., et al. (1993) show that deletion of the 3' noncoding region of the Arabidopsis *GLABROUS1* (GL1) gene negatively affects GL1 function. However, to date, identifying and specific regulatory regions and incorporating them into a robust trait delivery platform has not been accomplished.

Important aspects of the present invention are based on the discovery that DNA sequences from the MADS gene family are exceptionally useful in the development of robust expression cassettes that express recombinant genes in the reproductive tissues of plants.

SUMMARY OF THE INVENTION

The present invention includes a number of different aspects, including specific regulatory control of transgene expression in plants by identifying regulatory sequences from the MADS gene family and incorporating such sequences into expression cassettes for expression of recombinant gene products in the reproductive tissue of plants.

The present invention relates to a method of constructing expression cassettes by identifying the target gene, using the relevant cDNA sequence to annotate the gDNA sequence for the purpose of identifying regulatory sequences of the target gene, and incorporating one or more of the regulatory sequences into an expression cassette with a nucleic acid molecule. A plant transformed with an expression cassette of the invention expresses the product of the nucleic acid molecule in a manner that mimics the expression of the target gene.

The present invention relates to the specific regulatory control of transgene expression in plants, and includes targeting transgene expression to developing reproductive tissue in maize, rice and other monocots. Use of the expression cassettes of the present invention includes expressing a glucose or sucrose transporter to increase reproductive sink strength. Sink strength can also be increased by flower-specific expression of an invertase gene or one or more of the trehalose metabolism genes. The invention further encompasses enhancing the capacity for small molecule uptake via increased expression of specific transporters.

DEFINITIONS

The terms "open reading frame" and "ORF" refer to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence. The terms "initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides ('codon') in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation).

The term "abiotic stress" refers to nonliving environmental factors such as frost, drought, excessive heat, high winds, etc., that can have harmful effects on plants.

The term "nucleic acid" refers to a polynucleotide of high molecular weight which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and a base which is either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of information

contained within DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers. Unless otherwise indicated, a particular nucleic acid sequence of this invention also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer, et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka, et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini, et al., Mol. Cell. Probes 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

"Operably-linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably-linked with a coding sequence or functional RNA when it is capable of affecting the expression of that coding sequence or functional RNA (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences in sense or antisense orientation can be operably-linked to regulatory sequences.

"Promoter" refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. "Promoter regulatory sequences" consist of proximal and more distal upstream elements. Promoter regulatory sequences influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, untranslated leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences that may be a combination of synthetic and natural sequences. An "enhancer" is a DNA sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. The meaning of the term "promoter" includes "promoter regulatory sequences."

"Primary transformant" and "T0 generation" refer to transgenic plants that are of the same genetic generation as the tissue that was initially transformed (i.e., not having gone through meiosis and fertilization since transformation). "Secondary transformants" and the "T1, T2, T3, etc. generations" refer to transgenic plants derived from primary transformants through one or more meiotic and fertilization cycles. They may be derived by self-fertilization of primary or secondary transformants or crosses of primary or secondary transformants with other transformed or untransformed plants.

"Gene" refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory sequences. The term "Native gene" refers to a gene as found in nature. The term "chimeric gene" refers to any gene that contains 1) DNA sequences, including regulatory and coding sequences, that are not found together in nature, or 2) sequences encoding parts of proteins not naturally adjoined, or 3) parts of promoters that are not naturally adjoined. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or comprise regulatory sequences and coding sequences derived from the same source, but arranged in a manner different from that found in nature. A "transgene" refers to a gene that has been introduced into the genome by transformation and is stably maintained. Transgenes may include, for example, genes that are either heterologous or homologous to the genes of a particular plant to be transformed. Additionally, transgenes may comprise native genes inserted into a non-native organism, or chimeric genes. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism but one that is introduced into the organism by gene transfer.

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components.

"Intron" refers to an intervening section of DNA which occurs almost exclusively within a eukaryotic gene, but which is not translated to amino acid sequences in the gene

product. The introns are removed from the pre- mature mRNA through a process called splicing, which leaves the exons untouched, to form an mRNA. For purposes of the present invention, the definition of the term "intron" includes modifications to the nucleotide sequence of an intron derived from a target gene, provided the modified intron does not significantly reduce the activity of its associated 5' regulatory sequence.

"Exon" refers to a section of DNA which carries the coding sequence for a protein or part of it. Exons are separated by intervening, non- coding sequences (introns). For purposes of the present invention, the definition of the term "exon" includes modifications to the nucleotide sequence of an exon derived from a target gene, provided the modified exon does not significantly reduce the activity of its associated 5' regulatory sequence.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B are schematic representations of the OsMADS5 cDNA and of the annotation of the OsMADS5 gDNA with OsMADS5 cDNA sequence.

FIG. 2A is a schematic representation of the OsMADS5 assembly vector.

FIG. 2B is a schematic representation of the OsMADS5 binary vector.

FIG. 3 includes scanned images showing the histochemical localization of OsMADS5-driven GUS activity in T1 maize 5 days before pollination in (A) the longitudinal section of the ear (B) cross sections of the ear, (C) ear node (ear removed), (D) node below ear node, (E) tassel (F) leaf and (G) silk.

FIG. 4 includes scanned images showing the histochemical localization of OsMADS5-driven GUS activity in T1 maize after pollination in the (A) longitudinal and (B) cross sections of ear tissue harvested at 1 day after pollination; and (C) longitudinal and (D) cross sections of ear tissue harvested at two days after pollination.

FIG. 5 includes scanned images showing histochemical localization of OsMADS5-driven GUS expression in T1 maize during kernel maturation in the (A) cross and (B) longitudinal sections of ear tissue harvested at 5 days after pollination; in the (C) cross and (D) longitudinal sections of ear tissue harvested at 10 days after pollination; and the (E) cross and (F) longitudinal sections of ear tissue harvested at 20 days after pollination

FIGS. 6A and 6B are schematic representations of the OsMADS 6 cDNA and of the annotation of the OsMADS6 gDNA with OsMADS6 cDNA sequence.

FIG. 7A is a schematic representation of the OsMADS6 assembly vector.

FIG. 7B is a schematic representation of the OsMADS6 binary vector.

FIG. 8 includes scanned images showing the histochemical localization of OsMADS6-driven GUS expression in T1 maize vegetative tissue in the (A) silk at 2 days before pollination, and in the (B) leaf disc, (C) tassels and (D,E) longitudinal and cross section of the ear node at 8 days before pollination.

FIG. 9 includes scanned images showing the histochemical localization of OsMADS6-driven GUS expression in T1 Maize ear tissue in the (A) longitudinal and (B) cross sections of the developing ear at 8 days before pollination; and in the (C) longitudinal and (D) cross section of the developing ear at 2 days before pollination.

FIG. 10 includes scanned images showing the histochemical localization of OsMADS6-driven GUS expression in T1 maize ear tissue in the (A) cross and (B) longitudinal sections at 1 day of pollination, and in the (C) cross and (D) longitudinal sections at 3 days after pollination.

FIG. 11 include photocopies showing the histochemical localization of OsMADS6-driven GUS expression in T1 maize ear tissue during maturation in the (A) longitudinal and (B) cross sections at 5 days after pollination, in the (C) longitudinal and (D) cross sections at 14 days after pollination, and in the (E) longitudinal and (F) cross sections and (G) kernel at 25 days after pollination.

FIG. 12 is a schematic representation of the annotation of the OsMADS8 gDNA with OsMADS8 cDNA sequence

FIG. 13A is a schematic representation of the OsMADS8 assembly vector.

FIG. 13B is a schematic representation of the OsMADS8 binary vector.

FIG. 14 includes scanned images showing the histochemical localization of OsMADS8-driven GUS expression in T1 maize in the (A) tassel, (B) leaf punch, (C) silk detached from florets, (D) ear node, (E) node beneath ear node, (F) cross section and (G) longitudinal section through the developing ear from tissue taken around 5 days before pollination.

FIG. 15 includes scanned images showing the histochemical localization of OsMADS8-driven GUS expression in T1 maize in (A) the cross and (B) longitudinal sections through the developing ear, and (C) the stained florets from tissue taken 3 days before pollination, and in the (E) cross and (F) longitudinal sections through the ears taken the day of pollination, and (F) cross and (G) longitudinal sections through ears taken 1 day after pollination, and (H) cross and (I) longitudinal sections through the ear taken 6 days after pollination.

FIG. 16 includes scanned images showing the histochemical localization of OsMADS8-driven GUS expression in T1 maize in (A) cross and (B) longitudinal sections through the developing ear taken 11 days after pollination, and (C) cross and (D) longitudinal sections

through the developing ear taken 14 days after pollination, and (E) cross and (F) longitudinal sections through the developing ear taken 20 days after pollination.

FIG. 17 is a schematic representation of the annotation of the OsMADS13 gDNA with OsMADS13 cDNA sequence.

FIG. 18 is a schematic representation of the OsMADS13 Assembly Vector.

FIG. 19 is a schematic representation of the OsMADS13 Binary Vector.

FIG. 20 includes scanned images showing the histochemical localization of OsMADS13-driven GUS activity in T1 maize taken 5 days before pollination in the first fully expanded leaf, the (B) silk; (C) tassel florets; (D) longitudinal section through developing ear; and (E) cross-section through developing ear.

FIG. 21 includes scanned images showing the histochemical localization of OsMADS13-driven GUS activity in T1 maize taken approximately 5 days before pollination in (A) the first fully expanded leaf; (B) silk; (C) tassel florets; and in (D) the stalk section through the node from which the ear emerged.

FIG. 22 includes scanned images showing the histochemical localization of OsMADS13-driven GUS activity in T1 maize siblings taken from the longitudinal sections of developing ears approximately 7 days before pollination of (A) Sibling #18 and (B) sibling #5 and from (C) the cross section and (D) longitudinal section of Sib #4 at 5 days before pollination.

FIG. 23 includes scanned images showing the histochemical localization of OsMADS13-driven GUS activity in a maturing maize ear harvested 4 days after pollination (A, B), and harvested (C) 6-7 days after pollination.

FIG. 24 is a scanned images showing the histochemical localization of OsMADS13-driven GUS activity in a maturing maize OsMADS 13 ear harvested 21 days after pollination.

FIG. 25 includes scanned images of T1 seed from plants transformed with (A) OsMADS5, (B) OsMADS6, (C) OsMADS8 and (D) OsMADS13. GUS expression was assessed by histochemical localization. Seeds were germinated and grown for 10 days in axenic culture. Seedlings were vacuum infiltrated with histochemical reagent and incubated at 37°C for 24 hours, then cleared with ethanol. There is very slight staining in the aerial tissue in (A), (C) and (D). Otherwise GUS activity was not detected. No GUS activity was observed in the roots.

FIG. 26 shows an alignment of T6PP protein sequences.

FIG. 27A shows the OsMADS6-OsT6PP-Assembly vector.

FIG. 27B shows the OsMADS6-OsT6PP-Binary vector.

FIG. 28 shows the effect of the OsMADS6-T6PP-3 transgene on yield in a drought stress study.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes a method for constructing expression cassettes based on identifying a target gene and incorporating into the expression cassettes modified regulatory elements of the selected target gene. For example, regulatory elements from genes that are expressed in roots, stalks, leaves, or reproductive tissues that provide insect resistance, herbicide tolerance, or abiotic stress tolerance are incorporated into expression cassettes for the purpose of producing a transgenic event in a plant that closely mimics the expression profile of the original target gene. Thus, the target gene may be identified from gene expression data.

The present invention is also directed to expression cassettes that incorporate the regulatory mechanisms of target genes of interest to express in plants the products of nucleic acid molecules of interest in a manner that mimics the expression profile of the original target genes.

The present invention further includes expression cassettes that incorporate 5'-MADS gene regulatory sequences to express the products of nucleic acid molecules in plant reproductive tissues, and further includes expression cassettes incorporating both MADS 5'- and 3'-regulatory sequences.

The present invention also includes expression cassettes that incorporate 5'-MADS gene regulatory sequences, and further incorporate a 5'-MADS gene exon.

The present invention also includes expression cassettes that incorporate 5'-MADS gene regulatory sequences, and further incorporates a 5'-MADS gene exon, and a 5'-MADS gene intron.

The present invention further includes expression cassettes that incorporate 5'-MADS gene regulatory sequences, and further incorporates a 5'-MADS gene exon, a 5'-MADS gene intron, and a second exon.

The present invention also includes and further includes expression cassettes incorporating both MADS 5'- and 3'-regulatory sequences, wherein said 3'-regulatory sequence include the 3'-non-translated sequence, and the 3'-nontranscribed sequence.

For purposes of this invention, the definition of the term "3'-non-translated sequence" includes includes modifications to the nucleotide sequence of a 3'-non-translated sequence

derived from a target gene, provided the modified 3'-non-translated sequence does not significantly reduce the activity of its associated 3' regulatory sequence.

For purposes of this invention, the definition of the term "3'-nontranscribed sequence" includes modifications to the nucleotide sequence of a 3'-nontranscribed sequence derived from a target gene, provided the modified 3'-nontranscribed sequence does not significantly reduce the activity of its associated 3' regulatory sequence. The 3'-nontranscribed sequence extends approximately 0.5 to 1.5 kb downstream of the transcription termination site.

The present invention also includes expression cassettes incorporating both MADS 5'- and 3'-regulatory sequences, wherein said 3'-regulatory sequence includes the 3'-non-translated sequence, and the 3'-nontranscribed sequence, and may further include an intron of said MADS gene.

In general MADS genes contribute to the development of plant reproductive structures (De Bodt et al., 2003). For example, the DoMADS3 gene is expressed specifically in pedicel tissue (Yu and Goh, 2000). The genes of the OsMADS gene family were selected for expression cassette development because they encode MADS-transcription factors that are expressed in young rice flowers (Kang and An, 1997). The proteins encoded by genes of the OsMADS gene family are similar to the orchid DoMADS3 gene (GenBank accession AF198176). The present invention recognizes that one method of stabilizing or increasing yield in monocots such as maize is to increase sink strength in reproductive tissue. Thus, transgenic methods for production of plants having increased sink strength in reproductive tissue would benefit from the use of promoters that result in specific expression in a plant's reproductive tissues. The present invention therefore includes the use of OsMADS gene 5'- and 3'-regulatory sequences in expression cassettes to target transgene expression to developing reproductive tissues. The MADS genes from which gene regulatory sequences were identified and utilized according to the present invention encode the following list of MADS proteins (TABLE 1). The MADS proteins are compared by percent identity and similarity to the protein encoded by the DoMADS3 gene.

TABLE 1

	Whole Protein			MADS Domain Only	
MADS gene	identity	similarity	gaps	identity	similarity
AB003322	42%	58%	0%	68%	78%
AB003324	59%	74%	3%	80%	95%

AB003328	48%	64%	0%	77%	91%
AF077760	40%	59%	2%	64%	80%
AF095645	40%	61%	5%	64%	90%
AF139664	50%	66%	1%	78%	95%
AF139665	48%	66%	0%	80%	95%
AF141964	40%	60%	11%	66%	86%
AF141965	51%	67%	0%	84%	91%
AY174093	42%	63%	0%	63%	87%
AF204063	60%	72%	3%	91%	98%
AF345911	50%	68%	5%	80%	95%
AF424549	39%	59%	2%	63%	87%
AJ293816	35%	52%	8%	65%	79%
AY115556	39%	61%	1%	60%	85%
AY177695	39%	58%	0%	66%	87%
AY177696	38%	62%	4%	61%	87%
AY177698	41%	61%	3%	68%	87%
AY177699	37%	59%	3%	63%	78%
AY177700	41%	61%	0%	66%	87%
AY177702	38%	59%	5%	70%	89%
AY224482	38%	59%	5%	70%	89%
AY250075	42%	67%	5%	64%	88%
L37527	37%	60%	5%	63%	85%
L37528	45%	68%	1%	84%	94%
U78891	62%	75%	4%	94%	99%
U78782 (OsMADS6)	58%	69%	4%	91%	99%
U78892 (OsMADS8)	60%	73%	6%	94%	99%
U78890 (OsMADS5)	57%	72%	2%	92%	97%
AF151693 (OsMADS13)	46%	67%	1%	84%	94%
AF095646	55%	67%	6%	94%	99%

The present invention therefore includes an expression cassette for expression of a nucleic acid molecule product primarily in the reproductive tissue of a plant comprising a promoter, a first exon; a first intron, and a second exon of a MADS gene, wherein said promoter, first exon, intron, and second exon are the 5'-regulatory sequence of said expression cassette; wherein said 5'-regulatory sequence is engineered to include a translational initiation codon at approximately the 3'-end of said 5'-regulatory sequence, and not to contain restriction endonuclease sites that hinder manipulation by recombinant DNA methods or additional translation initiation codons upstream of said translation initiation codon; a 3'-regulatory sequence of a MADS gene that does not to contain restriction endonuclease sites that hinder manipulation by recombinant DNA methods; and a nucleic acid molecule operably linked to said 5'-regulatory sequence and said 3'- regulatory sequence.

Recombinant DNA methods require the presence of specific restriction endonuclease sites at the termini of the DNA molecules to be joined. The most efficient practice requires the sites in one molecule complement the sites in the other molecule. For example, a plasmid

with SacI and NotI restriction endonuclease sites is required to clone a gene of interest with SacI and Not I restriction endonuclease sites at its termini. Ideally, these sites are unique, that is they should not occur at any other place in either molecule. If these sites occur internally, they hinder manipulation by recombinant DNA methods and should be eliminated. Site-directed mutagenesis is one method of eliminating such sites. Techniques such as partial digestion followed by gel-purification of the appropriately sized fragment will also accomplish this without eliminating the internal restriction endonuclease sites, but are far less efficient and therefore less desirable.

The present invention recognizes that chemical synthesis, that is use of synthetic chemical technology as opposed enzyme-mediated technology, of a polynucleotide molecule can replace or substitute for recombinant DNA methods in the construction of a polynucleotide molecule comprising a specific nucleotide sequence.

The present invention further includes a method for constructing an expression cassette comprising the steps of selecting a target gene based on its expression data or its encoded protein's similarity to a protein encoded by another gene of interest; identifying the open reading frame on said target gene cDNA; identifying the positions of the translational start codon, translational stop codon, the first intron, first exon, second exon, the 3'-untranslated sequence and the 3'-nontranscribed sequence of said target gene gDNA by using the cDNA to annotate the target gene gDNA; incorporating into an expression cassette a 5'-regulatory sequence comprising said promoter, first exon, first intron, and second exon and a 3'-regulatory sequence comprising the 3'-untranslated sequence and the 3'-nontranscribed sequence; and operably linking a nucleic acid molecule to said 5'-regulatory sequence and said 3'-regulatory sequence of said expression cassette, wherein said nucleic acid molecule is expressed in a manner that mimics the expression profile of said target gene of interest.

Example 1: Method of constructing expression cassettes comprising regulatory sequences from the MADS gene family.

1. Identifying target MADS genes.

2. Identifying high quality sequence for both the target's genomic DNA (gDNA) and cDNA.

3. Identifying the target gene's open reading frame on the cDNA. In general this is the longest open reading frame.

4. Using a candidate gene's cDNA sequence to annotate gDNA sequence and marking positions of the translation start codon, translation stop codon, introns, exons, the 5'-untranslated leader and the 3'-untranslated sequence. As is known in the art, marking the

translation start codon and the translation stop codon identifies the 5'-regulatory sequence and the 3'-regualtory sequence of the gene. According to the present invention, the promoter, which includes the promoter regulatory sequence, is the sequence that extends approximately 1.5 to 2.5 kb upstream from the translation start codon, wherein the 3'-regulatory sequence of the present invention includes the 3'-untranslated sequence located immediately downstream of the translation stop codon and all or a part of the 3'-nontranscribed sequence, which extends 0.5 to 1.5 kb downstream of the transcription termination site. In one embodiment of the invention, the 5'-regulatory sequence includes the promoter, the first exon, the first intron and the second exon.

By way of example only, FIG. 1A and 1B illustrates annotating the OsMADS5 gDNA with the OsMADS5 cDNA sequence. The gDNA contigs (AB026295 from GenBank and CL000624.108 (SEQ ID NO. 500) plus CL019873.131 (SEQ ID NO. 521) were aligned with OsMADS5 cDNA sequence (GenBank accession U78890). The cDNA sequence is broken into corresponding exons. The exons are labeled according to cDNA base numbers. Both sequences align precisely and the intervening sequences (introns) are flanked by GT..AG borders. Gaps in between exons represent introns. The AB026295 fragment is a portion of the entire bacterial artificial chromosome (BAC) sequence. The AB026295 (promoter) is an additional fragment from that BAC which defines sequence used for promoter development.

5. Designing expression cassettes that incorporate the following components from a MADS gene(s):

a. The promoter, a sequence that begins at the translation start codon and extends approximately 1.5 to 2.5 kb upstream of the translation start codon.

b. The first exon

c. The first intron

d. The 5'-most portion of the second exon

e. The terminus, including the 3'-untranslated sequence and the 3'-nontranscribed sequence, which extends 0.5 to 1.5 kb downstream of the transcription termination site. The terminus can further include an intron.

For simplicity the "5'-regulatory sequence" of the present invention includes components a-d and the "3'-regulatory sequence" of the present invention refers to component e.

6. Amplifying the 5'-regulatory sequence from the appropriate gDNA template by high-fidelity PCR and cloning into a suitable bacterial vector.

The 5'-regulatory sequence from rice genomic DNA (gDNA) is amplified using high-fidelity PCR. A 50 μL reaction mixture contains 100 ng rice gDNA, 200 μM dNTPs (dATP, dCTP, dGTP, TTP), 1 μL 20 μM each of oligonucleotide primers designed to amplify the 5'-regulatory gDNA (Table 2), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase (Roche Diagnostics, Cat. No. 1 759 078). The thermocycling program is 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. The 5'-regulatory gDNA product is cloned with the TOPO XL PCR cloning kit (Invitrogen, Cat. No.K4750-20). pCR-XL-TOPO-5'-regulatory-gDNA is identified by digesting 5 μL pCR-XL-TOPO-5'-regulatory-gDNA miniprep DNA (prepared using the QIAprep Spin Miniprep procedure from Qiagen, Cat. No. 27106) with EcoRI (New England Biolabs) in a 20 μL reaction containing 2 μg BSA and 2 μL 10X EcoRI restriction endonuclease buffer (New England Biolabs). The reaction is incubated at 37°C for 2 hours and the pCR-XL-TOPO-5'-regulatory-gDNA (EcoRI) products are resolved on 1% TAE agarose. The pCR-XL-TOPO-5'-regulatory-gDNA clone is sequenced using the ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer).

7. Amplifying the "3'-regulatory sequence" from the appropriate gDNA template by high-fidelity PCR and clone into a suitable bacterial vector.

The 3'-regulatory sequence from rice gDNA is amplified using high-fidelity PCR. The 50 μL reaction mixture consists of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM each of the oligonucleotide primers designed to amplify the 3'-regulatory gDNA (Table 2), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program is 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. The 3'-regulatory gDNA product is cloned with the TOPO XL PCR cloning kit (Invitrogen, Cat. No.K4750-20) following manufactures' instructions. The pCR-XL-TOPO-3'-regulatory-gDNA is identified by digesting 5 μL pCR-XL-TOPO-3'-regulatory-gDNA miniprep DNA with EcoRI in a 20 μL reaction containing 2 μg BSA and 2 μL 10X EcoRI restriction endonuclease buffer. The reaction is incubated at 37°C for 2 hours and the pCR-XL-TOPO-3'-regulatory-gDNA (EcoRI) products are resolved on 1% TAE agarose. The pCR-XL-TOPO-3'-regulatory-gDNA clone is then sequenced.

8. Assembling the 5'-regulatory sequence and 3'-regulatory sequence in any bacterial plasmid.

The expression cassettes of the present invention were assembled in the vector pNOV6901, also known as the "Assembly Vector". This vector contains the coding sequence for GUS reporter gene (which is disrupted by an intron to prevent bacterial expression) flanked at its 5'- and 3'-termini by unique restriction sites (polylinkers) to facilitate recombinant DNA procedures. Any number of other vectors may be used as is known to those persons skilled in the art.

9. Incorporating restriction sites in the expression cassettes, as necessary, to facilitate recombinant DNA procedures.

The "engineered" translation initiation codon, below, is the ATG in the NcoI restriction site (CCATGG). If there are any NcoI restriction sites in the "expression cassette 5'-regulatory sequence" they must be eliminated by mutagenesis. Likewise, restriction sites that are used to assemble the expression cassette must be eliminated by mutagenesis. Incorporation of the first intron in the "expression cassette 5'-regulatory sequence" requires the sequence be modified to avoid creating fusions between native coding sequence, which is normally translated into protein encoded by target gene, and the "gene of interest" (nucleic acid molecule) to be driven by the expression cassette. This is accomplished by any of a number of mutagenic procedures, including the procedure performed by the Stratagene QuikChange Multi Site-Directed Mutagenesis Kit (Cat. No. 200513). Modifications to the expression cassette 5'-regulatory sequence include:

a. Modifying the target gene's natural translation initiation codon so that the target gene's protein coding sequence is silent.

b. Modifying any other translation initiation codons that exist in the sequence between the "silenced" translation initiation codon and the "engineered" translation initiation codon to insure such codons are not operable.

c. Modifying any NcoI sites in the 5'-regulatory sequence.

d. Modifying restriction endonuclease sites, as necessary, to facilitate expression cassette assembly.

In this embodiment of the invention, the procedure does not eliminate nucleotides. Rather, it modifies them to preserve the length of the 5'-regulatory sequence in the expression cassette, yet still silencing the candidate gene's protein coding sequence. However, it is contemplated that one or more of the nucleotides could be eliminated to silence undesired protein expression, provided that 5'- and 3'-regulatory sequences of the cassette continue to enhance expression of the candidate gene in plant reproductive tissue. Furthermore, those skilled in the art do not consider it unreasonable to alter the sequence of nucleotides in a

polynucleotide molecule comprising a regulatory sequence so long as the modified regulatory sequence retains a majority of the activity associated with the original regulatory sequence.

Table 2 lists the primers designed to accomplish this task for each 5'-regulatory sequence derived from the MADs gene family. The Stratagene QuikChange Multi Site-Directed Mutagenesis Kit uses each gene's pCR-XL-TOPO-5'-regulatory-gDNA clone as a template and the primers listed to mutagenize that clone according to the present invention. The primers must contain a 5'-phosphate to work. Furthermore, alterations may require more than one round of mutagenesis. The modified pCR-XL-TOPO-5'-regulatory clone is sequenced using the ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer).

10. Modifying, in some cases, the 3'-regulatory sequence to eliminate restriction endonuclease sites to facilitate recombinant DNA procedures. The "engineered" translation initiation codon is the ATG in the NcoI restriction site (CCATGG). If there are any NcoI restriction sites in the "expression cassette 3'-regulatory sequence" they must be eliminated by mutagenesis. Again, this is accomplished any of a number of mutagenic procedures. The present invention therefore includes:

a. Modifying any NcoI sites in the 3'-regulatory sequence.

b. Modifying restriction endonuclease sites, as necessary, to facilitate expression cassette assembly.

In this embodiment of the present invention, the procedure does not eliminate nucleotides. Rather, it modifies them to preserve the length of the 3'-regulatory sequence in the expression cassette. However, it is contemplated that one or more of the nucleotides could be eliminated to silence undesired protein expression, provided that 5'- and 3'-regulatory sequences of the cassette continue to enhance expression of the candidate gene in plant reproductive tissue. Furthermore, those skilled in the art do not consider it unreasonable to alter the sequence of nucleotides in a polynucleotide molecule comprising a regulatory sequence so long as the modified regulatory sequence retains a majority of the activity associated with the original regulatory sequence. Table 2 lists the primers designed to accomplish this task for each 3'-regulatory sequence derived from the MADS gene family. Each gene's pCR-XL-TOPO-3'-regulatory-gDNA clone is used as a template and the primers listed are used to mutagenize these clones. The modified pCR-XL-TOPO-3'-regulatory clones are sequenced.

11. Cloning the 3'-regulatory sequence into pNOV6901, using PCR with the modified pCR-XL-TOPO-3'-regulatory clone as template and the appropriate primer set disclosed in Table 2.

These primers are a 5'-oligonucleotide primer that introduces unique restriction site from the GUS 3'-terminal polylinker in pNOV6901 and 3'-oligonucleotide primer that introduces a rare cutting restriction site (either AscI, PacI, SgfI or RsrII) followed by a restriction endonuclease site unique to the 3'-terminal polylinker. In Table 2, these primers are listed as "primers to clone 3'-regulatory sequence in pNOV6901".

High-fidelity PCR is used to amplify the 3'-regulatory sequence from the modified pCR-XL-TOPO-3'-regulatory clone. A 50 μL reaction mixture consists of 1 μL miniprep DNA, 200 μM dNTPs, 1 μL each of 20 μM oligonucleotide primers (Table 2), 5 μL 10X Cloned PFU buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252). The thermocycling program was 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The amplified 3'-regulatory sequence DNA fragment is recovered using the QIAquick PCR purification kit (Qiagen, Cat. No.28106). The recovered 3'-regulatory sequence DNA fragment is precipitated with 20 μg glycogen, 0.3 M CH_2COONa (pH 5.2) and 2.5 volumes ethanol at -20°C for more than 2 hours. The 3'-regulatory sequence DNA fragment is recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The 3'-regulatory sequence DNA fragment is digested in a 20 μL reaction containing 2 μg BSA, 2 μL of the appropriate 10X restriction endonuclease buffer and 2 μL of the appropriate restriction endonuclease(s). The reaction is incubated at 37°C for more than 6 hours. The digested 3'-regulatory sequence DNA products are resolved on 1.0% TAE agarose and the appropriate 3'-regulatory sequence (digested) band is excised. The 3'-regulatory sequence (digested) DNA is extracted and recovered using the QIAquick Gel extraction kit (Qiagen, Cat. No.28704). The recovered 3'-regulatory sequence (digested) DNA is ethanol precipitated with glycogen carrier. The 3'-regulatory sequence (digested) DNA fragment is recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg of pNOV6901 miniprep DNA is digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL of the appropriate 10X restriction endonuclease buffer (used to generate the 3'-gene regulatory sequence,) and 2 μL of the appropriate restriction endonuclease (used to generate the 3'-gene regulatory sequence). The reaction mixture is incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL calf-intestinal alkaline phosphatase (CIP-New England Biolabs) and 8 μL ddH_2O is added to the reaction mixture and incubated at 37°C for 30

minutes. The pNOV6901 (digested/CIP) DNA is resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (digested/CIP) band is excised. The pNOV6901 (digested/CIP) DNA is extracted and recovered using the QIAquick Gel extraction kit (Qiagen, Cat. No.28704). The recovered pNOV6901 (digested/CIP) DNA is ethanol precipitated with glycogen carrier. The pNOV6901 (digested/CIP) DNA is recovered by micro centrifugation, washed with 70% ethanol, and dried under vacuum and resuspend in 5 μL ddH_2O.

4.0 μL 3'-regulatory sequence (digested) is ligated to 4.0 μL pNOV6901 (digested/CIP) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL-New England Biolabs). The ligation mixture is incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture is transformed into 50 μL Top10 competent cells (Invitrogen, Cat. No. C4040-03). The pNOV6901-3'-regulatory-sequence recombinants are verified by digesting 2 μL pNOV6901-3'-regulatory-sequence miniprep DNA with 1 μL of the appropriate restriction endonuclease in 10 μL reactions containing 1 μg BSA and 1 μL of the appropriate 10X restriction endonuclease buffer. Digests are incubated at 37°C for 2 hours then resolved on 1% TAE agarose. The positive pNOV6901-3'-regulatory-sequence recombinants are sequenced.

12. Cloning the 5'-regulatory sequence into pNOV6901-3'-regulatory-sequence, using PCR with the modified pCR-XL-TOPO-5'-regulatory clone as template and the appropriate primer set disclosed in Table 2.

These primers are a 5'-oligonucleotide primer that introduces the same rare cutting restriction site used for the 3'-regulatory sequence preceded by a unique restriction endonuclease site in the 5'-terminal polylinker of pNOV6901 and a 3'-oligonucleotide primer that introduces an NcoI site preceded by a Kozak sequence (CCACCATGG) at the "engineered" translation initiation codon. Table 2 lists these primers as "primers to clone 5'-regulatory sequence in pNOV6901".

High-fidelity PCR is used to amplify the 5'-regulatory sequence from the modified pCR-XL-TOPO-5'-regulatory clone. A 50 μL reaction mixture consists of 1 μL miniprep DNA, 200 μM dNTPs, 1 μL each of 20 μM oligonucleotide primers (Table 2), 5 μL 10X Cloned PFU buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252). The thermocycling program was 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The amplified 5'-regulatory sequence DNA fragment is recovered using the QIAquick PCR purification kit. The recovered 5'-regulatory sequence DNA fragment is ethanol precipitated with glycogen

carrier. The 5'-regulatory sequence DNA fragment is recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The 5'-regulatory sequence DNA fragment is digested in a 20 μL reaction containing 2 μg BSA, 2 μL of the appropriate 10X restriction endonuclease buffer and 2 μL of the appropriate restriction endonuclease(s). The reaction is incubated at 37°C for more than 6 hours. The digested 5'-regulatory sequence DNA products are resolved on 1.0% TAE agarose and the appropriate 5'-regulatory sequence (digested) band is excised. The 5'-regulatory sequence (digested) DNA is extracted and recovered using the QIAquick Gel extraction kit (Qiagen, Cat. No.28704). The recovered 5'-regulatory sequence (digested) DNA is ethanol precipitated with glycogen carrier. The 5'-regulatory sequence (digested) DNA fragment is recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg of the pNOV6901-3'-regulatory-sequence miniprep DNA is digested in a 20 μL reaction containing 2 μg BSA, 2 μL of the appropriate 10X restriction endonuclease buffer and 2 μL of the appropriate restriction endonuclease. The reaction is incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O are added to the reaction and it is further incubated at 37°C for 30 minutes. The pNOV6901-3'-regulatory-sequence (digested/CIP) DNA is resolved on 1.0% TAE agarose and the pNOV6901-3'-regulatory-sequence (digested/CIP) band is excised. The pNOV6901-3'-regulatory-sequence (digested/CIP) DNA is extracted and recovered. The recovered pNOV6901-3'-regulatory-sequence (digested/CIP) DNA is ethanol precipitated with glycogen carrier. The pNOV6901-3'-regulatory-sequence (digested/CIP) DNA is recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL of the 5'-regulatory sequence (digested) is ligated to 4.0 μL pNOV6901-3'-regulatory-sequence (digested/CIP) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The ligation mixture is incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture is transformed into 50 μL Top10 competent cells. The pNOV6901-3'/5'-regulatory-sequence recombinants are verified by digesting 2 μL pNOV6901-3'/5'-regulatory-sequence miniprep DNA with 1 μL of the appropriate restriction endonuclease in 10 μL reaction mixtures containing 1 μg BSA and 1 μL of the appropriate 10X restriction endonuclease buffer. Digests are incubated at 37°C

for 2 hours then pNOV6901-3'/5'-regulatory-sequence (digested) DNA is resolved on 1% TAE agarose. The positive pNOV6901-3'/5'-regulatory-sequence recombinants are sequenced.

The expression cassette of the present invention includes a GUS reporter construct in the Assembly Vector. It is flanked by the engineered, rare-cutting restriction site. In this embodiment of the present invention the GUS reporter gene can be replaced with any gene of interest using methods known to those individuals skilled in the art.

13. The expression cassette can now be mobilized into the agrobacterium binary vector pNOV6900, by digesting the assembly vector with the rare-cutting enzyme and purifying the cassette DNA.

2 μg pNOV6900 is digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL of the appropriate 10X restriction endonuclease buffer and 2 μL of the appropriate restriction endonuclease. The reaction mixture is incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O are added to the reaction and it is further incubated at 37°C for 30 minutes. 2 μg of the pNOV6901-3'/5'-regulatory-sequence miniprep DNA is digested in a 20 μL reaction containing 2 μg BSA, 2 μL of the same 10X restriction endonuclease buffer used for pNOV6900 and 2 μL of the same restriction endonuclease used for pNOV6900. The reaction is incubated at 37°C for more than 6 hours.

The digested plasmid DNA, pNOV6900 (digested/CIP) and pNOV6901-3'/5'-regulatory-sequence (digested) are resolved on 1.0% TAE agarose and the 9.2 kb pNOV6900 (digested/CIP) and the appropriate pNOV6901-3'/5'-regulatory-sequence (digested) bands are excised. The pNOV6900 (digested/CIP) and the pNOV6901-3'/5'-regulatory-sequence (digested) DNAs are extracted and recovered. The recovered pNOV6900 (digested/CIP) and the pNOV6901-3'/5'-regulatory-sequence (digested) DNAs are ethanol precipitated with glycogen. The pNOV6900 (digested/CIP) and the pNOV6901-3'/5'-regulatory-sequence (digested) DNA fragments are recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended each in 5 μL ddH_2O.

4.0 μL of the pNOV6900 (digested/CIP) is ligated to 4.0 μL pNOV6901-3'/5'-regulatory-sequence (digested) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The ligation mixture is incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture is transformed into 50 μL Top10 competent cells. The pNOV6900-pNOV6901-3'/5'-regulatory-sequence recombinants are

verified by digesting 7.5 μL pNOV6900-pNOV6901-3'/5'-regulatory-sequence miniprep DNA with 1.0 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer 4 (New England Biolabs). Digests are incubated at 37°C for 2 hours then resolved on 1% TAE agarose. The junction sequence of positive pNOV6900-pNOV6901-3'/5'-regulatory-sequence recombinants is verified.

14. The expression cassette can now be transformed into agrobacterium and then transformed into plants in accordance with methods known to those persons skilled in the art.

TABLE 2

Primers for constructing expression cassettes comprising regulatory sequences from the MADS gene family

target gene	primers to clone 5'-regulatory sequence name	sequence
AB003322	AB003322-1 (SEQ. ID No. 133)	5'-CGTTGCCCAGCGAGGAGCATGCGTAAAATC-3'
	AB003322-2 (SEQ. ID No. 134)	5'-GATTTCATTCATACTGTCCAACAGAAGGCA-3'
AB003324	AB003324-1 (SEQ. ID No. 145)	5'-TCTAAATAGGGCCCAACATACTCA-3'
	AB003324-2 (SEQ ID No. 146)	5'-TCAAGCGTCTTAAGCATGCTGAAATATGA-3'
AB003328	AB003328-1 (SEQ. ID No. 158)	5'-AACAAAACCAATAATCTCCAATGCCC-3'
	AB003328-2 (SEQ. ID No. 158)	5'-CATCTTCGATCGCCGATGAACCAACC-3'
AB077760	AB077760-1 (SEQ. ID No. 167)	5'- ATTCGTTGGGGGTGACATGACTAGTC-3'
	AB077760-2 (SEQ. ID No. 168)	5'- CAAAGATCCCCTTGATGCTGCAGCAG-3'
AB095645	AB095645-1 (SEQ. ID No. 179)	5'-CTCGTCGCCGTCGTTGGCTCGGCGT-3'
	AB095645-2 (SEQ. ID No. 180)	5'-CAAGATTCTTGATAGCCTTATACATG-3'
AF139664	AF139664-1 (SEQ. ID No. 193)	5'-TGCATCCTTACAAAGAGACAGATAGATC-3'
	AF139664-2 (SEQ. ID No. 194)	5'-CAAGGATTTTGTCCATACTGTGAAAAATG-3'
AF139665	AF139665-1 (SEQ. ID No. 207)	5'- ACCTGGGGTTTGGAAATGGGGAGCG-3'
	AF139665-2 (SEQ. ID No. 208)	5'- CAAGGATCCCTTCCATACTACAGAAGAAG-3'
AF141964	AF141964-1 (SEQ. ID No. 218)	5'-AAAACACTGATTAAAGGGTTGTTGAAAGGAAAACACC-3'
	AF141964-2 (SEQ. ID No. 219)	5'-CATAGGTACCTTCAATGCTGAAAAAGAAAACAAGGCA-3'
AF141965	AF141965-1 (SEQ. ID No. 232)	5'-TGTTTCTCGATTAGGCTACAAGTTAAC-3'
	AF141965-2 (SEQ. ID No. 233)	5'-GATGGTTTTCTGTAGGCTGCAGAACAG-3'
AF174093	AF174093-1 (SEQ. ID No. 246)	5'-AAAATTCACAAGTATAATTCGTCAC-3'
	AF174093-2 (SEQ. ID No. 247)	5'-CAGCTCCATCAAGCTTAACAAAGCA-3'
AF204063	AF204063-1 (SEQ. ID No. 258)	5'-ACAAATTTGTTGTACCACCTACCTAGGGGT-3'

	AF204063-2 (SEQ. ID No. 259)	5'-CAAGGTTTTGTACATGCTGGAAGTGAA-3'
AF345911	AF345911-1 (SEQ. ID No. 271)	5'-GAAAATCTCAAGGTTTCGAAAACGAC-3'
	AF345911-2 (SEQ. ID No. 272)	5'-CAAGGATTTTGTCCATCCTGCAGGGAAAAC-3'
AF424549	AF424549-1 (SEQ. ID No. 283)	5'-TACAAAGTGCTGGAAGTGATAGTATGT-3'
	AF424549-2 (SEQ. ID No. 284)	5'-GATCCCCTTGATGCTGCAGCAGGATGCA-3'
AJ293816	AJ293816-1 (SEQ. ID No. 295)	5'-CAAACATTTTAAACTTTAACCATTAATAG-3'
	AJ293816-2 (SEQ. ID No. 296)	5'-CAATAATCTGTTCCATGCTTCATCAATG-3'
AY115556	AY115556-1 (SEQ. ID No. 305)	5'-GATATAAAAACCTACTTTATGTTCATG-3'
	AY115556-2 (SEQ. ID No. 306)	5'-CAGCTCCTCCATGGTTCTGTTCAAAGAAATC-3'
AY177695	AY177695-1 (SEQ. ID No. 316)	5'-TCAGTCCATCATTTCGTCTACAACTAA-3'
	AY177695-2 (SEQ. ID No. 317)	5'-TATTATTGATTTCATGCTAACAAAAAG-3'
AY177696	AY177696-1 (SEQ. ID No. 330)	5'-CAAGCAAAGAAACAAATTTCGCAATTAG-3'
	AY177696-2 (SEQ. ID No. 331)	5'-ATAACTGACTTCATGCTGCATATTTGCA-3'
AY177698	AY177698-1 (SEQ. ID No. 342)	5'-AAGATTTGAACTACTGCCTTGTCTTC-3'
	AY177698-2 (SEQ. ID No. 343)	5'-CAAAGATATTTGCAATCCTGCCAAAAG-3'
AY177699	AY177699-1 (SEQ. ID No. 356)	5'-TCCGGTCGGCCCTCGTCCTCCCCGT-3'
	AY177699-2 (SEQ. ID No. 357)	5'-CAAACTCCCTCACGCTGCGCCAAGAAAG-3'
AY177700	AY177700-1 (SEQ. ID No. 366)	5'-ATAAGTATTTCAGAAAGCTGAAGTTGA-3'
	AY177700-2 (SEQ. ID No. 367)	5'-CAAGTTATGCATATTTCTTGCATTTTG-3'
AY177702	AY177702-1 (SEQ. ID No. 379)	5'-TCTTTTCGCAAACTAAACAAGGCCT-3'
	AY177702-2 (SEQ. ID No. 380)	5'-CACAGTTTTCATGCTAGCATGCAAGTAAAG-3'
AY224482	AY224482-1 (SEQ. ID No. 392)	5'-TCTTTTCGCAAACTAAACAAGGCCTTAACA-3'
	AY224482-2 (SEQ. ID No. 393)	5'-TATCACAGTTTTCATGCTAGCATGCAAGT-3'
AY250075	AY250075-1 (SEQ. ID No. 405)	5'-GAAATGGTTTCATTTTGGGACAAGTTATTG-3'
	AY250075-2 (SEQ. ID No. 406)	5'-CAAGGATTCGCTCCATACTATCAATAAAATATG-3'
L37527	L37527-1 (SEQ. ID No. 419)	5'-GCCAAGAGAGCCCCCTTGCTGCTGGT-3'
	L37527-2 (SEQ. ID No. 420)	5'-CAAGATCCTTGACAGCCTAAAACGCCA-3'
L37528	L37528-1 (SEQ. ID No. 432)	5'-ACCACCTCAATCTCCACTGTTTGATTG-3'
	L37528-2 (SEQ. ID No. 433)	5'-CAGCTCAGATCGGTCTGGACACAAAC-3'
U78891	U78891-1 (SEQ. ID No. 443)	5'-TCTTCTTCTTGCCTTCATTTGAGTTAATTACA-3'
	U78891-2 (SEQ. ID No. 444)	5'-CAAGCGTTTTAGTCATGCTACAAAGTTC-3'
	AF095646-1 (SEQ ID No. 508)	5'-TGTTGCATGCATGCGTACTGGTGATGGCCGCA-3'
	AF095646-2 (SEQ ID No. 509)	5'-AATGTCTTGTTTATTCTGCAAACAAAAATAGG-3'

primers to clone 3'-regulatory sequence	
name	sequence
AB003322-3 (SEQ. ID No. 135)	5'-TAACTGAAAGGAAAAAAAAAGCATCCTTGC-3'
AB003322-4 (SEQ. ID No. 136)	5'-GAATAAATCTGGCAACTGAAATCCAACCAT-3'
AB003324-3 (SEQ. ID No. 147)	5'-CTAGCTAGCTAGCTACCGTTTCAGCTTC-3'
AB003324-4 (SEQ. ID No. 148)	5'-TGGAGATCGACCACAATTAAATTCGT-3'
AB003328-3 (SEQ. ID No. 160)	5'-TAAGTAACAGGCCAGGAATAAGCTGG-3'
AB003328-4 (SEQ. ID No. 161)	5'-ATTGCCGGGCTATAGCCTTTCTCCCT-3'
AB077760-3 (SEQ. ID No. 169)	5'- TGATATATCATCGCCGCCGCCGCCG-3'
AB077760-4 (SEQ. ID No. 170)	5'- CCGTGGTACTGAAATCGAAAAAAGAAATG-3'
AB095645-3 (SEQ. ID No. 181)	5'-TAAGCTGCTAGGTTGCCCCGCCACT-3'
AB095645-4 (SEQ. ID No. 182)	5'-GCACGGCTACCTCTCGCCGGAGTACG-3'
AF139664-3 (SEQ. ID No. 195)	5'-TAAGGAGGCTTCAGATCCATACCAG-3'
AF139664-4 (SEQ. ID No. 196)	5'-GGATAAACATTGTGAAGCAACATTTC-3'
AF139665-3 (SEQ. ID No. 209)	5'- TGAAGGCATCTGTTGATCTCAAACGTC-3'
AF139665-4 (SEQ. ID No. 210)	5'- ATGAACTCCACCTCGGGAACTCAGCCT-3'
AF141964-3 (SEQ. ID No. 220)	5'-TGAGCAGGAAGCACAGGTGTCCTGT-3'
AF141964-4 (SEQ. ID No. 221)	5'-ATGAGATACAATCTAGTACAACGAAT-3'
AF141965-3 (SEQ. ID No. 234)	5'-TGAAGAAGGCCAGCCACAGCAACAGCTG-3'
AF141965-4 (SEQ. ID No. 235)	5'-CCTTATCGAATATTCAAATCTCGATG-3'
AF174093-3 (SEQ. ID No. 248)	5'-TGACTTCCTGGAAGCAGTAGGAAC-3'
AF174093-4 (SEQ. ID No. 249)	5'-CCCCTTCTCCTCCTCCGGGAAGAAG-3'
AF204063-3 (SEQ. ID No. 260)	5'-TGATGTGTGTGTTCAGTTCAGGCTT-3'
AF204063-4 (SEQ. ID No. 261)	5'-CTGCATATACTTGCAACATTGCAATTTTA-3'
AF345911-3 (SEQ. ID No. 273)	5'-TAAGATGATCATCGTCGTCGTCGTCG-3'
AF345911-4 (SEQ. ID No. 274)	5'-TTGACTTAAATGGCAAGATAAATTAGATATAG-3'
AF424549-3 (SEQ. ID No. 285)	5'-TGAGATATCATCGCCGCCGCCGCCGCCG-3'
AF424549-4 (SEQ. ID No. 286)	5'-TTTTATTATCTTGGCGAGGCCCAAGCACCTTTG-3'
AJ293816-3 (SEQ. ID No. 297)	5'-TGATGGCTGGAAACTAAAACTGAGAGGA-3'
AJ293816-4 (SEQ ID No. 298)	5'-TTTGAAACATTTCGGCCCTGGCTCTCCT-3'
AY115556-3 (SEQ. ID No. 307)	5'-TAACACTAATAATGGCCTGGGGGATAC-3'
AY115556-4 (SEQ. ID No. 308)	5'-AACTTTGCACGAATGATTAAATTGCAT-3'
AY177695-3 (SEQ. ID No. 318)	5'-TAACACATGATCAAGTGTTAAAAACAG-3'
AY177695-4 (SEQ. ID No. 319)	5'-GGCTCGGTCAAACCAGCGTGGTGAGCT-3'

AY177696-3 (SEQ. ID No. 332)	5'-TGACCAGAAAAACATTGTTTTGCTAC-3'
AY177696-4 (SEQ. ID No. 333)	5'-TTTGGGATTGGACTGCATCCCAGGAA-3'
AY177698-3 (SEQ. ID No. 344)	5'-TGATTGCCCTCTTTCCATCCCAATAGG-3'
AY177698-4 (SEQ. ID No. 345)	5'-GTTAACCTAAAAGAAATATTGTTCAG-3'
AY177699-3 (SEQ. ID No. 358)	5'-TAGAAACAGATGGACGCTTGACGTTCA-3'
AY177699-4 (SEQ. ID No. 359)	5'-AGGCTTGGTGCAAGGATACGAAATCTTG-3'
AY177700-3 (SEQ. ID No. 368)	5'-TAGCCGTCAAAGGACCTTGGTCAATTC-3'
AY177700-4 (SEQ. ID No. 369)	5'-TAATTTTACTAGTACTTTTACTTTGAC-3'
AY177702-3 (SEQ. ID No. 381)	5'-TAAGACTATGCCGTACAAGCTGGACGA-3'
AY177702-4 (SEQ. ID No. 382)	5'-CCGTGGTTGTCACAGAAGACCAATCTATAC-3'
AY224482-3 (SEQ. ID No. 394)	5'-TAAGACTATGCCGTACAAGCTGGACGA-3'
AY224482-4 (SEQ. ID No. 395)	5'-CCGTGGTTGTCACAGAAGACCAATCTATAC-3'
AY250075-3 (SEQ. ID No. 407)	5'-TAGAAGATGTTCAGATGAAATGGTCCCT-3'
AY250075-4 (SEQ. ID No. 408)	5'-AAAAAAATCACCGGCGTCAAATATTTAGGGA-3'
L37527-3 (SEQ. ID No. 421)	5'-TAGGCTGCTGAGCACTGCCAATTTG-3'
L37527-4 (SEQ. ID No. 422)	5'-TGCTGGTGCTCGAGGTGCTGAGCGCG-3'
L37528-3 (SEQ. ID No. 434)	5'-TAGTTTTGGTGTAGACACCGTACGTAC-3'
L37528-4 (SEQ. ID No. 435)	5'-TGAAAGCTACATTTTAGCCTTGTATTTG-3'
U78891-3 (SEQ. ID No. 445)	5'-TGAACAGTGCGTGCATGAACACCTACATG-3'
U78891-4 (SEQ. ID No. 446)	5'-GATGTTTTTTTTTGTCTTTTGATGCAAGGCC-3'
AF095646-3 (SEQ ID No. 510)	5'-TGAAGTCCAAGCTTGCTAATAAAAACGCTG-3'
AF095646-4 (SEQ ID No. 511)	5'-AAGGGTAGGGCTGCACGAAATGC-3'

primers to mutagenize 5'-regulatory sequence	
name	**sequence**
AB003322-5 (SEQ. ID No. 137)	5'-CGCGCGGCGGCGATCGCGCGGGAGAGGCGG-3'
AB003322-6 (SEQ. ID No. 138)	5'-CTTCTGTTGGACAGTATCAATGAAATC-3'
AB003322-7 (SEQ. ID No. 139)	5'-ATTTCATCCATGCTACAATTTATTTT-3'
AB003322-8 (SEQ. ID No. 140)	5'-AGAAGGCCGAGGAGGTCTCCGTGCTC-3'
AB003324-5 (SEQ. ID No. 149)	5'-TCGAGCGGGAGATCGAGATCGGGCGAGGCA-3'
AB003324-6 (SEQ. ID No. 150)	5'-AATTCATATTTCAGCATCCTTAAGAC-3'
AB003324-7 (SEQ. ID No. 151)	5'-ACTGTGCAGATGAGGTCATGCGCTCAT-3'
AB003324-8 (SEQ. ID No. 152)	5'-AGGCCTACGAGCTGTCCATCCTCTGCGA-3'
AB003324-9 (SEQ. ID No. 153)	5'-ACGCGTCCGGCCATCGCTGCCTAGCTAG-3'
AB077760-5 (SEQ. ID No. 171)	5'- GCTGCAGGGGGCGGCCATCGGGAGGGGCAAGATCGA-3'
AB077760-6 (SEQ. ID No. 172)	5'- CGCACGGGGATCATCAAGAAGGCCAG-3'
AB077760-7 (SEQ. ID No. 173)	5'- TCGCCATCATCATCTTCTCCTCCAC-3'
AB077760-8 (SEQ. ID No. 174)	5'- ATGAAGAAGGCCAGGGTGCTCACCGTGCTCT-3'

AB095645-5 (SEQ. ID No. 183)	5'- AGGAGGCGGGATCGGGCGCGGGAAG-3'
AB095645-6 (SEQ. ID No. 184)	5'- GAAACGTGGGCCATCGCCATTGAGCGAAA-3'
AB095645-7 (SEQ. ID No. 185)	5'- TTCCTCTTCCTCCCATCGCGAACTCCT-3'
AB095645-8 (SEQ. ID No. 186)	5'- TGCCGTTTCTGGAGCTGCCCTACTGA-3'
AF139664-5 (SEQ. ID No. 197)	5'- AGAACAGGAGGAAGAAGGGGCGGGGCAA-3'
AF139664-6 (SEQ. ID No. 198)	5'- TACGCCACCGACTCAAGGTACGTACGTAC-3'
AF139664-7 (SEQ. ID No. 199)	5'- TCATTTTTCACAGTATCGACAAAATCCT-3'
AF139664-8 (SEQ. ID No. 200)	5'-TGGGCAGGAAAAGGCATGGCCCACCCCCA-3'
AF139664-9 (SEQ. ID No. 201)	5'-TGCTTTGATTAATACAGCTCGCAGCGAC-3'
AF139665-5 (SEQ. ID No. 211)	5'- CCGCCGACGACGATCGGGAGAGGGCCGGT-3'
AF139665-6 (SEQ. ID No. 212)	5'- TCTTCTGTAGTATCGAAGGGATCCTTG-3'
AF139665-7 (SEQ. ID No. 213)	5'- TCGCCGCGCGCGCGCGATGGGCGAGCT-3'
AF141964-5 (SEQ. ID No. 222)	5'-GGGTTTTTCGGGGCGGGGAAGGCGCGGAGGGGGA-3'
AF141964-6 (SEQ. ID No. 223)	5'-TGAAGTTGTCCATGCTTACTAATACTCAAA-3'
AF141964-7 (SEQ. ID No. 224)	5'-ATTGGTTTTCCCATGCGTGTGATGGATATTC-3'
AF141964-8 (SEQ. ID No. 225)	5'-GACCAAAACCAGCCCATGCTGCATGGTACACTATTAGGCT-3'
AF141964-9 (SEQ. ID No. 226)	5'-CCTAATAAGAAACCGATGGGTATAAAATGGAGA-3'
AF141965-5 (SEQ. ID No. 236)	5'-GAGAGATCGGGATCGATCGTGCGGGGGA-3'
AF141965-6 (SEQ. ID No. 237)	5'-TCCAAGCGCCGGAAAGGCCTCCTCAAGAA-3'
AF141965-7 (SEQ. ID No. 238)	5'-GAAAATGGAGGATGCAGAATATATATCCT-3'
AF141965-8 (SEQ. ID No. 239)	5'-AACAACTGTTCCAAGGAGCATGTCCAC-3'
AF141965-9 (SEQ. ID No. 240)	5'-CTTCTCGCGCGCCCGCGATTTCCGTT-3'
AF141965-10 (SEQ. ID No. 241)	5'-TCTCCCCCCGCGGCGGCCTCTACGA-3'
AF174093-5 (SEQ. ID No. 250)	5'-GGCGAGGTCGCGTTGGGGCAAGGGAA-3'
AF174093-6 (SEQ. ID No. 251)	5'-GAAGATCGAGATCAAGAGGATCGAGGAC-3'
AF174093-7 (SEQ ID No. 252)	5'-CGTGCTGTGCGAAGCGCAGGTCGGC-3'
AF174093-8 (SEQ ID No. 253)	5'-AAGAAGGCGAACCAGCTCGCCGTGC-3'
AF204063-5 (SEQ. ID No. 262)	5'-AGGAGGAGGAAGAAGATCGGGAGGGGGAA-3'
AF204063-6 (SEQ. ID No. 263)	5'-TCTCCAGCTCATCTTGGTACGTATAGCA-3'
AF204063-7 (SEQ. ID No. 264)	5'-TTCACTTCCAGCATCTACAAAACCTTG-3'
AF204063-8 (SEQ. ID No. 265)	5'-ATCTTCTCCGGCCGCCGCCGCCTCTT-3'
AF204063-9 (SEQ. ID No. 266)	5'-GTCCAGCTAGCCAAGGATGGATATATTA-3'
AF345911-5 (SEQ. ID No. 275)	5'-AGCGGCGAGGATCGGGCGGGGGAAGGT-3'
AF345911-6 (SEQ. ID No. 276)	5'-GTTTTCCCTGCAGGATCGACAAAATCCT-3'
AF345911-7 (SEQ. ID No. 277)	5'-CGGCCGCTTCCATCGATTTCAGCTGCT-3'
AF424549-5 (SEQ. ID No. 287)	5'-AGGGGGCGGCGATGGGGAGGGGCAAGAT-3'
AF424549-6 (SEQ. ID No. 287)	5'-CCGCACGGGGATCATCAAGAAGGCCAG-3'
AF424549-7 (SEQ. ID No. 289)	5'-AGGTCGCCATCATCAAGTTCTCCTCCA-3'
AF424549-8 (SEQ. ID No. 290)	5'-TTGCATCTATCCATCGTTAAAACAAGTC-3'
AJ293816-5 (SEQ. ID No. 299)	5'-TGTGTGATCTGATAAGGCTGGCGGCGGCGGT-3'
AJ293816-6 (SEQ. ID No. 300)	5'-TACATTGATGAAGCATCGAACAGATTATTG-3'

AY115556-5 (SEQ. ID No. 309)	5'-CTTATCTTGATCGATCGCGCGAGGCAAG-3'
AY115556-6 (SEQ. ID No. 310)	5'-TGAACAGAACCATCGAGGAGCTG-3'
AY115556-7 (SEQ. ID No. 311)	5'-AGCTCTGATATAGCATGGAGGTTAATTG-3'
AY177695-5 (SEQ. ID No. 320)	5'-TCGATCGATTGAAGAAGGGGAGAGGGA-3'
AY177695-6 (SEQ. ID No. 321)	5'-GACAACACGAAGAACCGGCAGGTGAC-3'
AY177695-7 (SEQ. ID No. 322)	5'-CGCGGCGGGCTGATCAAGAAGGCCCGGGAG-3'
AY177695-8 (SEQ. ID No. 323)	5'-CTTTTTGTTAGCATCAAATCAATAATA-3'
AY177695-9 (SEQ. ID No. 324)	5'-TGAAGAGCAAACCATCGAGCTAACAAACACA-3'
AY177695-10 (SEQ. ID No. 325)	5'-TCAGGGATGACAACACGATGGCACCATCAGTCAT-3'
AY177696-5 (SEQ. ID No. 334)	5'-TAGCGGCGAAGAAGATCGGGAGGGGGAAGA-3'
AY177696-6 (SEQ. ID No. 335)	5'-GAGGTCGGCCTCAAGATCTTCTCCAG-3'
AY177696-7 (SEQ. ID No. 336)	5'-CAAATATGCAGCATCAAGTCAGTTAT-3'
AY177696-8 (SEQ. ID No. 337)	5'-CAGCATGGGCCATCGCCAGCTCTTCTCT-3'
AY177698-5 (SEQ. ID No. 346)	5'-ACCAACCTGATCAAAGGGGCGTGGGA-3'
AY177698-6 (SEQ. ID No. 347)	5'-TCCAAGAGGAGGATCGGGCTGCTCAAGAAA-3'
AY177698-7 (SEQ. ID No. 348)	5'-AGGCACTGGCAAGAAGTACGAGTACT-3'
AY177699-5 (SEQ. ID No. 360)	5'-ATCGGCCAGAATCGGGAGGGGGCGCA-3'
AY177700-5 (SEQ. ID No. 370)	5'-AAGAGTGAAACAACAAGGTCAGAGGA-3'
AY177700-6 (SEQ. ID No. 371)	5'-GAAAGGTGCAAATCCGACGAATAGAG-3'
AY177700-7 (SEQ. ID No. 372)	5'-ATTAGCTACTAAAGGGTGTGTTTCCA-3'
AY177700-8 (SEQ. ID No. 373)	5'-AAAATGCAAGAAATTTGCATAACTTG-3'
AY177702-5 (SEQ. ID No. 382)	5'-GAGAGCGAGGAGATCGGGAGGGGGAAGA-3'
AY177702-6 (SEQ. ID No. 384)	5'-TTACTTGCATGCTAGCTTGAAAACTGT-3'
AY177702-7 (SEQ. ID No. 385)	5'-ATCCTAAATTTTCATCGATGGCATCTAG-3'
AY177702-8 (SEQ. ID No. 386)	5'-GATCAGGACCATGCCAGTCTGATGGCCAA-3'
AY177702-9 (SEQ. ID No. 387)	5'-GCACAAGGAAAGCCTTGGAACATCTT-3'
AY224482-5 (SEQ. ID No. 396)	5'-GAGAGCGAGGAGATCGGGAGGGGGAAGA-3'
AY224482-6 (SEQ. ID No. 397)	5'-CTTGCATGCTAGCTTGAAAACTGTGA-3'
AY224482-7 (SEQ. ID No. 398)	5'-CTAAATTTTCATGCATGGCATCTAG-3'
AY224482-8 (SEQ. ID No. 399)	5'-TGATCAGGACCATCGCAGTCTGATGG-3'
AY224482-9 (SEQ. ID No. 400)	5'-CAAGGAAAGCCAAGGAACATCTTTAC-3'
AY250075-5 (SEQ. ID No. 409)	5'-AGAGAGAGAGATGAAGGGGAGGGGGAA-3'
AY250075-6 (SEQ. ID No. 410)	5'-TATTGATAGTATCGAGCGAATCCTTG-3'
AY250075-7 (SEQ. ID No. 411)	5'-CAGTTGGTTGCCAAGGCGCGTTGCCGA-3'
AY250075-8 (SEQ. ID No. 412)	5'-GAGCCTCGGCCATGCCCAGCTCCTTCCT-3'
AY250075-9 (SEQ. ID No. 413)	5'-CTATTATTTTAAGAAAGCATGGATTATATAG-3'
AY250075-10 (SEQ. ID No. 414)	5'-TGAGACAGACCCATGCCTGGGCCTAC-3'
L37527-5 (SEQ. ID No. 423)	5'-GAGGAGTTGGATATCGGGCGCGGCAAGAT-3'
L37527-6 (SEQ. ID No. 424)	5'-ACCTCCATTCCTTGCATGGCGGCCT-3'

L37527-7 (SEQ. ID No. 425)	5'-TACTCCACTCCAACGATGGCGTCCAGT-3'
L37528-5 (SEQ. ID No. 436)	5'-AGCATACCCATCCATCTTGAACATGATGGT-3'
L37528-6 (SEQ. ID No. 437)	5'-CATCCATCTTGAACATCTTGGTAAATTCTGGCT-3'
U78891-5 (SEQ. ID No. 447)	5'-AGATCGATCGGGATCGGGAGGGGTCGGGT-3'
U78891-6 (SEQ. ID No. 448)	5'-GAACTTTGTAGCATCACTAAAACGCT-3'
U78891-7 (SEQ. ID No. 449)	5'-GTCCATGAAACAAACCGATGGTAATTGC-3'
AF095646-5 (SEQ ID No. 512)	5'-CAAAAGTTGAGGGATTGGATCGATCAGA-3'
AF095646-6 (SEQ ID No. 513)	5'-GATTGGATCGATCAGAGATCGGGAGGGGAAGGGT-3'
AF095646-7 (SEQ ID No. 514)	5'-GTTTTGTTTCCTTCGATGGGATGCGTATTC-3'
AF0956461-8 (SEQ ID No. 515)	5'-GAAGGCGTACGAGGTCTCCGTGCTCTG-3'

primers to mutagenize 3'-regulatory sequence	
name	**sequence**
AB003328-5 (SEQ. ID No. 162)	5'- CGTATTGTCGTCCATGCATGCGAAATGCTA-3'
AB095645-9 (SEQ. ID No. 187)	5'- GTAAAATTGCAGATCGATGGATGTCTCCA-3'
AB095645-10 (SEQ. ID No. 188)	5'- GAACCTCTCCATGCCGTGCACCCCG-3'
AF139664-10 (SEQ. ID No. 202)	5'-ACCTGACCCGGTCCCGTCGCCTGCTGCT-3'
AF141964-10 (SEQ. ID No. 227)	5'-AGAGAAAAGAGTTGCCTTGGCCTCTGGCTCTGC-3'
AF345911-8 (SEQ. ID No. 278)	5'-AGAACTATTCCAAGGTAATTGTACCATC-3'
AY177698-8 (SEQ. ID No. 349)	5'-AGGAGGCCAACCTGCATGGCTACGTTCTT-3'
AY177698-9 (SEQ. ID No. 340)	5'-GCTCGTTTGATCCAAGGACAAGATCA-3'
AY177698-10 (SEQ. ID No. 351)	5'-TTCCTAAGGACACGATGGAGTCTGGA-3'
AY177699-6 (SEQ. ID No. 361)	5'-AGTCGCCCCCGGCCTTTTAAGAGCCGCA-3'
AY177700-9 (SEQ. ID No. 374)	5'-TAGGATTCATAACGATGGACATGTTC-3'
L37527-8 (SEQ. ID No. 426)	5'-ACGGCAGCGGCCCGTCCGGCAGCTCCGGGA-3'
L37527-9 (SEQ. ID No. 427)	5'-GAACCTCTCCATCGCGCCGCCCCGGGGGCT-3'
L37528-7 (SEQ. ID No. 438)	5'-ATGTGAGGACCATCGATTGATGCATTG-3'
U78891-8 (SEQ. ID No. 450)	5'-ACCTACATGCCCGCATGGCTACCATGAT-3'
U78891-9 (SEQ. ID No. 451)	5'-TCGTCGTTTTCTCGATGGCCTTGCAT-3'
AF095646-9 (SEQ ID No. 516)	5'-CCCTTCCTTACCCATGCTGGTAACAAATATG-3'

primers to clone 3'-regulatory sequence in pNOV6901

name	sequence
AB003322-9 (SEQ. ID No. 141)	5'-TAATAAGAGCTCTGAAAGGAAAAAAAAAGCA-3'
AB003322-10 (SEQ. ID No. 142)	5'-ATATATGCGGCCGCGGTCCGAATAAATCTGGCAACTGA-3'
AB003324-10 (SEQ. ID No. 154)	5'-ATATATGCGGCCGCGGTCCGTGGAGATCGACCACAATTAAATTC-3'
AB003324-11 (SEQ. ID No. 155)	5'-TAGGTAGAGCTCCTAGCTAGCTAGCTACCGTTTCA-3'
AB003328-6 (SEQ. ID No. 163)	5'- TAATAAGAGCTCGTAACAGGCCAGGAATAAGCT-3'
AB003328-7 (SEQ. ID No. 164)	5'- ATATATGCGGCCGCGGTCCGATTGCCGGGCTATAGCCTT-3'
AB077760-11 (SEQ. ID No. 175)	5'- ATGCATGAGCTCTATATCATCGCCGC-3'
AB077760-12 (SEQ. ID No. 176)	5'- ATATATGCGGCCGCGGTCCGTGGTACTGAAATC-3'
AB095645-13 (SEQ. ID No. 189)	5'- TAATAAGAGCTCGCTGCTAGGTTGCCCCGCCACT-3'
AB095645-14 (SEQ. ID No. 190)	5'- TAATAAGCGGCCGCGGTCCGCACGGCTACCTCTCGCCGGA-3'
AF139664-11 (SEQ. ID No. 203)	5'- TAATAAGAGCTCGGAGGCTTCAGATCCATACCA-3'
AF139664-12 (SEQ. ID No. 204)	5'- ATATATGCGGCCGCGGTCCGGATAAACATTGTGAAGCAAC-3'
AF139665-8 (SEQ. ID No. 214)	5'-TGATGAGCGGCCGCAGGCATCTGTTGATCTCA-3'
AF139665-9 (SEQ ID No. 215)	5'-TATATAACTAGTGCGGTCCGATGAACTCCACCTCGGGAAC-3'
AF141964-11 (SEQ. ID No. 228)	5'-TGATGAGCGGCCGCAGGAAGCACAGGTGT-3'
AF141964-12 (SEQ. ID No. 229)	5'-ATATATCCCGGGCGGTCCGATGAGATACAATCTAGTAC-3'
AF141965-11 (SEQ. ID No. 242)	5'-TGATGAGCGGCCGCAGAAGGCCAGCCACA-3'
AF141965-12 (SEQ. ID No. 243)	5'-TATATACCCGGGCGGTCCGCCTTATCGAATATTCA-3'
AF174093-10 (SEQ. ID No. 254)	5'-TGATGAGAGCTCCTTCCTGGAAGCAGTAG-3'
AF174093-11 (SEQ. ID No. 255)	5'-ATATATGCGGCCGCGGTCCGCCCCTTCTCCTCCTCCGGGA-3'
AF204063-10 (SEQ. ID No. 267)	5'-TATATACCCGGGCGGTCCGCTGCATATACTTGCAACA-3'
AF204063-11 (SEQ. ID No. 268)	5'-TGATGAGCGGCCGCTGTGTGTGTTCAGTTCAG-3'
AF345911-9 (SEQ. ID No. 279)	5'-TAATAAGCGGCCGCGATGATCATCGTCGTCGT-3'
AF345911-10 (SEQ. ID No. 280)	5'-TATATACCCGGGCGGTCCGTTGACTTAAATGGCAAG-3'
AF424549-9 (SEQ. ID No. 291)	5'-TGATGAGCGGCCGCGATATCATCGCCGCCG-3'
AF424549-10 (SEQ. ID No. 292)	5'-TATATACCCGGGCGGTCCGTTTTATTATCTTGGCGA-3'
AJ293816-7 (SEQ. ID No. 301)	5'-TGATGAGCGGCCGCTGGCTGGAAACTAAAACT-3'
AJ293816-8 (SEQ. ID No. 302)	5'-TATATACCCGGGCGGTCCGTTTGAAACATTTCGGCCCT-3'
AY115556-8 (SEQ. ID No. 312)	5'-TAATAAGCGGCCGCACTAATAATGGCCTGG-3'
AY115556-9 (SEQ. ID No. 313)	5'-TATATACCCGGGCGGTCCGAACTTTGCACGAATG-3'
AY177695-11 (SEQ ID No. 325)	5'-TAATAAGAGCTCCACATGATCAAGTGTTAAAAAC-3'
AY177695-12 (SEQ. ID No. 326)	5'-TATATAGCGGCCGCGGTCCGGCTCGGTCAAACCAGCGT-3'
AY177696-9 (SEQ. ID No. 338)	5'-TGATGAGAGCTCCCAGAAAAACATTGTTTTG-3'

AY177696-10 (SEQ. ID No. 339)	5'-TATATAGCGGCCGCGGTCCGTTTGGGATTGGAC-3'
AY177698-11 (SEQ. ID No. 325)	5'-TGATGAGCGGCCGCTTGCCCTCTTTCCATC-3'
AY177698-12 (SEQ. ID No. 353)	5'-TATATACCCGGGCGGTCCGTTAACCTAAAAGAAAT-3'
AY177699-7 (SEQ. ID No. 362)	5'-TAGTAGGCGGCCGCAAACAGATGGACGCTTGA-3'
AY177699-8 (SEQ. ID No. 363)	5'-TATATACCCGGGCGGTCCGAGGCTTGGTGCAAG-3'
AY177700-10 (SEQ. ID No. 375)	5'-TAGTAGGCGGCCGCGTCAAAGGACCT-3'
AY177700-11 (SEQ. ID No. 376)	5'-TATATACCCGGGCGGTCCGTAATTTTACTAGTACT-3'
AY177702-10 (SEQ. ID No. 388)	5'-TAATAAGAGCTCGACTATGCCGTACAAGC-3'
AY177702-11 (SEQ. ID No. 389)	5'-ATATATGCGGCCGCGGTCCGTGGTTGTCACAG-3'
AY224482-10 (SEQ. ID No. 401)	5'-TAATAAGAGCTCGACTATGCCGTACAAGC-3'
AY224482-11 (SEQ. ID No. 402)	5'-TATATAGCGGCCGCGGTCCGTGGTTGTCACAGA-3'
AY250075-11 (SEQ. ID No. 415)	5'-TAGTAGAGCTCAAGATGTTCAGATGAAATG-3'
AY250075-12(SEQ. ID No. 416)	5'-TATATAGCGGCCGCGGTCCGAAAAAAATCACCGGCGT-3'
L37527-10 (SEQ. ID No. 428)	5'-TAGTAGAGCTCGCTGCTGAGCACTGCCA-3'
L37527-11 (SEQ. ID No. 429)	5'-TATATAGCGGCCGCGGTCCGTGCTGGTGCTCGAGGT-3'
L37528-8 (SEQ. ID No. 439)	5'-TAGTAGAGCTCTTTTGGTGTAGACACC-3'
L37528-9 (SEQ. ID No. 440)	5'-ATATAGCGGCCGCGGTCCGTGAAAGCTACATTTTAGC-3'
U78891-10 (SEQ. ID No. 452)	5'-TGATGAGCTCACAGTGCGTGCATGAACA-3'
U78891-11 (SEQ. ID No. 453)	5'-TATATAGCGGCCGCGGTCCGATGTTTTTTTTTGTCTTTTGATGC-3'
AF095646-10 (SEQ ID No. 517)	5'-ATATATCTCGAGCGGACCGTGTTGCATGCATGCGTACTGGTGA-3'
AF095646-11 (SEQ ID No. 518)	5'-ATATATCCATGGTGGGTTTATTCTGCAAACAAAAATAG-3'

primers to clone 5'-regulatory sequence in pNOV6901	
name	**sequence**
AB003322-10 (SEQ. ID No. 142)	5'-TATATACCATGGTGGTGATACTGTCCAACAGAAGG -3'
AB003322-11 (SEQ. ID No. 143)	5'-ATATATGGATCCGGACCGTTGCCCAGCGAGGAGCATG -3'
AB003324-12 (SEQ. ID No. 156)	5'-ATATATGGATCCGGACCGTCTAAATAGGGCCCAACATAC-3'
AB003324-13 (SEQ. ID No. 157)	5'-TATATACCATGGTGGCTTAAGGATGCTGAAATATGA-3'
AB003328-8 (SEQ. ID No. 165)	5'- TATATAGGATCCGGACCGAACAAAACCAATAATCTCCAATG-3'
AB003328-9 (SEQ. ID No. 166)	5'- TATATACCATGGTGGATCGCCGATGAACCAACCAAC-3'
AB077760-9 (SEQ. ID No. 177)	5'- ATATATGTCGACGGACCGATTCGTTGGGGGTGACATGAC-3'
AB077760-10 (SEQ. ID No. 178)	5'- TACAGTACCATGGTGGCCCTTGATGCTGCAGCAGGAT-3'
AB095645-11 (SEQ. ID No. 191)	5'- GAGAGACCTGCAGGCGGACCGCTCGTCGCCGTCGTTGGCTCGGCGT-3'
AB095645-12 (SEQ. ID No. 192)	5'- ATATATCCATGGTGGTTGATAGCCTTATACATGTCCC-3'

AF139664-13 (SEQ. ID No. 205)	5'- ATATATGGATCCGGACCGTGCATCCTTACAAAGAGACA-3'
AF139664-14 (SEQ. ID No. 206)	5'- TATATACCATGGTGGTTGTCCATACTGTGAAAAATG-3'
AF139665-10 (SEQ. ID No. 216)	5'-ATATATGTCGACGGACCGACCTGGGGTTTGGAAATG-3'
AF139665-11 (SEQ. ID No. 217)	5'-TATATACCATGGTGGCCTTCCATACTACAGAAG-3'
AF141964-13 (SEQ. ID No. 230)	5'-ATATATCCTGCAGGCGGACCGAAAACACTGATTAAAGGGT-3'
AF141964-14 (SEQ. ID No. 231)	5'-ATATATCCATGGTGGCCTTCAATGCTGAAAAAGAAAAC-3'
AF141965-13 (SEQ. ID No. 244)	5'-ATATATGGATCCGGACCGTGTTTCTCGATTAGGCTAC-3'
AF141965-14 (SEQ. ID No. 245)	5'-ATATATCCATGGTGGTCTGTAGGCTGCAGAACAGACAC-3'
AF174093-12 (SEQ. ID No. 256)	5'-TATATCCTGCAGGCGGACCGAAAATTCACAAGTATAATTC-3'
AF174093-13 (SEQ. ID No. 257)	5'-TATATACCATGGTGGTCAAGCTTAACAAAGCAT-3'
AF204063-12 (SEQ. ID No. 269)	5'-TATATAGGATCCGGACCGACAAATTTGTTGTACCAC-3'
AF204063-13 (SEQ. ID No. 270)	5'-ATATATCCATGGTGGTGTACATGCTGGAAGTGA-3'
AF345911-11 (SEQ. ID No. 281)	5'-TATATAGTCGACGGACCGAAAATCTCAAGGTTTC-3'
AF345911-12 (SEQ. ID No. 282)	5'-TATATACCATGGTGGTTGTCGATCCTGCAGGGAA-3'
AF424549-11 (SEQ. ID No. 293)	5'-TATATAGTCGACGGACCGTACAAAGTGCTGGAAGT-3'
AF424549-12 (SEQ. ID No. 294)	5'-TATATACCATGGTGGTGATGCTGCAGCAGGATGCA-3'
AJ293816-9 (SEQ. ID No. 303)	5'-TATATAGTCGACGGACCGCAAACATTTTAAACTTTAAC-3'
AJ293816-10 (SEQ. ID No. 304)	5'-TATATACCATGGTGGTGTTCGATGCTTCATCAATGT-3'
AY115556-10 (SEQ. ID No. 314)	5'-TATATACCTGCAGGCGGACCGATATAAAAACCTACTTTATG-3'
AY115556-11 (SEQ. ID No. 315)	5'-ATATATCCATGGTGGCGATGGTTCTGTTCAAAGAAATC-3'
AY177695-13 (SEQ. ID No. 328)	5'-TATATAGGATCCGGACCGTCAGTCCATCATTTCGTC-3'
AY177695-14 (SEQ. ID No. 329)	5'-TATATACCATGGTGGATTTGATGCTAACAAAAAGG-3'
AY177696-11 (SEQ. ID No. 340)	5'-TATATAGGATCCGGACCGCAAGCAAAGAAACAAATTTCG-3'
AY177696-12 (SEQ. ID No. 341)	5'-ATATACCATGGTGGCTTGATGCTGCATATTTG-3'
AY177698-13 (SEQ. ID No. 354)	5'-TATATAGGATCCGGACCGAAGATTTGAACTACTGCCT-3'
AY177698-14 (SEQ. ID No. 355)	5'-TATATACCATGGTGGTTTGCAATCCTGCCA-3'
AY177699-9 (SEQ. ID No. 364)	5'-TATATAGGATCCGGACCGTCCGGTCGGCCCTCGTCCT-3'
AY177699-10 (SEQ. ID No. 365)	5'-TATATACCATGGTGGCTCACGCTGCGCCA-3'
AY177700-12 (SEQ. ID No. 377)	5'-TATATAGGATCCGGACCGATAAGTATTTCAGAAAG-3'
AY177700-13 (SEQ. ID No. 378)	5'-TATATACCATGGTGGGCAAATTTCTTGCATTTTG-3'
AY177702-12 (SEQ. ID No. 390)	5'-TATATAGTCGACGGACCGTCTTTTCGCAAACTAAAC-3'
AY177702-13 (SEQ. ID No. 391)	5'-TATATACCATGGTGGTCAAGCTAGCATGCAAG-3'
AY224482-12 (SEQ. ID No. 403)	5'-TATATAGTCGACGGACCGTCTTTTCGCAAACTAAACAAGG-3'
AY224482-13 (SEQ. ID No. 404)	5'-TATATACCATGGTGGTTTTCAAGCTAGC-3'

AY250075-13 (SEQ. ID No. 417)	5'-TATATACCTGCAGGCGGACCGAAATGGTTTCATTTTGG-3'
AY250075-14 (SEQ. ID No. 418)	5'-TATATACCATGGTGGCGCTCGATACTATCA-3'
L37527-12 (SEQ. ID No. 430)	5'-TATATACCTGCAGGCGGACCGCCAAGAGAGCCCCCT-3'
L37527-13 (SEQ. ID No. 431)	5'-TATATACCATGGTGGTTGACAGCCTAAAACG-3'
L37528-10 (SEQ. ID No. 441)	5'-TATATAGGATCCGGACCGACCACCTCAATCTCCACT-3'
L37528-11 (SEQ. ID No. 442)	5'-TATATACCATGGTGGATCGGTCTGGACA-3'
U78891-12 (SEQ. ID No. 454)	5'-TATATAGGATCCGGACCGTCTTCTTCTTGCCTTCATTTG-3'
U78891-13 (SEQ. ID No. 455)	5'-TATATACCATGGTGGTTAGTGATGCTACAAAGTTC-3'
AF095646-12 (SEQ ID No. 519)	5'-TATATAGAGCTCAGTCCAAGCTTGCTAATAAAAACGCT-3'
AF095646-13 (SEQ ID No. 520)	5'-TATATACCCGGGCGGTCCGAAGGGTAGGGCTGCACGAA-3'

EXAMPLE 2:

1. Construction of the assembly vector pNOV6901 containing the β-glucuronidase (GUS) coding sequence.

A. Preparation of GUS coding sequence.

The β-glucuronidase (GUS) coding sequence Narasimhulu, et al 1996, Plant Cell, 8: 873-886, which includes an engineered intron, was amplified from pNOV5003 in a Pfuturbo polymerase (Stratagene, Cat. No. 600250) reaction. The reaction mixture consisted of 1 μL pNOV5003 miniprep DNA 200 μM dNTPs, 20 μM GUS5 oligonucleotide primer 5'-atggtacgtcctgtagaaacc-3' (SEQ ID NO 498), 20 μM GUS3 oligonucleotide primer 5'-gatcgagctctcattgtttgcctccctg-3' (SEQ ID NO 499), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600250) in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 10 cycles of (95°C for 5 seconds, 55°C for 10 seconds, 72°C for 2.5 minutes) then 20 cycles of (95°C for 5 seconds, 57°C for 15 seconds, 72°C for 2.5 minutes) then 72°C for 2.5 minutes. The 2.2 kb GUS PCR product was isolated and concentrated using the QIAEX II kit (Qiagen, Cat. No. 20021). The GUS PCR product was recovered in 15 μL ddH_2O and subsequently digested in a 20 μL reaction containing 1 μg BSA, 2 μL 10X restriction endonuclease buffer and 1 μL SacI. The reaction was incubated at 37°C for 2 hours. The GUS PCR product (SacI) was resolved on 1.5% TBE agarose and the 2.2 kb GUS PCR product (SacI) band was excised. The GUS PCR product (SacI) DNA was recovered from the agarose in 15 μL ddH_2O with the QIAEX II kit (Qiagen, Cat. No. 20021).

B. Preparation of the pSP73 vector.

An E. coli vector pSP73 (Promega, Cat. No. P2221) miniprep DNA was prepared. 1 μL of the miniprep DNA was digested in a 20 μL reaction mixture containing 1 μg BSA, 2 μL 10X restriction endonuclease, 1 μL SmaI and 1 μL SacI. The reaction was incubated at 25°C for 1.5 hours then 37°C for 1.5 hours. The pSP73 (SmaI/SacI) DNA was resolved on 1.5% TBE agarose and the 2.4 kb pSP73 (SmaI/SacI) band was excised. The pSP73 (SmaI/SacI) DNA was recovered from the agarose in 15 μL ddH_2O with the QIAEX II kit (Qiagen, Cat No. 20021).

C. Construction of pSP73-GUS

5 μL of pSP73 (SmaI/SacI) was ligated to 5 μL GUS PCR product (SacI) by mixing with an equal volume of Takara DNA Ligation Mix,Version II (Cat. No. TAK 6022) and incubating at 16°C for 30 minutes. 7.5 μL of the ligation mixture was transformed into 50 μL XL-1 supercompetent cells (Stratagene, Cat. No. 200236). pSP73-GUS recombinants were verified by digesting 2 μL pSP73-GUS miniprep DNA in a 20 μL reaction containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL XbaI and 1 μL SacI and the pSP73-GUS (XbaI/SacI) products were resolved on 1.5% TBE agarose. The positive pSP73-GUS recombinants were sequenced.

D. Addition of restriction endonuclease sites to pSP73-GUS

The pSP73-GUS construct lacks flexibility to clone 3'-regulatory sequence just after the GUS coding sequence. Additional restriction sites were added to the polylinker to increase flexibility at the 3'-terminus of the GUS coding sequence by ligating a synthetic adapter to the construct. The adapter (Synthetic Adaptor I) was made by combining 40 μL of 50 μM oligonucleotide PL-F 5'-Pccgcgggcggccgcactagtcccgggcccat-3' (SEQ ID NO. 456), 40 μL of 50 μM oligonucleotide PL-R 5'-Pcgatgggcccgggactagtgcggccgcccgcggagct-3' (SEQ ID NO. 457) --where P is a 5'-phosphate group--in a 100 μL mixture that is 25 mM in Tris-HCl (pH 8.0) and 10 mM in $MgCl_2$. The mixture was boiled for 5 minutes, removed from heat and naturally cooled to room temperature (about 60 minutes), yielding a 20 μM Synthetic Adaptor I solution.

The pSP73-GUS construct was prepared by digesting 14 μL of miniprep pSP73-GUS DNA with 1 μL SacI and 1 μL ClaI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The reaction mixture was incubated at 37°C for 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it

was further incubated at 37°C for 30 minutes. The pSP73-GUS (SacI/ClaI/CIP) DNA was resolved on 1% TAE agarose, excised, recovered and ethanol precipitated with glycogen carrier. The pSP73-GUS (SacI/ClaI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.5 μL of Synthetic Adaptor I solution was ligated to 2.5 μL pSP73-GUS (SacI/ClaI/CIP) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase and incubated more than 8 hours at 16°C. 4 μL of the ligation mixture was transformed into 50 μL XL-1 supercompetent cells (Stratagene, Cat. No. 200236). The pSP73-GUS-mod recombinants were verified by digesting 5 μL pSP73-GUS-mod miniprep DNA in a 20 μL reaction containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 1 μL NotI. The digests were resolved on 1.0% TAE agarose, and the sequence of positive pSP73-GUS-mod recombinants was verified. The finished clone was designated pNOV6901.

2. Construction of pNOV6900

It was necessary to construct an Agrobacterium binary vector to facilitate mobilization of expression cassettes constructed in pNOV6901 into plants. The pNOV2115 vector was modified by inserting an adaptor that introduces the PacI, SgFI and RsrII restriction endonuclease recognition sites. pNOV2115 miniprep DNA (14 μL) was digested with 1 μL KpnI and 1 μL HindIII in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. Digests were incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. pNOV2115 (KpnI/HindIII/CIP) was resolved on 1% TAE agarose, the 9.2 kb pNOV2115 (KpnI/HindIII/CIP) DNA band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV2115 (KpnI/HindIII/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

Additional restriction sites were added to pNOV2115 (KpnI/HindIII/CIP) by ligating the vector to Synthetic Adapter II. The Synthetic Adapter II was made by combining 37 μL of 150 μM oligonucleotide PL1 5'-Pgtaccggaccgcgatcgcttaatta-3' (SEQ ID NO 458), 37 μL of 150 μM PL2 oligonucleotide 5'-Pagcttaattaagcgatcgcggtccg-3' (SEQ ID NO 459)--where P is a 5'-phosphate group--in a 100 μL mixture that is 25 mM in Tris-HCl (pH 8.0) and 10 mM in

$MgCl_2$. The mixture was boiled for 5 minutes, removed from heat and naturally cooled to room temperature (about 60 minutes), yielding a 55 μM Synthetic Adapter II solution.

2.5 μL pNOV2115 (KpnI/HindIII/CIP) was ligated to 2.5 μL 55 μM Synthetic Adapter II solution by mixing with an equal volume of Takara DNA Ligation Mix,Version II (Cat. No. TAK 6022), and was incubated at 16°C for 30 minutes. 5.0 μL of ligation mixture was transformed into 50 μL DH5α competent cells (Invitrogen, Cat. No. 18258-012). pNOV2115-mod recombinants were verified by digesting 2 μL pNOV2115-mod miniprep DNA with KpnI, HindIII, PacI or RsrII in 10 μL reactions containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The sequence of positive pNOV2115-mod recombinants was verified. The finished clone was designated pNOV6900.

Example 3: Construction of the OsMADS5 Expression Cassette

A. Cloning the OsMADS5 5'-regulatory sequence

High-fidelity PCR was used to amplify the OsMADS5 5'-regulatory sequence from rice genomic DNA (gDNA). The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS5-P3 5'-tgagcaggtagccggcgaccaatcgcgag-3' (SEQ ID NO 460), 1 μL 20 μM oligonucleotide primer OsMADS#5-P2 5'-catactgttacaaaaaagaaaatcagtggaccac-3' (SEQ ID NO 461), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. The 5.4 kb DNA product, encoding the OsMADS5 5'-regulatory sequence, was cloned with the TOPO XL PCR cloning kit. The pCR-XL-TOPO-OsMADS5-5'-gDNA recombinants, containing the OsMADS5 5'-regulatory sequence, were identified by digesting 5 μL pCR-XL-TOPO-OsMADS5-5'-gDNA miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The reaction mixture was incubated at 37°C for 2 hours then the pCR-XL-TOPO-OsMADS5-5'-gDNA (EcoRI) products were resolved on 1% TAE agarose. Positive pCR-XL-TOPO-OsMADS5-5'-gDNA clones were sequenced.

B. Cloning the OsMADS5 3'-regulatory sequence

High-fidelity PCR was used to amplify the OsMADS5 3'-regulatory sequence from rice genomic DNA (gDNA). The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS#5-T1 5'-atgaattgcttatcacattaatggacatc-3' (SEQ ID NO. 462), 1 μL 20 μM oligonucleotide primer

OsMADS#5-T2 5'-caaaactacatcaagagccttggaattggtcc-3' (SEQ ID NO. 463), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 60°C for 30 seconds, 68°C for 6 minutes) followed by 68°C for 15 minutes. The 1.2 kb OsMADS5-3'-gDNA DNA product, encoding the OsMADS5 3'-regulatory sequence, was cloned with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Cat. No. K2875-20). pCR-Blunt II-TOPO-OsMADS5-3'-gDNA recombinants, with the OsMADS5 3'-regulatory sequence, were identified by digesting 5 μL pCR-Blunt II-TOPO-OsMADS5-3'-gDNA miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The reaction mixture was incubated at 37°C for 2 hours and then the pCR-Blunt II-TOPO-OsMADS5-3'-gDNA (EcoRI) products were resolved on 1% TAE agarose. Positive pCR-Blunt II-TOPO-OsMADS5-3'-gDNA clones were sequenced.

C. Construction of the OsMADS5 5'- regulatory sequence

The OsMADS5 5'- regulatory sequence for the expression cassette was made in several steps. The 3'-half (OsMADS-5Pb, about 3.03 kb) was produced by high-fidelity PCR from the pCR-XL-TOPO-OsMADS5-5'-gDNA clone described above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS5-5'-gDNA miniprep DNA, 200 μM dNTPs, 20 μM oligonucleotide primer OsMADS5-C3 5'-cagtgtcgacggggcgagggaaagtagagc-3' (SEQ ID NO 464), 20 μM oligonucleotide primer OsMADS5-C4 5'-cgatccatggtggatactgttacaaaaaagaaaatcagtg-3' (SEQ ID NO 465), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252) in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The OsMADS-5Pb DNA product was recovered using the QIAquick PCR purification kit (Qiagen, Cat. No. 28106). The recovered OsMADS-5Pb DNA was ethanol precipitated with glycogen carrier. The OsMADS-5Pb DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The OsMADS-5Pb was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI. The reaction mixture was incubated at 37°C for more than 6 hours. The OsMADS-5Pb (NcoI/SalI) DNA was resolved on 1.0% TAE agarose and the 3.03 kb OsMADS-5Pb (NcoI/SalI) DNA band was excised, recovered and ethanol precipitated with glycogen carrier. The OsMADS-5Pb (NcoI/SalI) DNA was

recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg of the pNOV6901 miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI . The reaction mixture was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901 (NcoI/SalI/CIP) DNA was resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (NcoI/SalI/CIP) band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901 (NcoI/SalI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL of the OsMADS-5Pb (NcoI/SalI) was ligated to 4.0 μL pNOV6901 (NcoI/SalI/CIP) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL) and incubated more than 8 hours at 16°C. 5.0 μL of the ligation mixture was transformed into 50 μL Top10 competent cells. pNOV6901-OsMADS-5Pb recombinants were verified by digesting 2 μL pNOV6901-OsMADS-5Pb miniprep DNA with 0.5 μL SalI, 0.5 μL NcoI in 10 μL reactions containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours and the pNOV6901-OsMADS-5Pb (NcoI/SalI) DNA was resolved on 1% TAE agarose. Positive pNOV6901-OsMADS-5Pb recombinants were sequenced.

The 5'-half (OsMADS-5Pa, about 2.4 kb) was produced by high-fidelity PCR from the pCR-XL-TOPO-OsMADS5-5'-gDNA clone described above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS5-5'-gDNA miniprep DNA, 200 μM dNTPs, 20 μM oligonucleotide primer OsMADS5-C1 5'-gactctcgaggcgcgcctgagcaggtagccggcgacc-3' (SEQ ID NO 466), 20 μM oligonucleotide primer OsMADS5-C2b 5'-gtgtgtctcgagctctctctagctctctctcgg-3' (SEQ ID NO 467), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252) in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The 2.4 kb OsMADS-5Pa DNA product was cloned with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Cat. No. K2875-20). pCR-Blunt II-TOPO-OsMADS-5Pa recombinants were

identified by digesting 5 μL pCR-Blunt II-TOPO-OsMADS-5Pa miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt II-TOPO-OsMADS-5Pa recombinants were sequenced.

2 μg of the pNOV6901-OsMADS-5Pb miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL XhoI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg of the pCR-Blunt II-TOPO-OsMADS-5Pa miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL SalI. The digest was incubated at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6901-OsMADS-5Pb (XhoI/CIP) and pCR-Blunt II-TOPO-OsMADS-5Pa (SalI), were resolved on 1.0% TAE agarose and the 7.7 kb pNOV6901-OsMADS-5Pb (XhoI/CIP) and the 2.4 kb OsMADS-5Pa (SalI) bands were excised, extracted, recovered and ethanol precipitated with glycogen. The pNOV6901-OsMADS-5Pb (XhoI/CIP) and OsMADS-5Pa (SalI) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended each in 5 μL ddH_2O.

4.0 μL of the pNOV6901-OsMADS-5Pb (XhoI/CIP) was ligated to 4.0 μL OsMADS-5Pa (SalI) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The reaction mixture was incubated more than 8 hours at 16°C. 5.0 μL of the ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS5P recombinants were verified by digesting 2 μL pNOV6901-OsMADS5P miniprep DNA with 0.5 μL XhoI, 0.5 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS5P recombinants were sequenced.

D. Construction of the OsMADS5 3'-regulatory sequence

The OsMADS-5 3'-regulatory sequence for the expression cassette was produced by high-fidelity PCR from the pCR-Blunt II-TOPO-OsMADS5-3'-gDNA clone, above. The reaction mixture consisted of 1 μL pCR-Blunt II-TOPO-OsMADS5-3'-gDNA miniprep DNA, 200

μM dNTPs, 20 μM oligonucleotide primer OsMADS5T-F 5'-cccgggccatgggggtctagaatgaattgcttatcacattaatgg-3' (SEQ ID NO 468), 20 μM oligonucleotide primer OsMADS5T-R 5'-cccgggcgcgccggatgagaacagctacatcc-3' (SEQ ID NO 469), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252) in a final volume of 50 μL. The thermocycling program was 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The OsMADS5T DNA product was recovered using the QIAquick PCR purification kit (Qiagen, Cat. No. 28106) and ethanol precipitated with glycogen carrier. The OsMADS5T DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The OsMADS5T DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL XmaI. The digest was incubated at 37°C for more than 6 hours. The OsMADS5T (XmaI) DNA was resolved on 1.0% TAE agarose and the 1.1 kb OsMADS5T (XmaI) band was excised, recovered and ethanol precipitated with glycogen carrier. OsMADS5T (XmaI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901-OsMADS5P miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL XmaI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901-OsMADS5P (XmaI/CIP) DNA was resolved on 1.0% TAE agarose and the 10.1 kb pNOV6901-OsMADS-5P (XmaI/CIP) band was excised, recovered and ethanol precipitated with glycogen carrier. pNOV6901-OsMADS-5P (XmaI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended each in 5 μL ddH_2O.

4.0 μL pNOV6901-OsMADS5P (XmaI/CIP) was ligated to 4.0 μL OsMADS5T (XmaI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL) and incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. Positive pNOV6901-OsMADS5P/OsMADS5T recombinants were verified by digesting 2 μL miniprep DNA with 1.0 μL AscI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction

endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS5P/OsMADS5T recombinants were sequenced. The construct was designated pNOV6901-OsMADS5P/OsMADS5T. The plasmid's QC number is 11084. 11084 contains the complete OSMADS5 expression cassette depicted by SEQ ID NO. 536.

E. Mobilization of the OsMADS5 GUS expression cassette into pNOV6900

2 μg pNOV6900 was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL AscI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pNOV6901-OsMADS5P/OsMADS5T miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL AscI. The digest was incubated at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6900 (AscI/CIP) and pNOV6901-OsMADS5P/OsMADS5T (AscI), were resolved on 1.0% TAE agarose and the 9.2 kb pNOV6900 (AscI/CIP) and the 8.7 kb pNOV6901-OsMADS5P/OsMADS5T (AscI) DNA bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6900 (AscI/CIP) and pNOV6901-OsMADS5P/OsMADS5T (AscI) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O each.

4.0 μL pNOV6900 (AscI/CIP) was ligated to 4.0 μL pNOV6901-OsMADS5P/OsMADS5T (AscI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase, which was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. pNOV6900-pNOV6901-OsMADS5P/OsMADS5T recombinants were verified by digesting 7.5 μL pNOV6900-pNOV6901-OsMADS5P/OsMADS5T miniprep DNA with 1.0 μL NcoI in 10 μL reactions containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6900-pNOV6901-OsMADS5P/OsMADS5T recombinants were sequenced. The finished clone was designated pNOV6911. The plasmid's QC number is 11085.

The engineered alterations in the OsMADS5P sequence include introduction of an XhoI site followed by an AscI site at the 5'-end of the OsMADS5P sequence, elimination the

natural translation start codon of the OsMADS5P sequence, elimination of undesired ORFs in the new leader sequence (5'-UTR) of the OsMADS5P sequence, insertion a Kozak sequence upstream of the new translation start codon of the OsMADS5P sequence and insertion of a new translation start codon downstream of the intron1/exon2 junction as an NcoI site in the OsMADS5P sequence. The engineered alterations in the OsMADS5T sequence include introduction of an XmaI site at the 5'-terminus of the OsMADS5T sequence and introduction of an AscI site at the 3'-terminus of the OsMADS5T sequence. In this configuration the GUS coding sequence can be replaced with any gene of interest flanked by NcoI restriction sites. The complete cassette can then be excised as an AscI fragment and cloned into pNOV6900.

The cassette was transformed into A188 X HyII maize and Kaybonnet rice using standard agrobacterium mediated methodology.

GUS Expression in T0 maize

Fifteen T0 transgenic maize lines were generated. Tassel spikelets and leaf punches were harvested just before pollen shed and histochemically screened for GUS activity. The ear from a plant containing multiple transgene copies was sacrificed to examine GUS expression in developing florets. Gus activity localized primarily to transmitting tissue at the base of each floret, and to a lesser extent, the vascular bundles in developing ears. GUS activity was also apparent in developing silks. These data indicate the cassette drives GUS expression primarily in female reproductive tissue.

GUS expression in T0 rice

Of forty T0 rice (cv. Kaybonnet) lines containing pNOV6911 (or 11085), eighteen independent transformants were histochemically stained for GUS expression. Only four events had detectable GUS activity in leaf tissue. In most events, activity in spikelets was localized to glume tips, and anthers to a much lesser extent.

GUS Expression in T1 maize

T1 progeny from three events were sown for expression analysis in vegetative and reproductive tissue. FIG. 3 summarizes data for tissue sampled about 5 days before pollination. FIGS. 3A and 3B show GUS activity is restricted to developing ears, particularly the vasculature along the outer ear and the transmitting tissue beneath florets. FIG 3B also shows GUS activity in tissue surrounding the ovule sac. GUS activity was undetectable in the ear node or the node beneath it, tassel, leaf or silk (FIGS. 3C-3G). FIGS. 4 and 5 provide further evidence for GUS activity in developing ears. The data show the pattern established at 5 days before pollination persists up to 2 days after pollination. GUS activity becomes restricted to transmitting tissue and maternal tissue at the base of developing kernels during

seed development. GUS protein is detectable throughout ovule and kernel development, up to 20 days after pollination. FIG. 25A shows very light staining in the aerial tissue, with no GUS activity in the roots

In summary, the present invention includes expression cassettes based on the *Oryza sativa* OsMADS5 gene. These cassettes consist of the gene's promoter including the first intron, 5'-UTR, 3'-UTR and 3'-nontranscribed sequence. The cassette's design facilitates replacement of the GUS coding sequence with any gene of interest. The cassette drives gene expression primarily in maternal reproductive tissue. Within developing ears, expression localizes to the outer vasculature along the long axis of the ear, the transmitting tissue in developing florets and kernels, tissue surrounding ovules and maternal tissue at the base of developing kernels. The expression cassettes of the present invention drive gene expression from a very early point in ovule development, perhaps from shortly after differentiation.

Example 4. Construction of the OsMADS6 expression cassette

A. Cloning of the OsMADS6 5'-regulatory sequence

Used high-fidelity PCR to amplify the OsMADS6 5'-regulatory sequence from rice genomic DNA (gDNA). The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS#6-P1 5'-ctaggacgatggtgtgatgtgggaacacg-3' (SEQ ID NO 470), 1 μL 20 μM oligonucleotide primer OsMADS#6-P2 5'-gtacctttctaaagtctttgttatgctgcac-3' (SEQ ID NO 471), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. Cloned the 4.5 kb OsMADS6-5'-gDNA DNA product with the TOPO XL PCR cloning kit. pCR-XL-TOPO-OsMADS6-5'-gDNA recombinants were identified by digesting 5 μL pCR-XL-TOPO-OsMADS6-5'-gDNA miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then the products were resolved on 1% TAE agarose. The positive pCR-XL-TOPO-OsMADS6-5'-gDNA clones were sequenced.

B. Cloning of the OsMADS6 3'-regulatory sequence

The OsMADS6 3'-regulatory sequence from rice genomic DNA (gDNA) was amplified using high-fidelity PCR. The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS#6-T1 5'-gctaagcagccatcgatcagctgtcag-3' (SEQ ID NO 470), 1 μL 20 μM oligonucleotide primer OsMADS#6-T2 5'-gatgccattgtgtaatgaatggaggagagc-3' (SEQ ID NO 471), 1 μL 10X Expand

High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 60°C for 30 seconds, 68°C for 6 minutes) followed by 68°C for 15 minutes. The 1.2 kb DNA product was cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-II-Blunt-OsMADS6-3'-gDNA recombinants were identified by digesting 5 μL pCR-II-Blunt-OsMADS6-3'-gDNA miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-II-Blunt-OsMADS6-3'-gDNA clones were sequenced.

C. Construction of the OsMADS6 5'- regulatory sequence

The OsMADS6 5'- regulatory sequence for the expression cassette was made in several steps. The 3'-half (OsMADS-6Pb, about 2.96 kb) was produced by high-fidelity PCR from the OsMADS6 5'-gene regulatory sequence clone, above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS6-5'-gDNA miniprep DNA, 200 μM dNTPs, 20 μM oligonucleotide primer OsMADS6-P3b 5'-cgagtcgacgaggggaagagttgagctgag -3' (SEQ ID NO 474), 20 μM oligonucleotide primer OsMADS6-P4c 5'-gactccatggtggttatgctgcacaaaaatg-3' (SEQ ID NO 475), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252) in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The DNA product was cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-TOPO-OsMADS6-Pb recombinants were identified by digesting 5 μL pCR-Blunt-II-TOPO-OsMADS6-Pb miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt-II-TOPO-OsMADS6-Pb recombinants were sequenced.

The 5'-half (OsMADS-6Pa, about 1.5 kb) was produced by high-fidelity PCR from the pCR-XL-TOPO-OsMADS6-5'-gDNA clone, above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS6-5'-gDNA miniprep DNA, 200 μM dNTPs, 20 μM oligonucleotide primer OsMADS6-C1b 5'-cagtgcatgcggaccgctaggacgatggtgtgatgtg-3' (SEQ ID NO 476), 20 μM oligonucleotide primer OsMADS6-Paa 5'-cctcgtcgactcgcccgatcgatcgaacg-3' (SEQ ID NO 477), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C

for 6 minutes) then 72°C for 10 minutes. The 1.5 kb OsMADS6-Pa DNA product was cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-TOPO-OsMADS6-Pa recombinants were identified by digesting 5 μL pCR-Blunt-II-TOPO-OsMADS6-Pa miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt-II-TOPO-OsMADS6-Pa recombinants were sequenced.

14 μL pCR-Blunt-II-TOPO-OsMADS6-Pb miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI. The digest was incubated at 37°C for more than 6 hours. The digested DNA was resolved on 1.0% TAE agarose and the 2.96 kb OsMADS6-Pb (SalI/NcoI) DNA band was excised, recovered and ethanol precipitated with glycogen. The OsMADS6-Pb (SalI/NcoI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901 miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The digested plasmid DNA was resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (SalI/NcoI/CIP) band was excised, recovered and ethanol precipitated with glycogen. The pNOV6901 (SalI/NcoI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL OsMADS6-Pb (SalI/NcoI) was ligated to 4.0 μL pNOV6901 (SalI/NcoI/CIP) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The ligation mixture was incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The recombinants were verified by digesting 2 μL pNOV6901-OsMADS6-Pb miniprep DNA with 0.5 μL SalI, 0.5 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS6-Pb recombinants were sequenced.

2 μg pNOV6901-OsMADS6-Pb miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL SphI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pCR-Blunt-II-TOPO-OsMADS6-Pa miniprep DNA was digested in a 20 μL reaction containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL SphI. The digest was incubated at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6901-OsMADS6-Pb (SalI/SphI/CIP) and pCR-Blunt-II-TOPO-OsMADS6-Pa (SalI/SphI), were resolved on 1.0% TAE agarose and the 7.7 kb pNOV6901-OsMADS6-Pb (SalI/SphI/CIP) and the 1.5 kb OsMADS6-Pa (SalI/SphI) bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901-OsMADS6-Pb (SalI/SphI/CIP) and OsMADS6-Pa (SalI/SphI) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and each resuspended in 5 μL ddH_2O.

4.0 μL pNOV6901-OsMADS6-Pb (SalI/SphI/CIP) was ligated to 4.0 μL OsMADS6-Pa (SalI/SphI) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The reaction mixture was incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS6P recombinants were verified by digesting 7.5 μL pNOV6901-OsMADS6P miniprep DNA with 0.5 μL SphI, 0.5 μL NcoI in 10 μL reactions containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. Digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS6P recombinants were sequenced.

D. Construction of the OsMADS6 3'- regulatory sequence

The OsMADS-6 3'-regulatory sequence for the expression cassette, about 1.3 kb, was produced by high-fidelity PCR from the pCR-II-Blunt-OsMADS6-3'-gDNA clone, above. The reaction mixture consisted of 1 μL pCR-II-Blunt-OsMADS6-3'-gDNA miniprep DNA, 200 μM dNTPs, 20 μM oligonucleotide primer OsMADS6-C4b 5'-acgtgagctcgctaagcagccatcgatcag-3' (SEQ ID NO 478), 20 μM oligonucleotide primer OsMADS6-C2 5'-actgcggaccgatgccattgtgtaatgaatgg-3' (SEQ ID NO 479), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase in a final volume of 50 μL. The

thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The 1.3 kb OsMADS6T DNA product was recovered and ethanol precipitated with glycogen. Recovered the OsMADS6T DNA by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The OsMADS6T DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, and 2 μL SmaI. The digest was incubated at 37°C for more than 6 hours. The OsMADS6T (SmaI) DNA was resolved on 1.0% TAE agarose and the 1.3 kb OsMADS6T (SmaI) band was excised, recovered and ethanol precipitated with glycogen carrier. The OsMADS6T (SmaI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901-OsMADS6P miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL SmaI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901-OsMADS6P (SmaI/CIP) DNA was resolved on 1.0% TAE agarose and the 9.7 kb pNOV6901-OsMADS6P (SmaI/CIP) band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901-OsMADS6P (SmaI/CIP) DNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL pNOV6901-OsMADS6P (SmaI/CIP) was ligated to 4.0 μL OsMADS6T (SmaI) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The reaction mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The recombinants were verified by digesting 2 μL pNOV6901-OsMADS6P/OsMADS6T miniprep DNA with 1.0 μL RsrII in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS6P/OsMADS6T recombinants were sequenced. Designated the vector pNOV6901-OsMADS6P/OsMADS6T. The plasmid's QC number is 11082. 11082 contains the OsMADS6 expression cassette depicted by SEQ ID NO. 537.

E. Mobilization of the OsMADS6 GUS expression cassette into pNOV6900

2 μg pNOV6900 was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pNOV6901-OsMADS6P/OsMADS6T miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours.

The pNOV6900 (RsrII/CIP) and the pNOV6901-OsMADS6P/OsMADS6T (RsrII) plasmid DNAs were resolved on 1.0% TAE agarose, and the 9.2 kb pNOV6900 (RsrII/CIP) and the 8.0 kb pNOV6901-OsMADS6P/OsMADS6T (RsrII) bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6900 (RsrII/CIP) and pNOV6901-OsMADS6P/OsMADS6T (RsrII) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O each.

4.0 μL pNOV6900 (RsrII/CIP) was ligated to 4.0 μL pNOV6901-OsMADS6P/OsMADS6T (RsrII) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6900-pNOV6901-OsMADS6P/OsMADS6T recombinants were verified by digesting 2 μL miniprep DNA with 1.0 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6900-pNOV6901-OsMADS6P/OsMADS6T recombinants were sequenced. The finished clone was designated pNOV6907. The plasmid's QC number is 11083.

The engineered alterations in the 5'- regulatory sequence derived from the OsMADS6 gene include introduction of an SphI site followed by an RsrII site at the 5'-end of OsMADS6P, elimination of the natural translation start codon in OsMADS6P, elimination of undesired open reading frames in the new 5'-untranslated leader sequence transcribed from OsMADS6P, insertion of a Kozak sequence upstream of the new translation start codon in OsMADS6P and insertion of the new translation start codon downstream of the intron1/exon2 junction in OsMADS6P as an NcoI site. The engineered alterations in the 3'-gene regulatory sequence derived from the OsMADS6 gene include introduction of a SacI site at the 5'-terminus of OsMADS6T and introduction of an RsrII site at the 3'-terminus of

OsMADS6T. In this configuration the GUS coding sequence can be replaced with any gene of interest flanked by NcoI/NotI or NcoI/SacI restriction sites. The complete cassette is mobilized, as an RsrII fragment, to the binary vector pNOV6900.

The cassette was transformed into A188 X HyII maize and Kaybonnet rice using standard agrobacterium mediated methodology.

GUS Expression in T0 maize

One hundred-two T0 transgenic maize lines were generated. Tassel spikelets were histochemically screened for GUS activity. Sixty-four events were positive for GUS activity in the tassel glume, and some also stained positive at the spikelet base. Fifty-six also showed GUS expression in leaf punches. Ears from several plants were sacrificed to examine GUS expression in developing florets. GUS activity localizes primarily to vascular bundles in developing ears, which appears connected to transmitting tissue in each floret. These data indicate the cassette drives GUS expression primarily in female reproductive tissue.

GUS expression in T0 rice

Forty-one T0 rice lines containing pNOV6907 were generated. Twenty independent transformants were histochemically stained for GUS expression. Light to strong GUS activity was detected in leaf tissue. In most events, activity in spikelets localized to glumes. Staining intensity varied significantly. Seed were collected for each line, but were not further analyzed.

GUS Expression in T1 maize

T1 progeny from two independent transformants were sown and analyzed in detail for GUS expression. FIGS. 8A-8C show there was no detectable GUS expression in silk, leaf and tassel. This indicates tassel and leaf expression observed in T0 plants may result from tissue culture associated with the transformation process. Dissected organs from T1 tassel spikelets had no apparent GUS activity (data not shown). FIGS. 8D-8E show GUS activity in the ear node and the developing ear shoot. FIG. 8D shows residual GUS activity in the central pith, and most activity in the developing ear shoot. Ear activity is confined to the node, the outer whorls and the central region. The pith beneath the ear node has no detectable GUS activity. FIG. 9 shows GUS activity in ears from 8 to 2 days prior to pollination. As in T0 ears, activity is confined to the vaculature, florets and transmitting tissue. Post-pollination GUS activity remains confined to the same tissues (FIG. 10). Activity in developing kernels appears restricted to maternal tissue. FIG. 11 shows this pattern persists through kernel development. No activity is detected in the endosperm or developing embryo, it localizes to the placental, funicular and hilar regions of developing kernels. GUS protein is detectable

throughout ovule and kernel development, up to 20 days after pollination. These data support the OsMADS6 cassette as a very good candidate for trait expression in developing florets and kernels. When driven by the OsMADS6-based expression cassette, genes that facilitate phloem unloading such as invertase or a sucrose transporter should prove effective in supporting early ear development by increasing sink strength. FIG. 25B shows very light or no staining in the aerial tissue, with no GUS activity in the roots

One embodiment of the invention is an expression cassette based on the *Oryza sativa* OsMADS6 gene. The expression cassette consists of the gene's promoter including the first intron, 5'-UTR, 3'-UTR and 3'-nontranscribed sequence. These components were assembled into a GUS expression cassette and tested in transgenic plants. The cassette's design facilitates replacement of the GUS coding sequence with any gene of interest. The expression cassette drives gene expression primarily in maternal reproductive tissue. Within developing ears, expression localizes to florets, maternal components of developing kernels, the placental or transmitting tissue and vasculature. The expression cassettes of the present invention further drive gene expression from a very early point in ovule development, perhaps from shortly after differentiation.

Example 5: Construction of the OsMADS8 expression cassette

A. Cloning of the OsMADS8 5'-regulatory sequence

The OsMADS8 5'-regulatory sequence from rice genomic DNA (gDNA) was amplified using high-fidelity PCR. The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS8.P1 5'-ggtatctttccaaagttctggtcatgctgc-3' (SEQ ID NO 522), 1 μL 20 μM oligonucleotide primer OsMADS8.P2 5'-ccatttttttgcgaaatgccaaatcctggc-3' (SEQ ID NO 523), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. The 5.2 kb OsMADS8-5'-gDNA DNA product was cloned with the TOPO XL PCR cloning kit. The pCR-XL-TOPO-OsMADS8-5'-gDNA was identified by digesting 5 μL pCR-XL-TOPO-OsMADS8-5'-gDNA miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-XL-TOPO-OsMADS8-5'-gDNA clones were sequenced.

B. Cloning of the OsMADS8 3'-regulatory sequence

The OsMADS8 3'-regulatory sequence from rice genomic DNA (gDNA) was amplified using high-fidelity PCR. The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS8.T1 5'-acgtgagctcactcctgaaggccgatgcgacaacc-3' (SEQ ID NO 480), 1 μL 20 μM oligonucleotide primer OsMADS8.T2 5'-agtcatcgatcatgacaaaatatcatgtttatttcgagg-3' (SEQ ID NO 481), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 60°C for 30 seconds, 68°C for 6 minutes) followed by 68°C for 15 minutes. Cloned the 2.04 kb OsMADS8-3'-gDNA DNA product with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-OsMADS8-3'-gDNA recombinants were identified by digesting 5 μL miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt-II-OsMADS8-3'-gDNA clones were sequenced.

C. Construction of the OsMADS8 5'- regulatory sequence

The OsMADS8 5'- regulatory sequence for the expression cassette was made in several steps. The 3'-half (OsMADS-8Pb, about 2.8 kb) was produced by high-fidelity PCR from pCR-XL-TOPO-OsMADS8-5'-gDNA, above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS8-5'-gDNA miniprep DNA, 200 μM dNTP mixture, 20 μM oligonucleotide primer OsMADS8-Pcc 5'-atcgccatggtggtcaagctgcaagtttcaaaaacac-3' (SEQ ID NO 482), 20 μM oligonucleotide primer OsMADS8-C3 5'-acgtgtcgacgagagggagggtgga-3' (SEQ ID NO 483), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The 2.8 kb OsMADS-8Pb DNA product was cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-TOPO-OsMADS-8Pb recombinants were identified by digesting 5 μL pCR-Blunt-II-TOPO-OsMADS-8Pb miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. Digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt-II-TOPO-OsMADS-8Pb clones were sequenced.

The 5'-half (OsMADS-8Pa, about 2.4 kb) was produced by high-fidelity PCR from pCR-XL-TOPO-OsMADS8-5'-gDNA, above. The reaction mixture consisted of 1 μL pCR-XL-TOPO-OsMADS8-5'-gDNA miniprep DNA, 200 μM dNTP mixture, 20 μM oligonucleotide primer OsMADS8-C5b 5'-tcctcctcctcctccacctcacct-3' (SEQ ID NO 484),

20 μM oligonucleotide primer OsMADS8-C1b 5'-aactaaatcgcctgcaggcggaccgttttttgcgaaatgcc-3' (SEQ ID NO 485), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase in a final volume of 50 μL. The thermocycling program was at 95°C for 30 seconds then 40 cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The 2.4 kb OsMADS-8Pa DNA product was cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-TOPO-OsMADS-8Pa recombinants were identified by digesting 5 μL pCR-Blunt-II-TOPO-OsMADS-8Pa miniprep DNA with EcoRI in a 20 μL reaction mixture containing 2 μg BSA and 2 μL 10X restriction endonuclease buffer. Digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pCR-Blunt-II-TOPO-OsMADS-8Pb clones were sequenced.

14 μL pCR-Blunt-II-TOPO-OsMADS-8Pb miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI. The digest was incubated at 37°C for more than 6 hours. The pCR-Blunt-II-TOPO-OsMADS-8Pb (SalI/NcoI) DNA was resolved on 1.0% TAE agarose and the 2.96 kb OsMADS-8Pb (SalI/NcoI) band was excised, recovered and ethanol precipitated with glycogen carrier. OsMADS-8Pb (SalI/NcoI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901 miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL SalI and 1 μL NcoI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901 (SalI/NcoI/CIP) plasmid DNA was resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (SalI/NcoI/CIP) DNA band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901 (SalI/NcoI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL OsMADS-8Pb (SalI/NcoI) was ligated to 4.0 μL pNOV6901 (SalI/NcoI/CIP) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL), which was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS-8Pb recombinants were verified by digesting 2 μL pNOV6901-OsMADS-8Pb miniprep DNA with 0.5 μL SalI, 0.5 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X

restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS-8Pb recombinants were sequenced.

An SbfI restriction site was introduced to pNOV6901-OsMADS-8Pb by ligating Synthetic Adapter III to the construct. Synthetic Adapter III was made by combining 40 µL of 50 µM oligonucleotide 8PA-1 5'-Pggagatcggg-3' (SEQ ID NO 486), 40 µL of 50 µM oligonucleotide 8PA-2 5'-Ptcgacccgatcacc-3' (SEQ ID NO 487)--where P is a 5'-phosphate group--in a 100 µL mixture that is 25 mM in Tris-HCl (pH 8.0) and 10 mM in $MgCl_2$. The mixture was boiled for 5 minutes, removed from heat and naturally cooled to room temperature (about 60 minutes). This yielded a 20 µM Synthetic Adapter III mixture.

pNOV6901-OsMADS-8Pb was prepared by digesting 14 µL pNOV6901-OsMADS-8Pb miniprep DNA with 2 µL SalI in a 20 µL reaction mixture containing 2 µg BSA and 2 µL 10X restriction endonuclease buffer. The digest was incubated at 37°C for 6 hours, then at 70°C for 20 minutes. Then 1 µL of the appropriate 10X restriction endonuclease buffer, 1 µL 1 Unit/µL CIP and 8 µL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901-OsMADS-8Pb (SalI/CIP) DNA was resolved on 1% TAE agarose, excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901-OsMADS-8Pb (SalI/CIP) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 µL ddH_2O.

4.5 µL Synthetic Adapter III mixture was ligated to 2.5 µL pNOV6901-OsMADS-8Pb (SalI/CIP) in a 10 µL ligation mixture containing 1 µL 10X T4 DNA ligase buffer and 1 µL T4 DNA ligase (400 U/µL), which was incubated more than 8 hours at 16°C. 4 µL of ligation mixture was transformed into 50 µL XL-1 supercompetent cells (Stratagene, Cat. No.200236). The pNOV6901-OsMADS-8Pb-SbfI recombinants were verified by digesting 7.5 µL pNOV6901-OsMADS-8Pb-SbfI miniprep DNA in a 10 µL reaction mixture containing 1 µg BSA, 1 µL 10X restriction endonuclease buffer and 1 µL SalI. The digests were incubated at 37°C for 2 hours then resolved on 1.0% TAE agarose. The pNOV6901-OsMADS-8Pb-SbfI recombinants that lost the SalI restriction site were digested with SbfI in a 10 µL reaction mixture containing 1 µg BSA, 1 µL 10X SEBuffer Y restriction endonuclease buffer and 1 µL SbfI (New England Biolabs). The digests were incubated at 37°C for 2 hours then resolved on 1.0% TAE agarose. Positive pNOV6901-OsMADS-8Pb-SbfI recombinants were sequenced.

2 μg pNOV6901-OsMADS-8Pb-SbfI miniprep DNA was disgested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL SbfI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pCR-Blunt-II-TOPO-OsMADS-8Pa miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL SbfI. The digest was incubated at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6901-OsMADS-8Pb-SbfI (SbfI/CIP) and pCR-Blunt-II-TOPO-OsMADS-8Pa (SbfI), were resolved on 1.0% TAE agarose and the 7.5 kb pNOV6901-OsMADS-8Pb (SbfI/CIP) and the 2.4 kb OsMADS-8Pa (SbfI) bands were excised, recovered and ethanol precipitated with glycogen carrier. The DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and each resuspended in 5 μL ddH_2O.

4.0 μL pNOV6901-OsMADS-8Pb (SbfI/CIP) was ligated to 4.0 μL OsMADS-8Pa (SbfI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL), which was incubated for more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS-8P recombinants were verified by digesting 7.5 μL pNOV6901-OsMADS-8P miniprep DNA with 0.5 μL SbfI, 0.5 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS-8P recombinants were sequenced.

D. Construction of the OsMADS8 3'- regulatory sequence

The OsMADS-8 3'-regulatory sequence for the expression cassette, about 2.1 kb, was produced by high-fidelity PCR from the pCR-Blunt-II-OsMADS8-3'-gDNA clone, above. The reaction mixture consisted of 1 μL pCR-Blunt-II-OsMADS8-3'-gDNA miniprep DNA, 200 μM dNTP mixture, 20 μM oligonucleotide primer OsMADS8-C2 5'-acgtcccgggcggaccgagtcatcgatcatgac-3' (SEQ ID NO 488), 20 μM oligonucleotide primer OsMADS8-C4 5'-tcgagcggccgcaggccgatgcgacaaccaataaaaac-3' (SEQ ID NO 489), 5 μL 10X cloned Pfu buffer and 2.5 Units of Pfuturbo DNA polymerase (Stratagene, Cat. No. 600252) in a final volume of 50 μL. The thermocycling program was 95°C for 30 seconds then 40

cycles of (95°C for 10 seconds, 50°C for 60 seconds, 72°C for 6 minutes) then 72°C for 10 minutes. The OsMADS-8T DNA product was recovered using the QIAquick PCR purification kit. The recovered OsMADS-8T DNA was ethanol precipitated with glycogen carrier. The OsMADS-8T DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The OsMADS-8T DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL NotI and 1 μL XmaI. The digest was incubated at 37°C for more than 6 hours. The OsMADS-8T (NotI/XmaI) DNA was resolved on 1.0% TAE agarose, excised, recovered and ethanol precipitated with glycogen carrier. The OsMADS-8T (NotI/XmaI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901-OsMADS-8P miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL NotI and 1 μL XmaI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901-OsMADS-8P (NotI/XmaI/CIP) plasmid DNA was resolved on 1.0% TAE agarose and the 9.9 kb pNOV6901-OsMADS-8P band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901-OsMADS-8P (NotI/XmaI/CIP) DNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL of the pNOV6901-OsMADS-8P (NotI/XmaI/CIP) was ligated to 4.0 μL OsMADS-8T (NotI/XmaI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL), which was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. pNOV6901-OsMADS-8P/OsMADS-8T recombinants were verified by digesting 2 μL pNOV6901-OsMADS-8P/OsMADS-8T miniprep DNA with 1.0 μL RsrII in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS-8P/OsMADS-8T recombinants were sequenced. The finished clone was designated pNOV6901-OsMADS-8P/OsMADS-8T. The plasmid's QC number is 11170. 11170 contains the complete OSMADS8 expression cassette depicted by SEQ ID NO. 538.

E. Mobilization of the OsMADS8 GUS expression cassette into pNOV6900

2 μg pNOV6900 was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pNOV6901-OsMADS-8P/OsMADS-8T miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated the reaction at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6900 (RsrII/CIP) and pNOV6901-OsMADS-8P/OsMADS-8T (RsrII), were resolved on 1.0% TAE agarose and the 9.2 kb pNOV6900 (RsrII/CIP) and the 9.5 kb pNOV6901-OsMADS-8P/OsMADS-8T (RsrII) bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6900 (RsrII/CIP) and the pNOV6901-OsMADS-8P/OsMADS-8T (RsrII) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and each resuspended in 5 μL ddH_2O.

4.0 μL of pNOV6900 (RsrII/CIP) was ligated to 4.0 μL pNOV6901-OsMADS-8P/OsMADS-8T (RsrII) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6900-pNOV6901-OsMADS-8P/OsMADS-8T recombinants were verified by digesting 7.5 μL pNOV6900-pNOV6901-OsMADS-8P/OsMADS-8T miniprep DNA with 1.0 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6900-pNOV6901-OsMADS-8P/OsMADS-8T recombinants were sequenced. The finished clone was designated pNOV6909. The plasmid's QC number is 11171.

The engineered alterations in the 5'- regulatory sequence derived from the OsMADS8 gene include introduction of an SbfI site followed by an RsrII site at the 5'-end of OsMADS8P, elimination of the natural translation start codon in OsMADS8P, elimination of undesired open reading frames in the new 5'-untranslated leader sequence transcribed from OsMADS8P, insertion of a Kozak sequence upstream of the new translation start codon in OsMADS8P and insertion of the new translation start codon downstream of the intron1/exon2 junction in OsMADS8P as an NcoI site. The engineered alterations in the 3'-

gene regulatory sequence derived from the OsMADS8 gene include introduction of a NotI site at the 5'-terminus of OsMADS8T and introduction of an RsrII site at the 3'-terminus of OsMADS8T. In this configuration the GUS coding sequence can be replaced with any gene of interest flanked by NcoI/NotI restriction sites. The complete cassette is mobilized, as an RsrII fragment, to the binary vector pNOV6900.

The cassette was transformed into A188 X HyII maize and Kaybonnet rice using standard agrobacterium mediated methodology.

GUS Expression in T0 maize

Forty T0 transgenic maize lines were generated. Tassel spikelets were histochemically screened for GUS activity. Twenty-nine events were positive for GUS activity. Thirteen also showed GUS expression in leaf punches. In general, the pattern revealed detectable GUS activity in tassels and little of no activity in leaf punches. The ear from one plant reflecting this pattern was sacrificed to examine GUS expression. Strong GUS expression is evident throughout the ear. These data indicate the cassette drives GUS expression primarily in female reproductive tissue.

GUS expression in T0 rice

Of thirty-six T0 rice lines containing pNOV6909, thirty-two independent transformants were histochemically stained for GUS expression. No GUS activity was detected in leaf tissue. In most events, activity localized to panicles and could be seen in anthers or the carpel base. Staining intensity varied significantly.

GUS Expression in T1 maize

T1 progeny from four independent transformants were sown and analyzed in detail for GUS expression. FIGS. 14A-14D show there was no detectable GUS expression in tassels, leaf tissue, developing silk or shoots. This indicates tassel and leaf expression observed in T0 plants may result from tissue culture associated with the transformation process. Dissected organs from T1 tassels indicated no apparent GUS expression (data not shown). FIG. 14D shows no GUS activity in the node attached to the developing ear shoot. The node below this also has no detectable GUS activity, but there is distinct activity in florets on the arrested ear (FIG. 14E). The expression cassette is activated very early in floret development. FIGS. 14F, 14G, 15A and 15B show GUS activity in the central pith and florets of the developing ear before pollination. This pattern persists from 5 days before pollination to one day before pollination. Central pith expression persists up to 1 day before pollination, after which GUS activity is no longer detected in this zone (FIGS. 15 and 16). FIGS. 15D-15G indicate some GUS activity in the ear's outer vasculature and the floret's transmitting tissue from the day of

pollination to 1 day after pollination. Afterwards, GUS activity is detected only in the maternal components of developing kernels. Data shown in FIGS. 15 and 16 cover development from 1 day before pollination to 20 days after pollination. GUS protein is detectable throughout ovule and kernel development, up to 20 days after pollination. These data support the OsMADS8 cassette as a very good candidate for trait expression in developing florets. When driven by the OsMADS8-based expression cassette, genes that facilitate phloem unloading such as invertase or a sucrose transporter should prove effective in supporting early ear development by increasing sink strength. FIG. 25C shows very light staining in the aerial tissue, with no GUS activity in the roots

In summary, the present invention includes expression cassettes based on the *Oryza sativa* OsMADS8 gene. It consists of the gene's promoter including the first intron, 5'-UTR, 3'-UTR and 3'-nontranscribed sequence. The cassette's design facilitates replacement of the GUS coding sequence with any gene of interest. The cassette targets gene expression primarily to developing florets and kernels, and the placental tissue beneath each floret. Post-fertilization, expression is detected in the aleurone, hilar region and pedicel. Developmentally, the cassette should drive gene expression from a very early point in ovule development, perhaps from shortly after differentiation.

Example 6: Construction of the OsMADS13 expression cassette

A. Cloning of the OsMADS13 5'-regulatory sequence

The OsMADS13 5'-regulatory sequence from rice genomic DNA (gDNA) was amplified using high-fidelity PCR. The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS13-C1 5'-gactgcatgcggaccgttccaaaattaagcacacacatttg-3' (SEQ ID NO 490), 1 μL 20 μM oligonucleotide primer OsMADS13-C2 5'-gactccatggcttcttgctctcaactgatcaac-3' (SEQ ID NO 491), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 68°C for 7.5 minutes) followed by 68°C for 10 minutes. The 1.9 kb OsMADS13-5'-gDNA DNA fragment was recovered and ethanol precipitated with glycogen carrier. The OsMADS13-5'-gDNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O. The OsMADS13-5'-gDNA fragment was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL NcoI. The digest was incubated at 37°C for more than 6 hours. The digest was resolved on 1.0% TAE agarose and the 1.9 kb OsMADS13-5'-

gDNA (NcoI) DNA band was excised, recovered and ethanol precipitated with glycogen carrier. The OsMADS13-5'-gDNA (NcoI) DNA as recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901 miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL SphI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901 (SphI/blunt) DNA was resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (SphI/blunt) band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901 (SphI/blunt) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O.

pNOV6901 (SphI/blunt) miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL NcoI. The digest was incubated at 37°C for more than 6 hours. The pNOV6901 (SphI/blunt/NcoI) plasmid DNA was resolved on 1.0% TAE agarose and the 4.7 kb pNOV6901 (SphI/blunt/NcoI) band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901 (SphI/blunt/NcoI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL OsMADS13-5'-gDNA (NcoI) was ligated to 4.0 μL pNOV6901 (SphI/blunt/NcoI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of the ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS13P recombinants were verified by digesting 2 μL pNOV6901-OsMADS13P miniprep DNA with 0.5 μL XhoI, 0.5 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS13P recombinants were sequenced.

B. Cloning of the OsMADS13 3'-regulatory sequence

The OsMADS13 3'-regulatory sequence from rice genomic DNA (gDNA) was amplified using high-fidelity PCR. The 50 μL reaction mixture consisted of 100 ng rice gDNA, 200 μM dNTPs, 1 μL 20 μM oligonucleotide primer OsMADS13-C3 5'-

tcgagcggccgctgacatggatatgatgatcag-3' (SEQ ID NO 492), 1 μL 20 μM oligonucleotide primer OsMADS13-C4 5'-acgtatcgatcggaccgcaacgcacgggcacccaac-3' (SEQ ID NO 493), 1 μL 10X Expand High Fidelity buffer and 1 μL Expand High Fidelity polymerase. The thermocycling program was at 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 60°C for 30 seconds, 68°C for 6 minutes) followed by 68°C for 15 minutes. The 1.2 kb OsMADS13-3'-gDNA DNA fragment was recovered and ethanol precipitated with glycogen carrier. The OsMADS13-3'-gDNA DNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 14 μL ddH_2O.

The OsMADS13-3'-gDNA fragment was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL NotI. The digest was incubated at 37°C for more than 6 hours then resolved on 1.0% TAE agarose and the 1.2 kb OsMADS13-3'-gDNA (NotI) DNA band was excised, recovered and ethanol precipitated with glycogen carrier. The OsMADS13-3'-gDNA (NotI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg pNOV6901-OsMADS13P miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL NotI and 1 μL SmaI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The pNOV6901-OsMADS13P (NotI/SmaI/CIP) DNA was resolved on 1.0% TAE agarose and the 6.6 kb band was excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6901-OsMADS13P (NotI/SmaI/CIP) DNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended each in 5 μL ddH_2O.

4.0 μL pNOV6901-OsMADS13P (NotI/SmaI/CIP) was ligated to 4.0 μL OsMADS13-3'-gDNA (NotI) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of the ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6901-OsMADS13P/OsMADS13T recombinants were verified by digesting 7.5 μL pNOV6901-OsMADS13P/OsMADS13T miniprep DNA with 1.0 μL

NotI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. Digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6901-OsMADS13P/OsMADS13T recombinants were sequenced. The finished clone was designated pNOV6904, which is also the plasmid's QC number. pNOV6904 contains the complete OSMAD13 expression cassette depicted by SEQ ID NO. 539.

C. Mobilization of the OsMADS13 GUS expression cassette into pNOV6900

2 μg pNOV6900 was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pNOV6901-OsMADS13P/OsMADS13T miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated the reaction at 37°C for more than 6 hours.

The digested plasmid DNAs, pNOV6900 (RsrII/CIP) and pNOV6901-OsMADS13P/OsMADS13T (RsrII), were resolved on 1.0% TAE agarose and the 9.2 kb pNOV6900 (RsrII/CIP) and the 5.3 kb pNOV6901-OsMADS13P/OsMADS13T (RsrII) bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6900 (RsrII/CIP) and pNOV6901-OsMADS13P/OsMADS13T (RsrII) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended each in 5 μL ddH_2O.

4.0 μL of pNOV6900 (RsrII/CIP) was ligated to 4.0 μL pNOV6901-OsMADS13P/OsMADS13T (RsrII) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6900-pNOV6901-OsMADS13P/OsMADS13T recombinants were verified by digesting 7.5 μL pNOV6900-pNOV6901-OsMADS13P/OsMADS13T miniprep DNA with 1.0 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6900-pNOV6901-OsMADS13P/OsMADS13T recombinants were sequenced. The finished clone was designated pNOV6905, which is also the plasmid's QC number.

The engineered alterations in the 5'-gene regulatory sequence derived from the OsMADS13 gene include introduction of an RsrII site at the 5'-end of OsMADS13P, insertion of a Kozak sequence upstream of the natural OsMADS13P translation start codon and modification of the natural OsMADS13P translation start codon so that it is contained within an NcoI site. The engineered alterations in the 3'-gene regulatory sequence derived from the OsMADS13 gene include introduction of a NotI site at the 5'-terminus of OsMADS13T and introduction of an RsrII site at the 3'-terminus of OsMADS13T. In this configuration the GUS coding sequence can be replaced with any gene of interest flanked by NcoI/NotI restriction sites. The complete cassette is mobilized, as an RsrII fragment, to the binary vector pNOV6900.

The cassette was transformed into A188 X HyII maize and Kaybonnet rice using standard agrobacterium mediated methodology.

GUS Expression in T0 maize

Sixty-seven T0 transgenic maize lines were generated. Tassel spikelets were histochemically screened for GUS expression. Fifty-six were positive for GUS activity. Thirty-five also showed GUS expression in leaf punches. Ten lines had no detectable GUS activity in tassels or leaf punches.

Two T0 lines were selected to analyze GUS expression in developing ears. Both lines had a GUS signal in tassel spikelets and no GUS signal in leaf punches. Ears were harvested approximately 7 days before silking and histochemically stained for GUS expression. Whole sections showed a strong GUS signal only in developing florets, whereas GUS activity is absent in surrounding ear tissue. These data indicate the OsMADS13 expression cassette functions to drive GUS expression in both male and female spikelets in T0 maize transformants.

GUS expression in T0 rice

Thirty-three T0 rice lines were produced. Fourteen independent transformants were histochemically stained for GUS expression GUS activity was primarily detected in spikelets. Some plants also had GUS activity in leaf tissue.

GUS Expression in T1 maize

T1 progeny from three independent transformants were sown and analyzed in detail for GUS expression. FIGS. 20A-20C and FIGS. 21A-21C show there was no detectable GUS expression in leaf tissue, developing silk or tassels. This indicates tassel expression observed in T0 plants may result from tissue culture associated with the transformation process. Dissected organs from T1 tassels indicated no apparent GUS expression (data not shown).

FIGS. 20D and 20E show GUS activity in the developing ear harvested about 5 days before pollination. The longitudinal section showed expression localized to developing ovules and transmitting or placental tissue. The cross section supports this and provides further evidence for expression in ear vasculature. FIG. 21 summarizes observed GUS expression in a second transgenic event. It also localizes to zones where ovules will likely develop. FIG. 22 demonstrates expression localizes to vasculature supplying the developing ear.

These data support the OsMADS13 cassette as a very good candidate for trait expression in developing ovules. When driven by the OsMADS13-based expression cassette, genes that facilitate phloem unloading such as invertase or a sucrose transporter should prove effective in supporting early ear development by increasing sink strength.

FIGS. 23 and 24 demonstrate the cassette continues to function post-fertilization. The observed GUS expression pattern at 4 and 6 days after pollination (FIG. 23). Late in kernel development (21 days after pollination) GUS expression remains localized to the pedicel and hilar regions (FIG. 24). It also appears in the aleurone. GUS protein is detectable throughout ovule and kernel development, up to 21 days after pollination. FIG. 25D shows very light staining in the aerial tissue, with no GUS activity in the roots.

The present invention includes an expression cassette based on the *Oryza sativa* OsMADS13 gene. The expression cassette includes the gene's promoter, including the first intron and the 5'-UTR, the 3'-UTR and the 3'-nontranscribed sequence. These components were assembled into a GUS expression cassette and tested in transgenic plants. The cassette's design facilitates replacement of the GUS coding sequence with any gene of interest. The cassette will target gene expression to the vasculature within the placental tissue below the floret of developing ear spikelets. Post-fertilization, expression is also expected in the aleurone, hilar region and pedicel. Developmentally, the cassette should drive gene expression from a very early point, more than 7 days before pollination, in ovule development.

Example 7: Identification of the OsT6PP cDNA sequence

The first vascular plant trehalose-6-phosphate phosphatase genes were cloned from *Arabidopsis thaliana* by complementation of a yeast *tps2* deletion mutant (Vogel et al. 1998). The genes designated *AtTPPA* and *AtTPPB* (GenBank accessions AF007778 and AF007779) were shown at that time to have trehalose-6-phosphate phosphatase activity. The AtTPPA and AtTTPB protein sequences were used in TBLASTN queries of maize and rice sequence databases. Sequence alignments organized the hits into individual genes. Three maize and three rice T6PP homologs were identified. The cDNA sequences corresponding to the

predicted protein sequence for each gene--ZmT6PP-1, -2 and -3 and OsT6PP-1, -2 and -3-- are shown in global alignment with the Arabidopsis T6PPs in Fig. 26.

The composition and method of the present invention includes using the OsMADS6 promoter operably linked to a nucleic acid molecule that when expressed in a plant cell, increases the expression of T6PP. By doing so, flux through the trehalose pathway is increased only in young developing ears where it functions to increase flux through central carbon metabolism.

The OsT6PP-3 cDNA sequence (SEQ ID NO. 531) is amplified using high-fidelity PCR. The 50 μL reaction mixture consists of 1 μL rice cDNA library (prepared from callus mRNA in Stratagene's Lambda Unizap Vector, primary library size >1 x 10^6 pfu, amplified library titer > 1 x 10^{12} pfu/mL), 200 μM dNTPs, 1 μL 20 μM of oligonucleotide primer T6PP-EC-5 (5'-catggaccatggatttgagcaatagctcac-3') SEQ ID NO. 528 and 1 μL 20 μM of oligonucleotide primer T6PP-EC-3 (5'-atcgcagagctcacactgagtgcttcttcc-3') SEQ ID NO. 529, 5 μL 10X Cloned PFU buffer and 2.5 Units of Pfuturbo DNA polymerase. The thermocycling program is 95°C for 2 minutes followed by 40 cycles of (94°C for 15 seconds, 50°C for 1 minute, 72°C for 1 minute) followed by 72°C for 10 minutes. The OsT6PP-3 product is cloned with the Zero Blunt TOPO PCR cloning kit. The pCR-Blunt-II-TOPO-OsT6PP-3 is identified by digesting 5 μL pCR-Blunt-II-TOPO-OsT6PP-3 miniprep DNA with EcoRI in a 20 μL reaction containing 2 μg BSA and 2 μL 10X EcoRI restriction endonuclease buffer. The reaction is incubated at 37°C for 2 hours and the pCR-Blunt-II-TOPO-OsT6PP-3 (EcoRI) products are resolved on 1% TAE agarose. The pCR-Blunt-II-TOPO-OsT6PP-3 clone is then sequenced. The OsT6PP-3 cDNA is flanked by NcoI/SacI restriction endonuclease sites.

To facilitate cloning into 11082, an internal NcoI site in OsT6PP was silenced using Stratagene's QuikChange Multi Site-Directed Mutagenesis Kit and the oligonucleotide primer T6PP-QC (5'-CTTTATTATGCTGGAAGTCATGGTATGGACATAATGGCACC-3') SEQ ID NO. 530.

Example 8: Construction of OsMADS6-T6PP

A. Construction of the OsMADS6-OsT6PP-3 expression cassette

The pCR-Blunt-II-TOPO-OsT6PP-3 clone (14 μL) DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL NcoI and 1 μL SacI. The digest was incubated at 37°C for more than 6 hours. The pCR-Blunt-II-TOPO-OsT6PP-3 (NcoI/SacI) DNA was resolved on 1.0% TAE agarose and the 1.3 kb

OsT6PP-3 (NcoI/SacI) band was excised, recovered and ethanol precipitated with glycogen carrier. The OsT6PP-3 (NcoI/SacI) DNA was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

2 μg 11082 miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer, 1 μL NcoI and 1 μL SacI. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. The 11082 (NcoI/SacI/CIP) DNA was resolved on 1.0% TAE agarose and the 8.1 kb 11082 (NcoI/SacI/CIP) band was excised, recovered and ethanol precipitated with glycogen carrier. The 11082 (NcoI/SacI/CIP) DNA fragment was recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O.

4.0 μL 11082 (NcoI/SacI/CIP) was ligated to 4.0 μL OsT6PP-3 (NcoI/SacI) in a 10 μL reaction mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 Units/μL). The reaction mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The recombinants were verified by digesting 7.5 μL 11082-OsT6PP-3 miniprep DNA with 1.0 μL RsrII in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive 11082-OsT6PP-3 recombinants were sequenced. The vector was designated OsMADS6-OsT6PP-Assembly and is shown in FIG. 27A.

B. Mobilization of the OsMADS6-OsT6PP-Assembly expression cassette into pNOV6900

2 μg pNOV6900 was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours, then at 70°C for 20 minutes. Then 1 μL of the appropriate 10X restriction endonuclease buffer, 1 μL 1 Unit/μL CIP and 8 μL ddH_2O were added to the reaction and it was further incubated at 37°C for 30 minutes. 2 μg pNOV6906-OsT6PP-Assembly miniprep DNA was digested in a 20 μL reaction mixture containing 2 μg BSA, 2 μL 10X restriction endonuclease buffer and 2 μL RsrII. The digest was incubated at 37°C for more than 6 hours.

The pNOV6900 (RsrII/CIP) and the pNOV6906-OsT6PP-Assembly (RsrII) plasmid DNAs were resolved on 1.0% TAE agarose, and the 9.2 kb pNOV6900 (RsrII/CIP) and the

6.8 kb pNOV6906-OsT6PP-Assembly (RsrII) bands were excised, recovered and ethanol precipitated with glycogen carrier. The pNOV6900 (RsrII/CIP) and pNOV6906-OsT6PP-Assembly (RsrII) DNA fragments were recovered by micro centrifugation, washed with 70% ethanol, dried under vacuum and resuspended in 5 μL ddH_2O each.

4.0 μL pNOV6900 (RsrII/CIP) was ligated to 4.0 μL pNOV6906-OsT6PP-Assembly (RsrII) in a 10 μL ligation mixture containing 1 μL 10X T4 DNA ligase buffer and 1 μL T4 DNA ligase (400 U/μL). The ligation mixture was incubated more than 8 hours at 16°C. 5.0 μL of ligation mixture was transformed into 50 μL Top10 competent cells. The pNOV6900-pNOV6906-OsT6PP-Assembly recombinants were verified by digesting 7.5 μL miniprep DNA with 1.0 μL NcoI in 10 μL reaction mixtures containing 1 μg BSA and 1 μL 10X restriction endonuclease buffer. The digests were incubated at 37°C for 2 hours then resolved on 1% TAE agarose. Positive pNOV6900-pNOV6906-OsT6PP-Assembly recombinants were sequenced. The finished clone was designated OsMADS6-OsT6PP-Binary and is shown in FIG 27B. The plasmid's QC number is 12194.

The OsMADS6-OsT6PP-3 expression cassette (SEQ ID NO. 533) was transformed into A188 maize using standard agrobacterium mediated methodology. Regenerated T0 shoots were screened transgene copy number and insert integrity using a Taqman™ assay. Events containing a single copy of the OsMADS6-OsT6PP-3 expression cassette and no other sequence derived from the binary vector were identified.

Expression cassette function in each transgenic Event was verified by RT-PCR. DNA-free total RNA template was prepared from 100 mg of T0 tassel tissue using the RNeasy Plant mini Kit. The RT-PCR assay was performed using the Qiagen One Step RT-PCR kit with 100 ng total RNA template, the T6PP-RTPCRF (5'-gacagaactgacgaagacgctttcaa-3') and 6906-tr (5'-ctccaacttctgacagctg-3') primers (SEQ ID NOS. 534, 535 respectively). This assay produces a transgene-specific 210 bp fragment.

Example 9: Greenhouse Growth Conditions

Corn seed is sown into 2.5 SVD pots (Classic 600, ~ 2 gallon nursery containers) in Universal mix (Sungrow Horticulture, Pine Bluff, AR). Universal mix is 45% Peat moss, 45% bark , 5% perlite, 5% vermiculite. Environmental conditions for greenhouse maize cultivation are typically 16 hour days (average light intensity 600 μmol $m^{-2}s^{-2}$), day time temperature of 80-86°F, night time temperature 70-76°F and relative humidity greater than 50%. Plants are placed on 2" platforms to avoid contact with the greenhouse floor. Plants are hand watered until daily irrigation as required, then they are placed on irrigation drip. The

irrigation schedule is 4 minutes every other day. Plants were routinely treated with insecticides to control pests.

Example 10: Evaluation of Transgenic Maize Expressing OsMADS6-OsT6PP-3 in the Greenhouse

The greenhouse evaluation is a controlled water-stress experiment that quantifies ovule viability in water-stressed and unstressed plants. Data from unstressed plants represent the genotype's potential to set seed under ideal conditions. Data from water-stressed plants quantify kernel abortion that results from drought at the time of flowering. The results of these experiments can be predictive of field performance. We used this tool to select transgenic events for field evaluations.

Transgenic maize segregating for a single copy of the OsMADS6-T6PP-3 transgene were sown as above. Taqman analysis was used to divide the progeny into homozygous or hemizygous (containing OsMADS6-OsT6PP-3) and azygous (lost the OsMADS6-OsT6PP-3) groups. These individuals were pollinated with JHAF031 maize pollen to generate hybrid seed (KPOO188RA x JHAF031) for the greenhouse experiment. The hybrid seed were sown as above. Seedlings were transferred to 600 pots, above, and maintained using standard greenhouse procedures until they reached the V6 growth stage (Ritchie et al., 1997). All plants were treated with the systemic pesticide, Marathon, to reduce susceptibility to pests. Water stress was gradually imposed, using salt as the osmoticum (Nuccio et al. 1998). The salt consisted of sodium chloride/calcium chloride at a 10:1 molar ratio, delivered in 0.5X Hoagland's Solution, to prevent sodium-induced disruption of potassium uptake. Salt concentration in the irrigant was increased from 50 mM to 100 mM to 150 mM every three days to give plants time to adjust to the salt. Plants were maintained on 150 mM salt solution through the flowering period, typically two weeks, after which pots were thoroughly flushed with water and plants were returned to normal irrigation. This protocol typically reduced kernel set by 40-60%, compared to control plants that received no salt.

Typically 15-20 seed per transgenic event were sown to generate a uniform seedling population. Plants were arranged in a complete, randomized block design consisting of six-eight replicates per treatment. Developing ears were covered with pollination bags before silk emergence. Pollen shed and silk emergence dates were recorded and individual ears were hand pollinated with donor pollen 5 days after silk emergence. Pollination bags were removed after completing all pollinations. Ears were harvested 30 days after pollinations, and dried for 4 days to 15% moisture content. Ears were shelled and the kernels were counted and weighed.

Example 11: Greenhouse Experiment

Two OsMADS6-T6PP-3 events were studied for their ability to set seed under water stress. Twenty-four hybrid seed (A188 x JHAF031) from each event were germinated. Taqman analysis was used to establish zygosity in each seedling. Hemizygotes and azygotes were analyzed using the greenhouse water stress protocol described above. In this experiment azygote plants served as the benchmark. In these greenhouse experiments, the hemizygote plants could not be distinguished from the azygote plants. On average the water stress reduced kernel set by 42%. The data in these greenhouse experiments indicate the OsMADS6-T6PP-3 expression cassette does not influence kernel set in maize in these particular greenhouse experiments and when evaluated by the above water stress protocols.

Example 12: Evaluation of Transgenic Maize Expressing OsMADS-T6PP-3 for Drought Stress tolerance in the Field

Hybrid seed were generated for each transgenic Event at the Syngenta Seeds field station in Kauai in late 2004. T1 seed obtained by selfing the T0 plant of the events was sown in four single-row plots, 12.7 feet long separated by 3 foot alleys with about 20 plants per row. Taqman analysis was used to divide the progeny into homozygous or hemizygous (containing OsMADS6-OsT6PP-3) and azygous (lost the OsMADS6-OsT6PP-3) groups. In two of the single- row plots, hemizygous and azygous plants were destroyed and homozygous plants were selfed for seed bulking and also testcrossed to NP2043BT11 and NP2044BT11. In the other two single-row plots homozygous and hemizygous plants were destroyed and azygous plants were selfed and also crossed to NP2043BT11 and NP2044BT11.. The azygous and hemizygous testcross seed of the events was used to conduct field trials.

A field evaluation was conducted to test transgene performance in a controlled drought experiment. The experiment was conducted at the Syngenta Crop Protection Facility in Visalia, California in the summer of 2004. The planting site typically gets less than 3" of rainfall during the summer. The NP2043BT11 testcross seed, generated above, was used in this study. This population also contained the BT transgene to control insect pressure. A split-block design, with watering regime as the main plots arranged in a randomised complete blocks and replicated three times, events as the subplots, and in cases where there was seed of the azgous and hemizygous hybrids, genotype as sub-sub-plots was used. Two watering regimes were attempted: water-stressed and well-watered. Each plot consisted of two-rows, 17.5 feet long planted with 40 seeds per row. Alleys between ranges were 2.5 feet. Furrow irrigation was used to water the fields. Each treatment block had a dedicated irrigation source situated at one end of the field. The replication were arranged in such a way that replication

one was closest to and replication three was the furthest from the irrigation source. After emergence, stand counts were taken and plots were thinned, as necessary, to establish field uniformity.

The well-watered block was thought to have been irrigated optimally throughout the experiment. The water-stress block was watered optimally until plants reached approximately V, at which time water was withheld. Plants were returned to optimal irrigation after 90% silk emergence.

After plants transitioned to reproductive development, the 50% pollen shed date, the 50% silk emergence date, and leaf scrolling at early-, mid- and late-flowering were recorded for each plot. Plot Barreness was recorded three weeks after silking.

Plots were combine-harvested and grain yield and grain moisture were recorded. The data from hemizygous plots were compared to azygous plots, or wild type plots where necessary, to gauge the transgene's effect on yield. Results for seven OsMADS6-T6PP-3 events are shown in Fig. 28. The data show the OsMADS6-T6PP-3 transgene has a positive effect on yield in four of the seven Events. The yield gain is evident in both unstressed and drought-stressed plots. For example in the drought-stressed treatment block the average yield for 5217 Events containing the transgene was 73 Bu/acre and the average yield for 5217 Events lacking the transgene was 54 Bu/acre. Results suggest the transgene improves kernel set by 25% in drought-stressed conditions. In the less stressed treatment block the average yield for 5217 Events containing the transgene was 132 Bu/acre and the average yield for 5217 Events lacking the transgene was 95 Bu/acre. Results suggest the transgene improves kernel set by nearly 28% in less stressed plants. The average yield calculated for each plot in the drought-stressed treatment block was 72 Bu/acre. The average yield calculated for each plot in the less stressed treatment block was 113 Bu/acre. The yield improvement due to the OsMADS6-T6PP-3 gene varies from Event to Event. It is observed in four of the seven Events tested, and is manifest in both less stressed and drought-stressed plants. Results from this field experiment demonstrate the effectiveness of the OsMADS-T6PP-3 transgene in stabilizing kernel set in drought stressed maize.

Example 13: Evaluation of Transgenic Maize Expressing OsMADS-T6PP-3 for Yield in the Field

Hybrid seed was generated for each transgenic Event at the Syngenta Seeds field station in Kauai in late 2004. T1 seed obtained by selfing the T0 plant of the events was sown in four single-row plots, 12.7 feet long separated by 3 foot alleys with about 20 plants

per row. Taqman analysis was used to divide the progeny into homozygous or hemizygous (containing OsMADS6-OsT6PP-3) and azygous (lost the OsMADS6-OsT6PP-3) groups. In two of the single- row plots, hemizygous and azygous plants were destroyed and homozygous plants were selfed for seed bulking also testcrossed to NP2043BT11 and NP2044BT11. In the other two single-row plots homozygous and hemizygous plants were destroyed and azygous plants were selfed and also crossed to NP2043BT11 and NP2044BT11. The azygous and hemizygous testcross seed of the events was used to conduct field trials.A series of yield trials were conducted in several mid-West locations to test transgene performance under conditions typically used by growers. The XPOO188RA x NP2043BT11 material, generated above, was used in late maturity zones and the XPOO188RA x JHAF431B material, generated above, was used in early maturity zones. These populations also contained the BT transgene to control insect pressure. The experimental design consisted of randomised complete blocks with three replications. Each experimental unit consisted of two-row plots, 17.5 feet long planted with 34 kernels per row. Ranges were separated by 3 foot alleys. Events for which there was seed of both the azygous and the hemizygous hybrids, randomization was restricted to keep the azygous and hemizygous hybrids of the events in neighboring plots. Most Events were evaluated in eight to nine locations. Event 5124 was evaluated in three locations. After emergence, stand counts were taken and plots were thinned, as necessary, to establish field uniformity. During the growing season plots were evaluated for intactness, greensnap, root lodging, heat units to 50% pollen shed and heat units to 50% silking.

Plots were Combine-harvested and grain yield and grain moisture were recorded. The data from hemizygous plots were compared to azygous plots, or wild type plots where necessary, to gauge the transgene's effect on yield. The data shows that the OsMADS6-T6PP-3 transgene does not significantly affect yield in this experiment. There are two factors to consider. First the standard deviation for grain yield in this experiment was 15-20% of the mean. This is not unusual. Second, growth conditions in the mid-West were ideal for maize in 2004. Depending on location yields in this experiment averaged from 90 to 130 Bu/acre. Results from this field experiment indicate the OsMADS-T6PP-3 transgene did not cause yield drag.

Example 14: Transformation

Once a nucleic acid sequence of the invention has been cloned into an expression system, it is transformed into a plant cell. The receptor and target expression cassettes of the present invention can be introduced into the plant cell in a number of art-recognized ways.

Methods for regeneration of plants are also well known in the art. For example, Ti plasmid vectors have been utilized for the delivery of foreign DNA, as well as direct DNA uptake via electroporation, microinjection, and microprojectiles. In addition, bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Below are descriptions of representative techniques for transforming both dicotyledonous and monocotyledonous plants, as well as a representative plastid transformation technique.

A. Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques that do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend on the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.* Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877 (1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Another approach to transforming plant cells with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792 all to Sanford et al. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

B. Transformation of monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent Applications EP 0 292 435, EP 0 392 225, and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 and Koziel *et al.* (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.* Plant Cell Rep 7: 379-384 (1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)). Furthermore, WO 93/21335 describes techniques for the transformation of rice via electroporation.

Patent Application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashiga & Skoog, Physiologia Plantarum 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos, which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 hours and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 hours (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one

month, developed shoots are transferred to larger sterile containers known as "GA7s" which contain half-strength MS, 2% sucrose, and the same concentration of selection agent.

Transformation of monocotyledons using *Agrobacterium* has also been described. *See,* WO 94/00977 and U.S. Patent No. 5,591,616, both of which are incorporated herein by reference. *See also,* Negrotto *et al., Plant Cell Reports* 19: 798-803 (2000), incorporated herein by reference.

Example 15: Use of expression cassettes of the present invention to confer abiotic stress tolerance in plants.

Once initiated, maize female spikelets are by definition metabolic sinks. They require a nutrient stream consisting of carbohydrate, amino acids, cofactors, minerals and other material from source tissues to fuel development. Source tissues include leaves, roots, the stalk and other vegetative plant parts. Much of what arrives at each spikelet is rapidly consumed, being converted to cell wall material, protein, lipids, nucleic acids etc. Very little is held in reserve.

The nutrient stream subsides during periods of abiotic stress. This stress is imposed by a number of stimuli including drought, cloud cover, temperature extremes and soil nutrient depletion. Spikelet development continues despite growing conditions, relying on reserves for energy and raw material. Reserves maintain development for, at most, a few days. If the abiotic stress period is prolonged reserves are depleted and spikelet development ceases. The result is kernel abortion and reduced yield.

The OsMADS expression cassettes of the present invention can be used to increase the sink strength in female spikelets by fusing them to genes that function to increase sink strength. These genes include a sucrose transporter, invertase, and trehalose metabolism genes. Many of these genes are not highly expressed in early spikelet development. Early and specific expression in the reproductive organs of plants, spikelets for example, of any of these genes will improve spikelet nourishment without detriment to other plant organs. Improved nutrition will enable spikelets to complete their developmental cycle and become competent for fertilization during ideal growth conditions and, importantly, during prolonged periods of abiotic stress.

Carbon arrives at developing spikelets as sucrose. Spikelets have limited ability to utilize sucrose because enzymes facilitating its entry into metabolism are not highly expressed. These enzymes include sucrose transporter(s) to aid uptake of sucrose unloaded from the phloem. The OsMADS expression cassettes can increase sucrose transporter levels in the transmitting and other maternal tissue. Imported sucrose fuels development and excess

sucrose is incorporated into starch and vacuolar reserves. Increased starch and sucrose reserves better enable spikelets to complete development during prolonged periods of abiotic stress.

Carbon nutrition can also be enhanced via increased invertase expression. This enzyme family cleaves sucrose into glucose and fructose. Both monosaccharides can be accumulated to high levels and rapidly enter carbon metabolism. The OSMADS expression cassettes of the present invention can be used to increase glucose and fructose levels in the apoplastic regions of spikelet and other maternal tissues via expression of an apoplastic or cell wall invertase. The monosaccharides enter cells and carbon metabolism more readily than sucrose. Facilitated sucrose utilization should increase sucrose unloading from the phloem, and carbon availability to developing spikelets.

Similarly, carbon nutrition in the cytosol of developing spikelets can be enhanced via expression of a cytosolic or neutral invertase. This enzyme cleaves sucrose in the cytosol, facilitating entry into carbon metabolism. The OSMADS expression cassettes of the present invention can increase neutral invertase expression in developing spikelets. The increased sucrose utilization in the cytosol, in transmitting and related spikelet tissue increases sucrose demand and thus, sucrose import from the apoplast.

Carbon availability and abiotic stress resistance in developing spikelets also can be enhanced via expression of a vacuolar or soluble acid invertase. This enzyme cleaves sucrose into fructose and glucose in the vacuole, making the carbon available for energy metabolism. Sucrose conversion into glucose and fructose also increases the solute potential of the cell, enabling it to maintain water and thus, turgor during periods of drought. This allows spikelets to continue developing despite decreased water availability. Again, the OSMADS expression cassettes of the present invention can increase expression of vacuolar or soluble acid invertase in developing spikelets for the purpose of enhancing abiotic stress tolerance.

The trehalose pathway functions to regulate carbon partitioning between primary metabolism and starch synthesis. Up-regulation of this pathway directs carbon towards starch synthesis. The OsMADS expression cassettes of the present invention can be used to drive expression of trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase and trehalase in developing spikelets, thereby increasing sink strength and starch synthesis in those tissues. Maintenance of a large starch pool better enables developing spikelets to withstand prolonged periods of abiotic stress and complete their development cycle.

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

5

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CLAIMS

1) An expression cassette for expression nucleic acid molecule product in the reproductive tissue of a plant comprising:
   a) a promoter, a first exon; a first intron, and a second exon of a MADS gene, wherein said promoter, first exon, intron, and second exon are the 5'-regulatory sequence of said expression cassette; wherein said 5'-regulatory sequence has been engineered to include a translational initiation codon at approximately the 3' end of said 5'-regulatory sequence, and not to contain restriction endonuclease sites that hinder manipulation by recombinant DNA methods or additional translation initiation codons upstream of said translation initiation codon;
   b) a 3'-regulatory sequence of a MADS gene; and
   c) a nucleic acid molecule operably linked to said 5'-regulatory sequence and said 3'-regulatory sequence.
2) The expression cassette of claim 1, wherein the product of said nucleic acid molecule when expressed in a plant makes said plant abiotic stress tolerant.
3) The expression cassette of claim 1, wherein when expressed in a plant the product of said nucleic acid molecule increases the sink strength in said reproductive tissue of said plant.
4) The expression cassette of claim 2, wherein said nucleic acid molecule is a sucrose transporter gene.
5) The expression cassette of claim 2, wherein said nucleic acid molecule is an invertase gene.
6) The expression cassette of claim 2, wherein said nucleic acid molecule is a RNA interference molecule directed against an invertase gene.
7) The expression cassette of claim 1, wherein said nucleic acid molecule is a T6PP gene.
8) The expression cassette of claim 7, wherein said nucleic acid molecule is depicted by SEQ ID NO. 531.
9) The expression cassette of claim 1, wherein said expression cassette is depicted by SEQ ID NO. 532.
10) The expression cassette of claim 1, wherein said expression cassette is depicted by SEQ ID NO. 536, 537, 538, or 539.
11) The expression cassette of claim 2, wherein said nucleic acid molecule is a trehalose metabolism gene.

12) The expression cassette of claim 2, wherein said nucleic acid molecule is a RNA interference molecule directed against a trehalose metabolism gene.

13) A recombinant vector comprising the expression cassette of claim 1.

14) A transgenic plant cell comprising the expression cassette of claim 1.

15) A transgenic plant comprising the transgenic plant cell of claim 10.

16) A transgenic plant according to claim 11, wherein said plant is a monocot plant.

17) A transgenic plant according to claim 11, wherein said plant is a maize plant.

18) A transgenic plant according to claim 11, wherein said plant is a rice plant.

19) Seed from the transgenic plant of claim 11.

20) Seed from the transgenic plant of claim 12.

21) Seed from the transgenic plant of claim 13.

22) Seed from the transgenic plant of claim 14.

23) Plants containing the expression cassette according to claim 1.

24) An expression cassette comprising a 5'-regulatory sequence selected from the group consisting of SEQ ID NOS. 6, 12, 20, 28, 506, 34, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 123, 127, 131, and 526 and a 3'-regulatory sequence selected from the group consisting of SEQ ID NOS. 10, 16, 24, 30, 507, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 124, 128, 132, and 527.

25) A method of constructing an expression cassette comprising the steps of:

a) selecting a target gene;

b) identifying the open reading frame on said target gene cDNA;

c) identifying the positions of the translational start codon, translational stop codon, the first intron, first exon, second exon, the 3'untranslated sequence and the 3'nontranscribed sequence of said target gene gDNA by using the cDNA to annotate said target gene gDNA;

d) constructing a 5'-regulatory sequence comprising said promoter, first exon, first intron, and second exon and a 3'-regulatory sequence comprising the 3'-untranslated sequence and the 3'-nontranscribed sequence; and

e) operably linking a nucleic acid molecule to said 5'-regulatory sequence and said 3'regulatory sequence of said expression cassette, wherein said nucleic acid molecule is expressed in a manner that mimics the expression of said target gene.

26) The method of constructing an expression cassette according to claim 21, wherein said target gene is a MADS gene.

27) The method of constructing an expression cassette according to claim 21, wherein said nucleic acid molecule is a sucrose transporter gene.

28) A method of constructing an expression cassette according to claim 21, wherein said nucleic acid molecule is an invertase gene.

29) A method of constructing an expression cassette according to claim 21, wherein said nucleic acid molecule is a trehalose metabolism gene.

30) A method of constructing an expression cassette according to claim 25, wherein said trehalose metabolism gene is a trehalose-6-phosphate synthase gene, a trehalose-6-phosphate phosphatase gene or trehalase gene.

31) A method of constructing an expression cassette according to claim 24, wherein said invertase gene is a soluble acid invertase gene, a soluble neutral invertase gene or an insoluble acid invertase gene.

32) The method of constructing an expression cassette according to claim 21, further comprising the step of engineering a translational initiation codon at approximately the 3' end of said 5' regulatory sequence.

33) The method of constructing an expression cassette according to claim 21, further comprising the step of engineering said expression cassette to not contain restriction endonuclease sites that hinder manipulation by recombinant DNA methods or additional translation initiation codons upstream of said translation initiation codon.

34) A method of conferring abiotic stress tolerance to a plant comprising the steps of constructing an expression cassette comprising 5'-regulatory and 3'-regulatory sequences of the MADs gene family.

35) A method of constructing an expression cassette according to claim 21, wherein said 5' regulatory sequence is selected from the group consisting of SEQ ID NOS. 6, 12, 20, 28, 506, 34, 39, 43, 47, 51, 55, 59, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 123, 127, 131, and 526.

36) A method of constructing an expression cassette according to claim 21, wherein said 3'-regulatory sequence is selected from the group consisting of SEQ ID NOS. 10, 16, 24, 30, 507, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 124, 128, 132, and 527.

37) A method of constructing an expression cassette according to claim 21, wherein said target gene is selected based on its expression data.

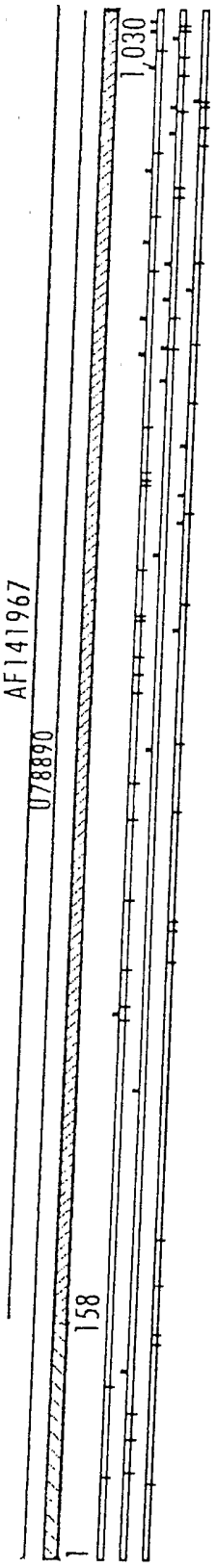

AF141967
U78890
1
158
1,030

FIG. 1A

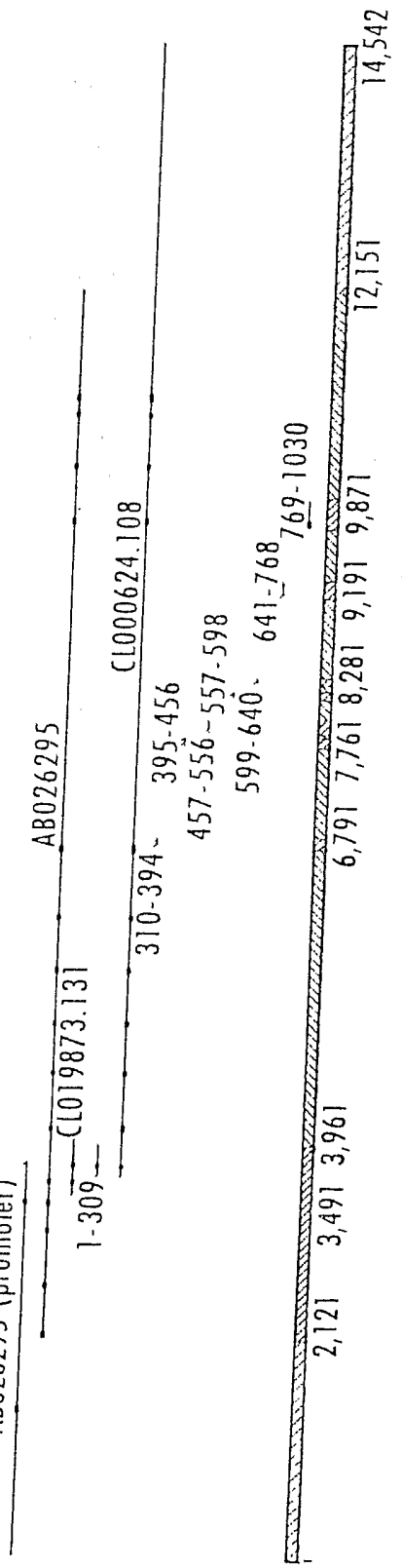

AB026295 (promoter)
AB026295
CL019873.131
1-309
CL000624.108
310-394
395-456
457-556
557-598
599-640
641-768
769-1030
1
2,121
3,491
3,961
6,791
7,761
8,281
9,191
9,871
12,151
14,542

FIG. 1B

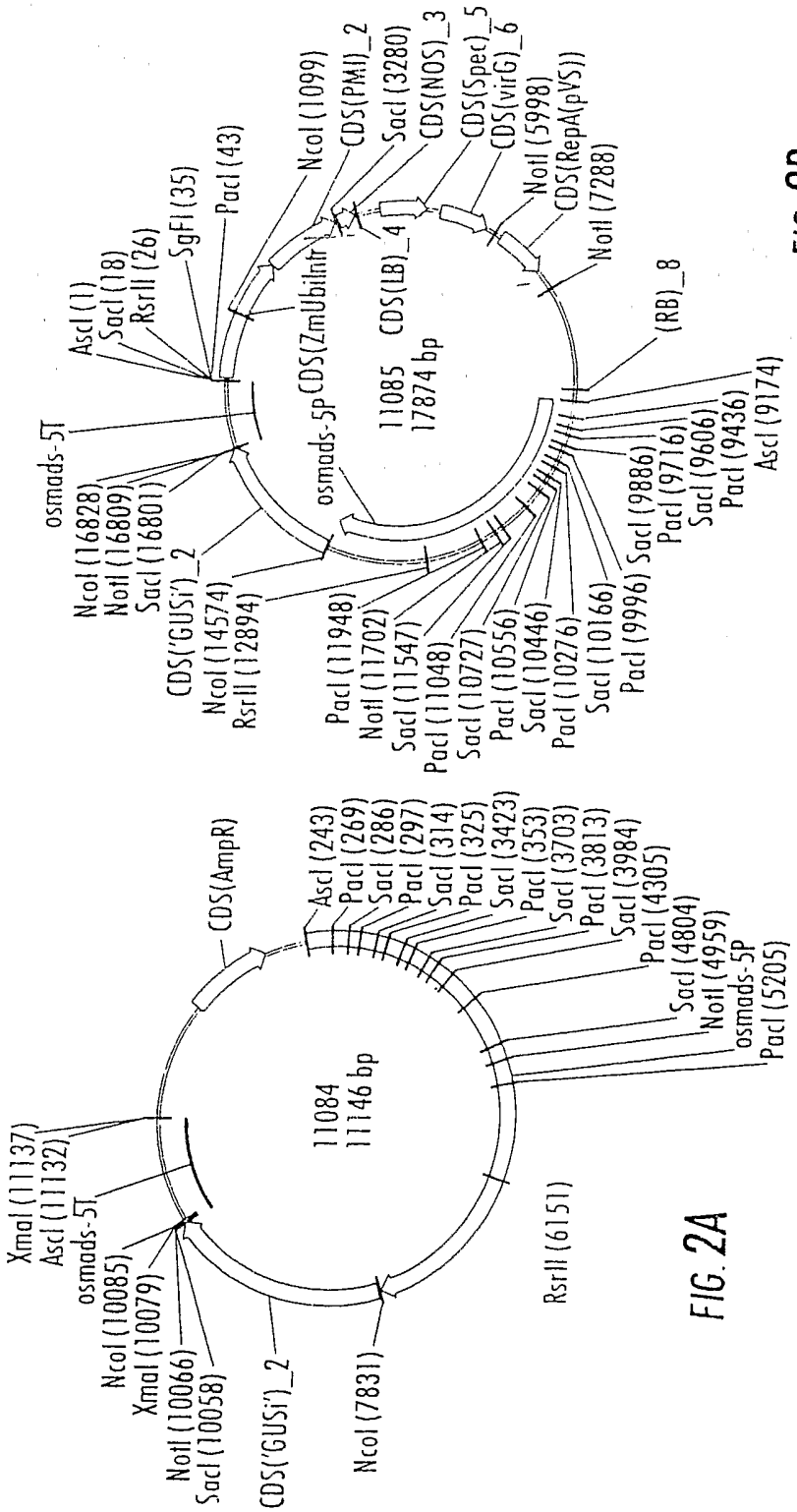

11084
11146 bp
CDS(AmpR)
AscI (243)
PacI (269)
SacI (286)
PacI (297)
SacI (314)
PacI (325)
SacI (3423)
PacI (353)
SacI (3703)
PacI (3813)
SacI (3984)
PacI (4305)
SacI (4804)
NotI (4959)
osmads-5P
PacI (5205)
RsrII (6151)
NcoI (7831)
CDS('GUSi')_2
NotI (10066)
SacI (10058)
XmaI (10079)
NcoI (10085)
osmads-5T
AscI (11132)
XmaI (11137)

FIG. 2A

11085
17874 bp
CDS(LB)_4
CDS(ZmUbiInt)
CDS(PMI)_2
SacI (3280)
CDS(NOS)_3
CDS(Spec)_5
CDS(virG)_6
NotI (5998)
CDS(RepA(pVS))
NotI (7288)
(RB)_8
AscI (9174)
PacI (9436)
SacI (9606)
PacI (9716)
SacI (9886)
PacI (9996)
SacI (10166)
PacI (10276)
SacI (10446)
PacI (10556)
SacI (10727)
PacI (11048)
SacI (11547)
NotI (11702)
PacI (11948)
RsrII (12894)
NcoI (14574)
CDS('GUSi')_2
SacI (16801)
NotI (16809)
NcoI (16828)
osmads-5T
AscI (1)
SacI (18)
RsrII (26)
SgFI (35)
PacI (43)
NcoI (1099)
osmads-5P

FIG. 2B



FIG. 3A

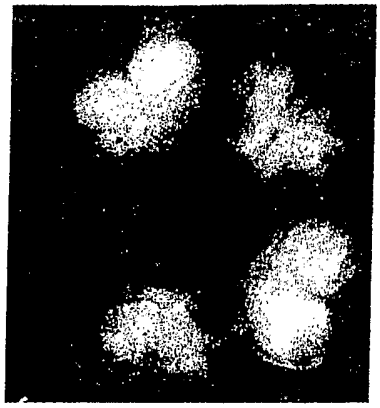


FIG. 3B

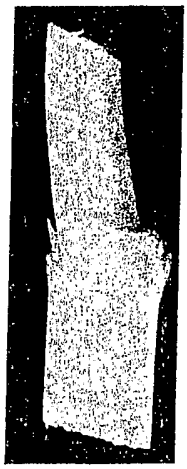


FIG. 3C



FIG. 3D



FIG. 3E

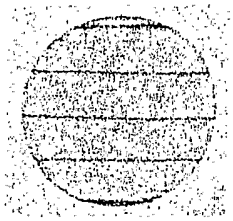


FIG. 3F

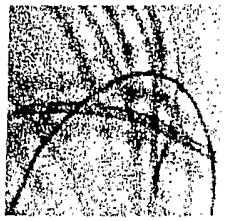


FIG. 3G

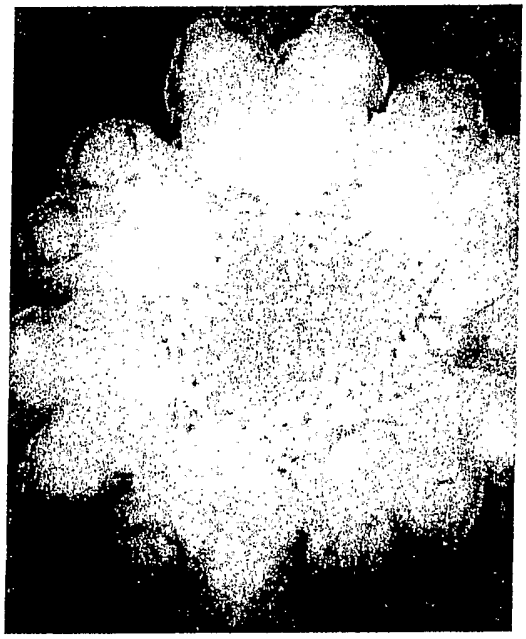


FIG. 4B



FIG. 4D

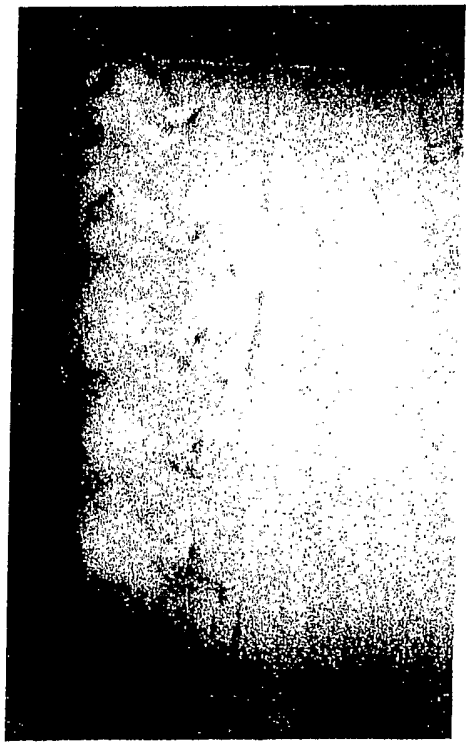


FIG. 4A

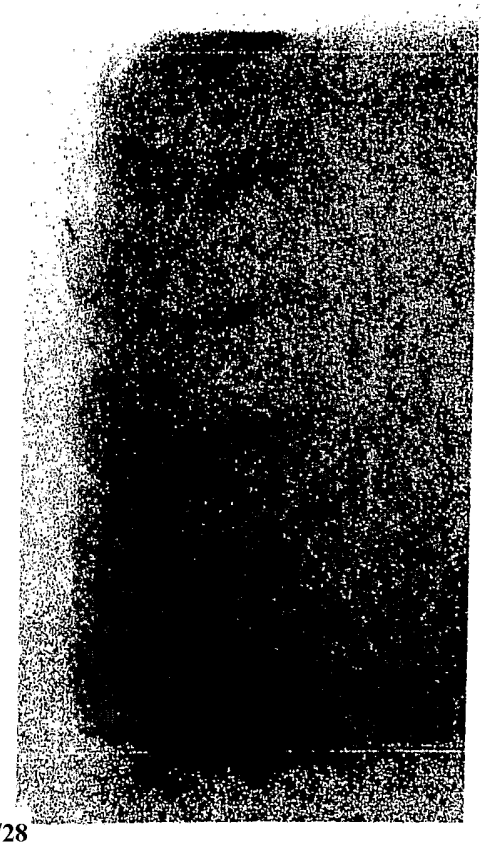


FIG. 4C

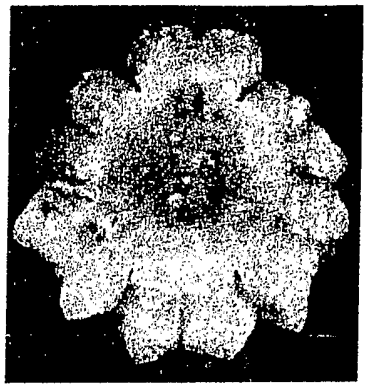


FIG. 5A

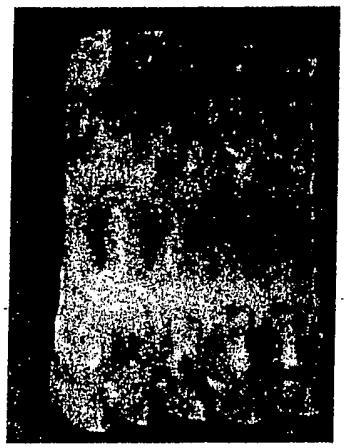


FIG. 5B



FIG. 5C

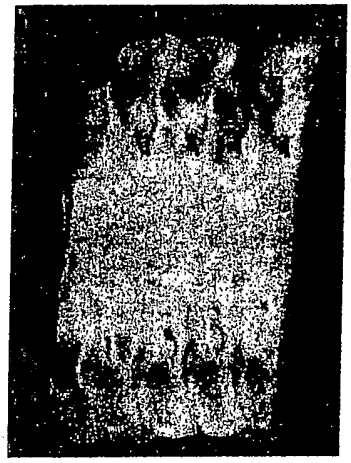


FIG. 5D

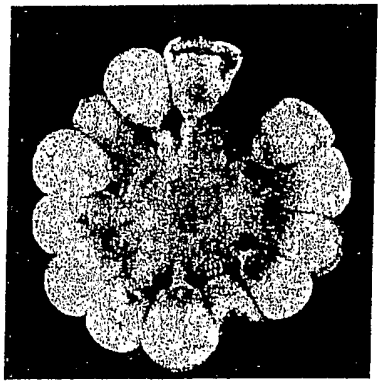


FIG. 5E



FIG. 5F

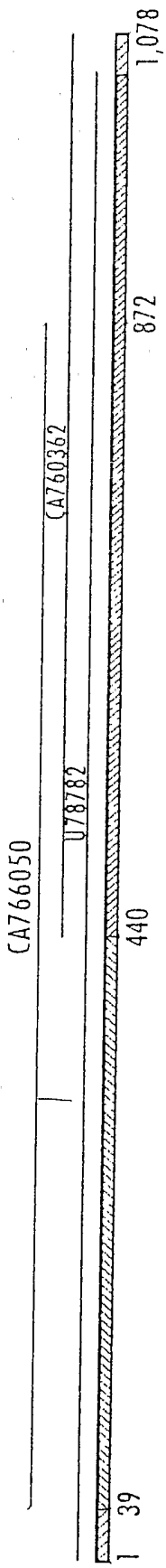

CA766050
U78782
CA760362
1
39
440
872
1,078

FIG. 6A

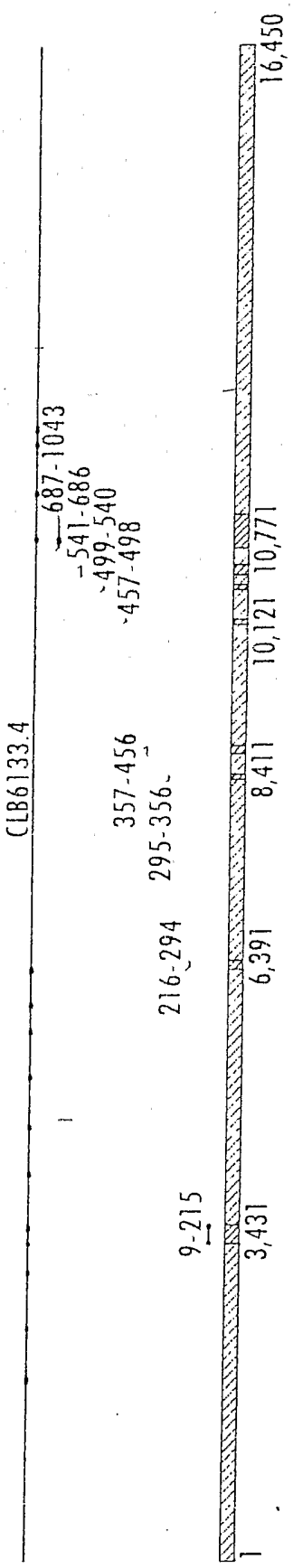

CLB6133.4
687-1043
541-686
499-540
457-498
357-456
295-356
216-294
9-215
1
3,431
6,391
8,411
10,121
10,771
16,450

FIG. 6B

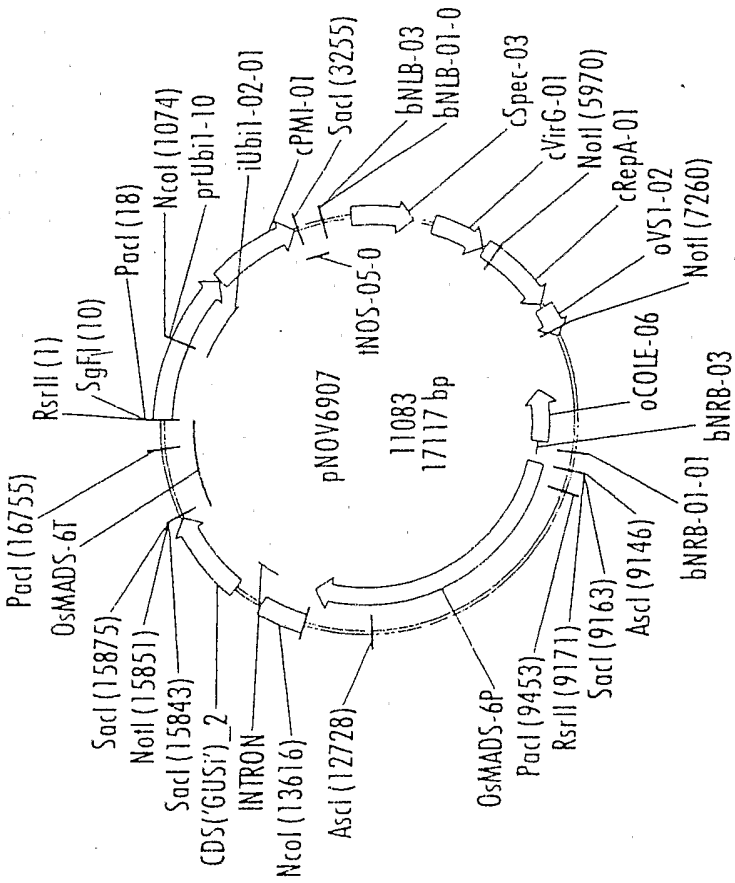

pNOV6907
11083
17117 bp
RsrII (1)
SgfI (10)
PacI (18)
NcoI (1074)
prUbi1-10
iUbi1-02-01
cPMI-01
SacI (3255)
tNOS-05-0
bNLB-03
bNLB-01-0
cSpec-03
cVirG-01
NotI (5970)
cRepA-01
oVS1-02
NotI (7260)
oCOLE-06
bNRB-03
bNRB-01-01
AscI (9146)
SacI (9163)
RsrII (9171)
PacI (9453)
OsMADS-6P
AscI (12728)
NcoI (13616)
INTRON
CDS('GUSi')_2
SacI (15843)
NotI (15851)
SacI (15875)
OsMADS-6T
PacI (16755)

FIG. 7B

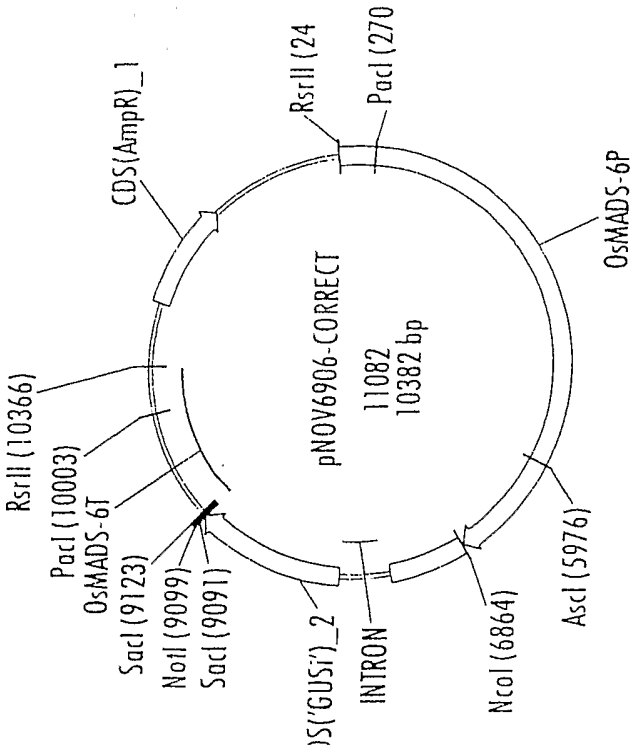

pNOV6906-CORRECT
11082
10382 bp
CDS(AmpR)_1
RsrII (24
PacI (270
OsMADS-6P
AscI (5976)
NcoI (6864)
INTRON
)S('GUSi')_2
SacI (9091)
NotI (9099)
SacI (9123)
OsMADS-6T
PacI (10003)
RsrII (10366)

FIG. 7A

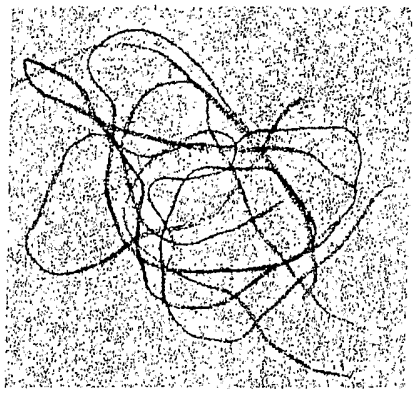


FIG. 8A

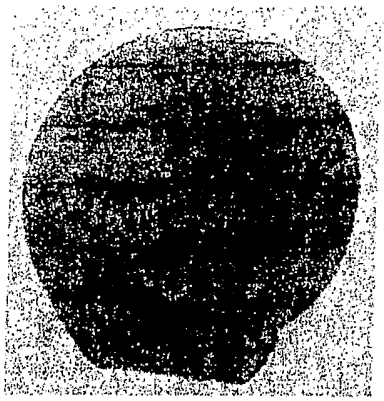


FIG. 8B



FIG. 8C

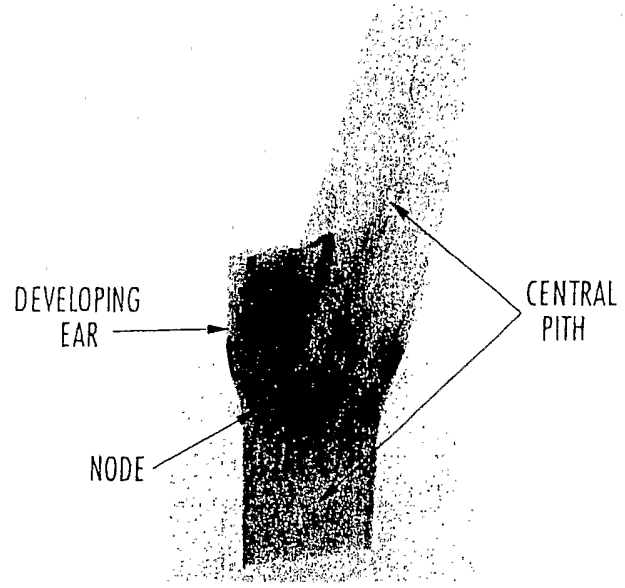


FIG. 8D



FIG. 8E

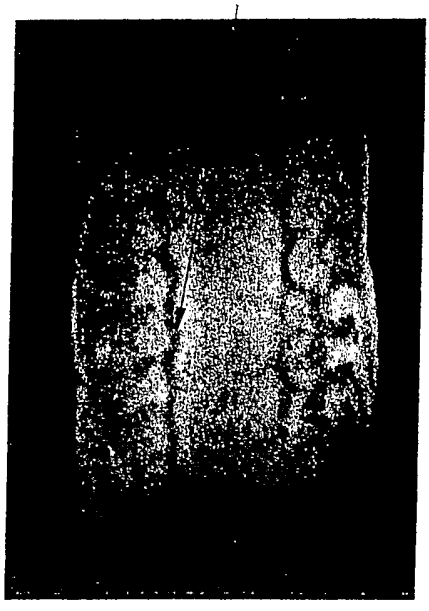


FIG. 9A



FIG. 9B

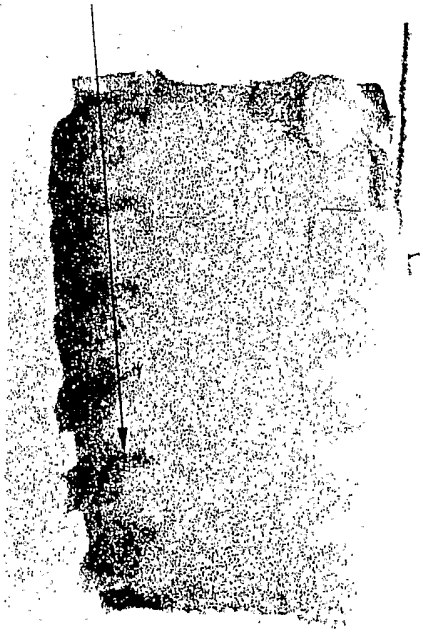


FIG. 9C

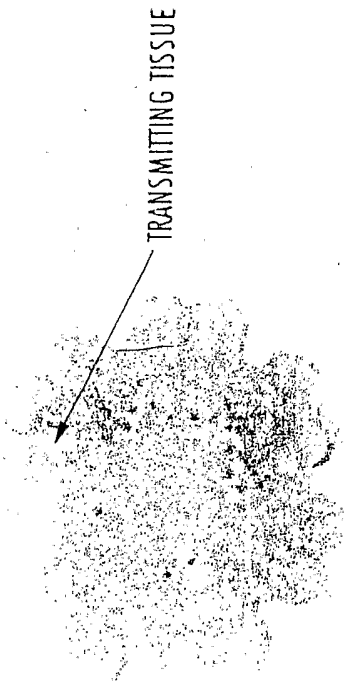


FIG. 9D



FIG. 10A

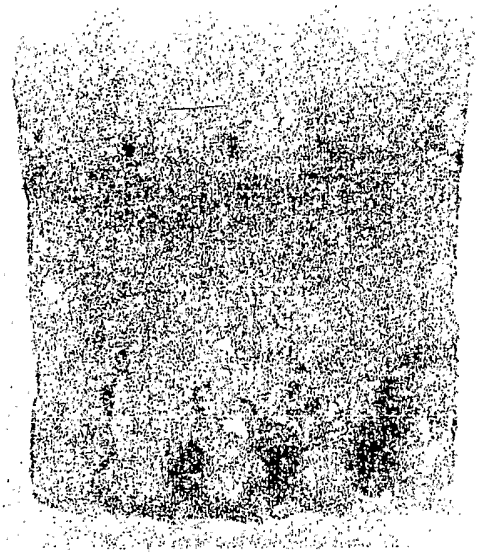


FIG. 10B



FIG. 10C

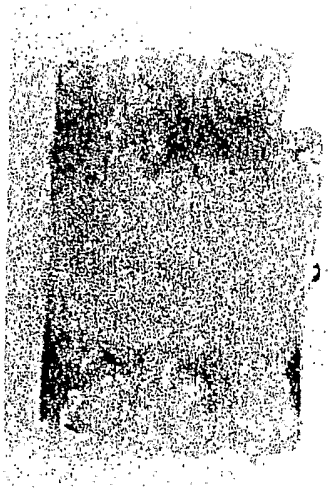


FIG. 10D



FIG. 11A

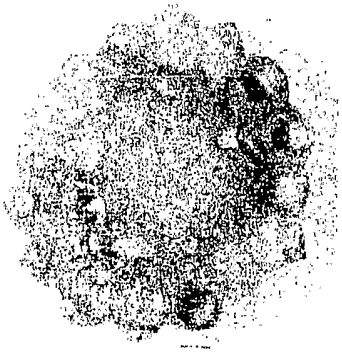


FIG. 11B



FIG. 11C

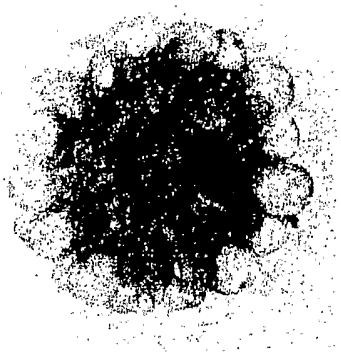


FIG. 11D



FIG. 11E

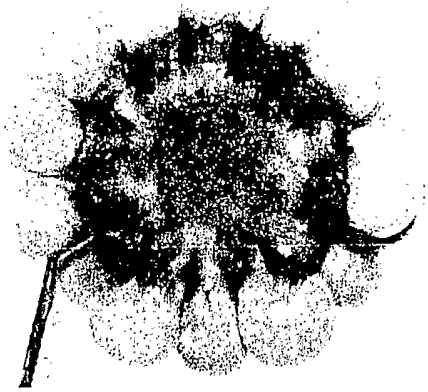


FIG. 11F

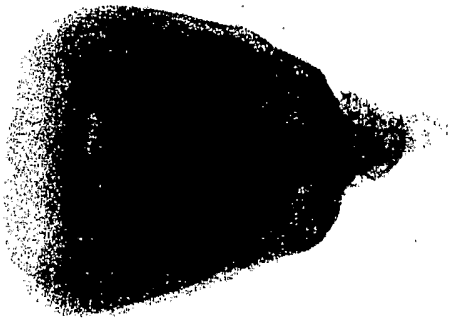


FIG. 11G

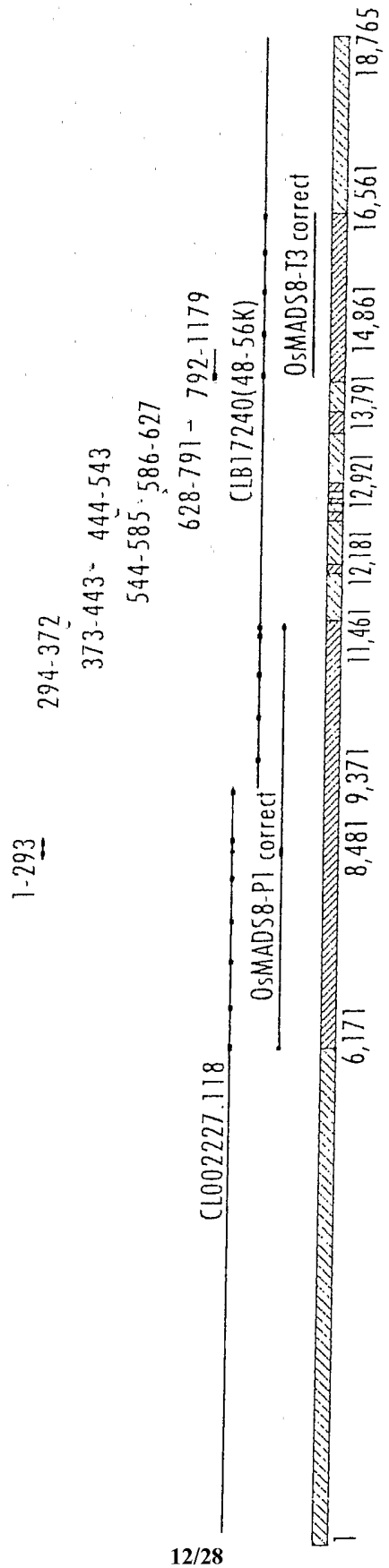

1-293
294-372
373-443
444-543
544-585
586-627
628-791
792-1179
CLB17240(48-56K)
OsMADS8-T3 correct
OsMADS8-P1 correct
CL002227.118
1
6,171
8,481
9,371
11,461
12,181
12,921
13,791
14,861
16,561
18,765

FIG. 12

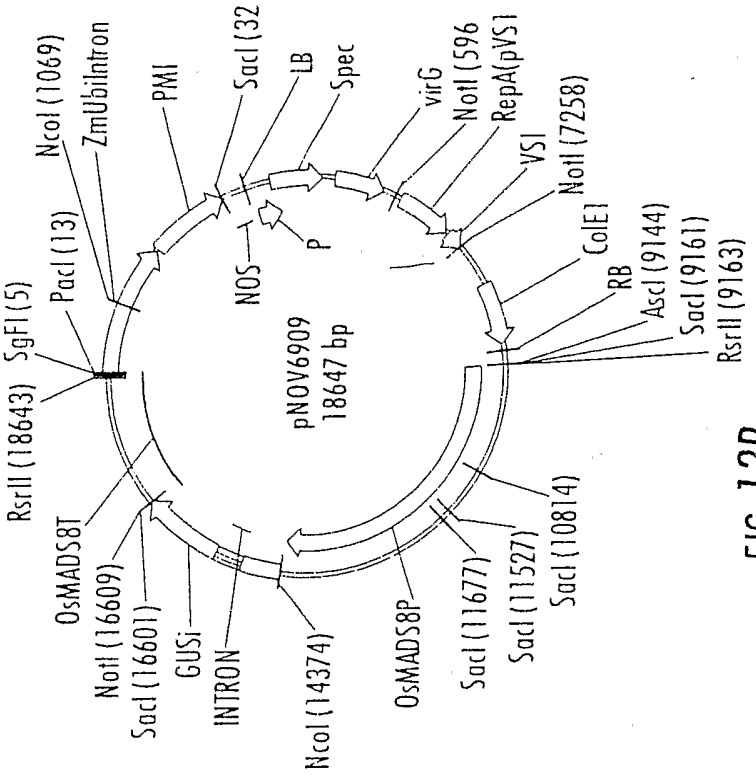


FIG. 13B

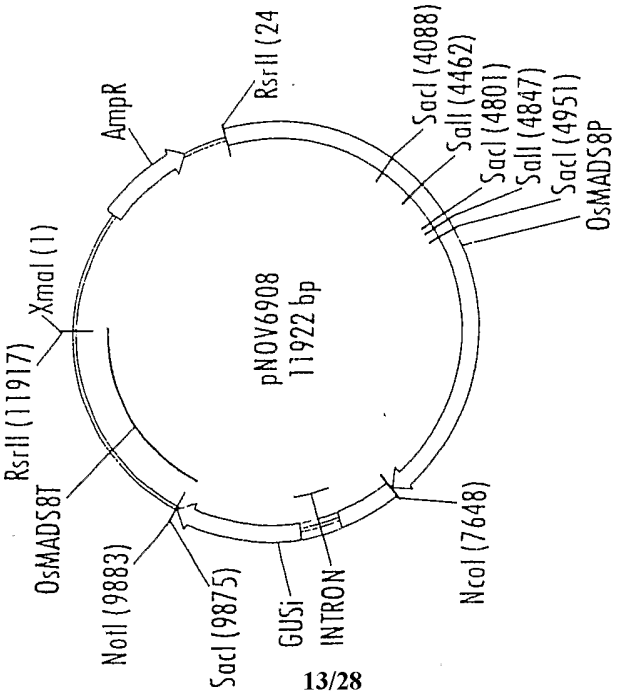


FIG. 13A

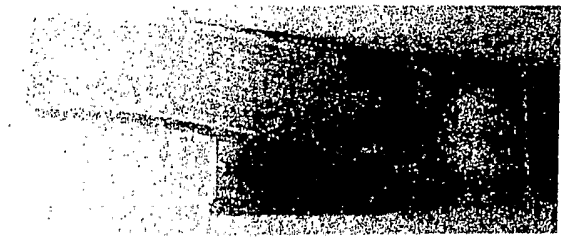


FIG. 14D

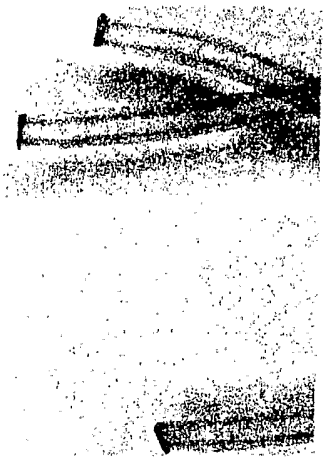


FIG. 14C

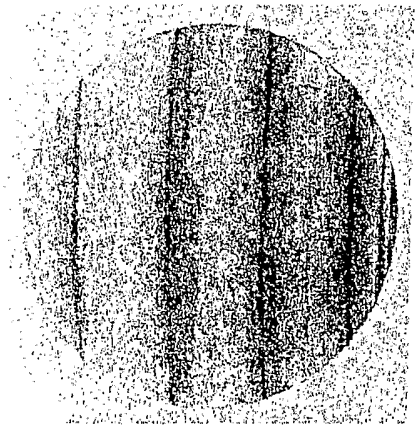


FIG. 14B



FIG. 14A



FIG. 14G

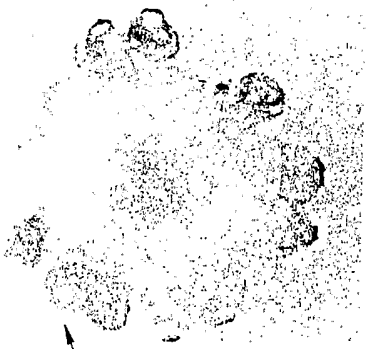


FIG. 14F

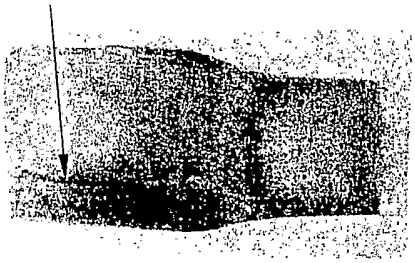


FIG. 14E

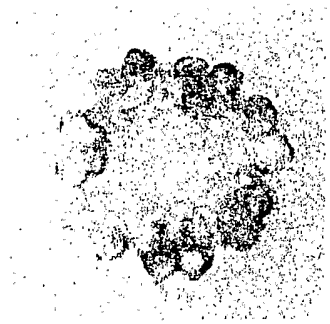


FIG. 15D

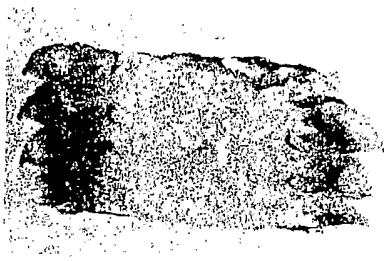


FIG. 15I

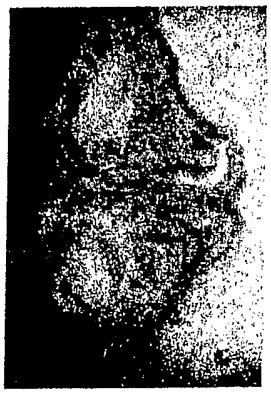


FIG. 15C

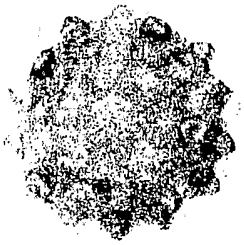


FIG. 15H

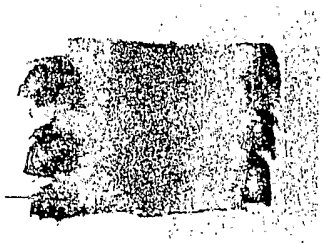


FIG. 15B

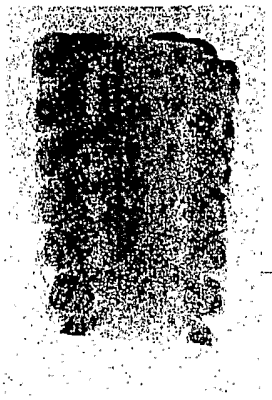


FIG. 15G



FIG. 15A

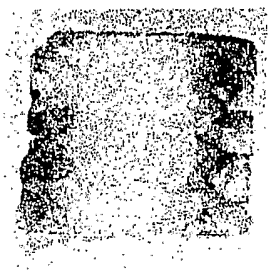


FIG. 15E



FIG. 15F

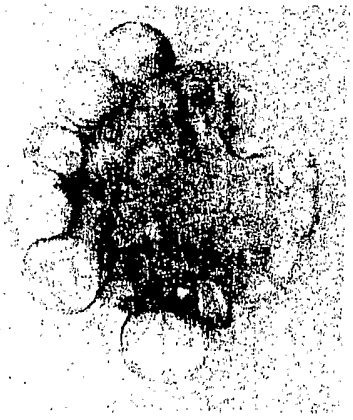


FIG. 16E

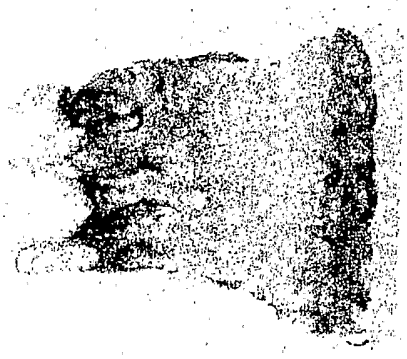


FIG. 16F



FIG. 16C

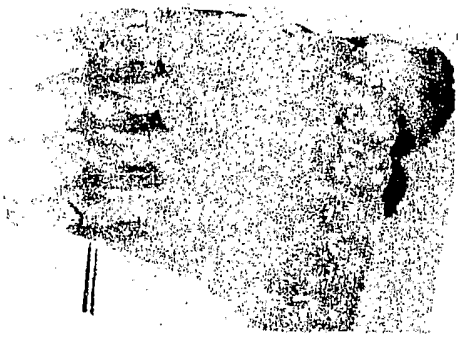


FIG. 16D



FIG. 16A

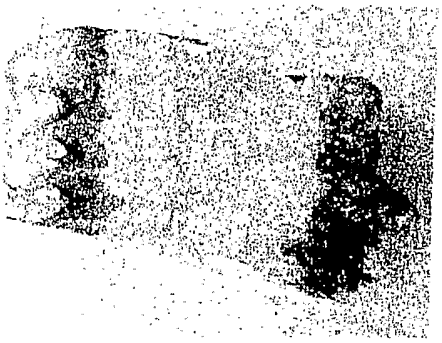


FIG. 16B

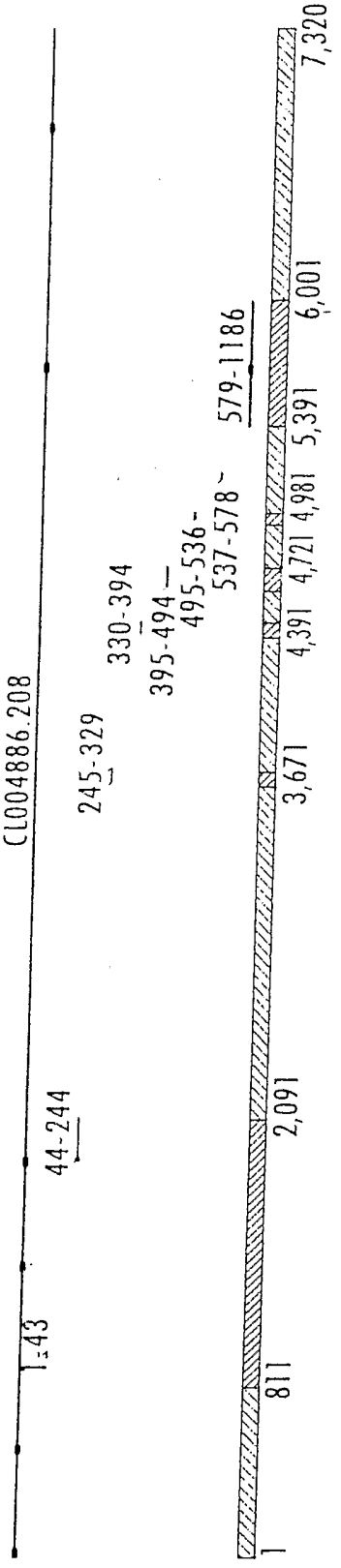

CL004886.208
1-43
44-244
245-329
330-394
395-494
495-536
537-578
579-1186
1
811
2,091
3,671
4,391
4,721
4,981
5,391
6,001
7,320

FIG. 17

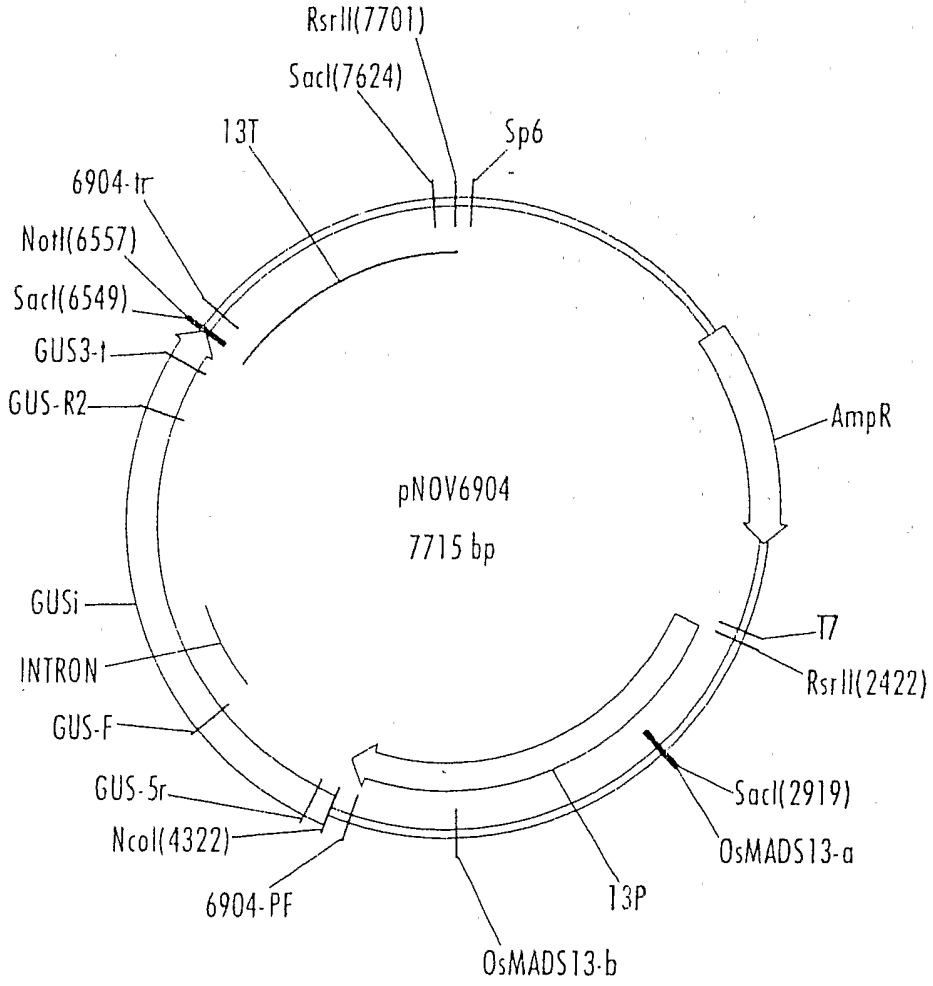

RsrII(7701)
SacI(7624)
T3T
Sp6
6904-tr
NotI(6557)
SacI(6549)
GUS3-t
GUS-R2
AmpR
pNOV6904
7715 bp
GUSi
INTRON
GUS-F
GUS-5r
NcoI(4322)
6904-PF
T3P
T7
RsrII(2422)
SacI(2919)
OsMADS13-a
OsMADS13-b

FIG. 18

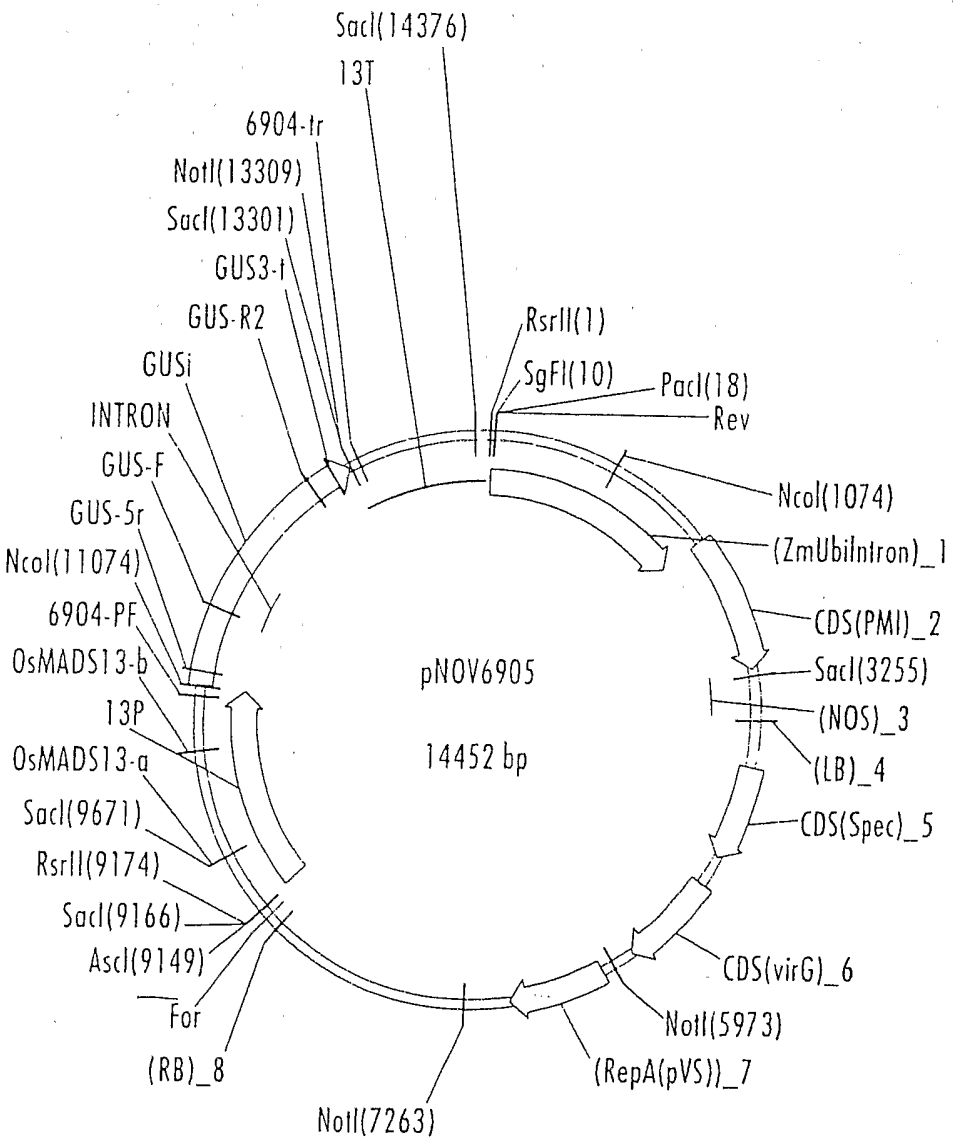

SacI(14376)
13T
6904-tr
NotI(13309)
SacI(13301)
GUS3-t
GUS-R2
GUSi
INTRON
GUS-F
GUS-5r
NcoI(11074)
6904-PF
OsMADS13-b
13P
OsMADS13-a
SacI(9671)
RsrII(9174)
SacI(9166)
AscI(9149)
For
(RB)_8
NotI(7263)
(RepA(pVS))_7
NotI(5973)
CDS(virG)_6
CDS(Spec)_5
(LB)_4
(NOS)_3
SacI(3255)
CDS(PMI)_2
(ZmUbiIntron)_1
NcoI(1074)
Rev
PacI(18)
SgFI(10)
RsrII(1)
pNOV6905
14452 bp

FIG. 19

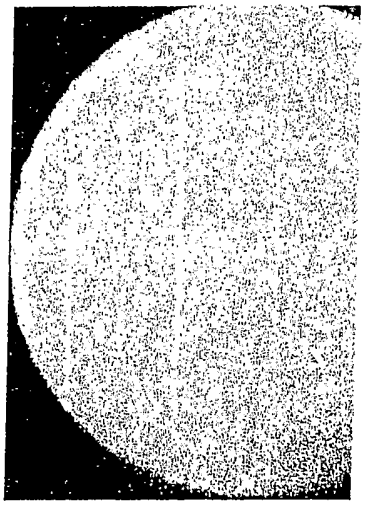


FIG. 20A

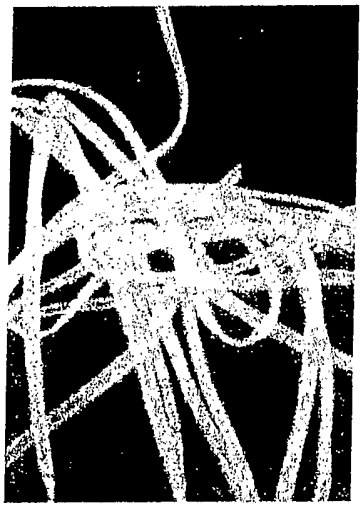


FIG. 20B



FIG. 20C

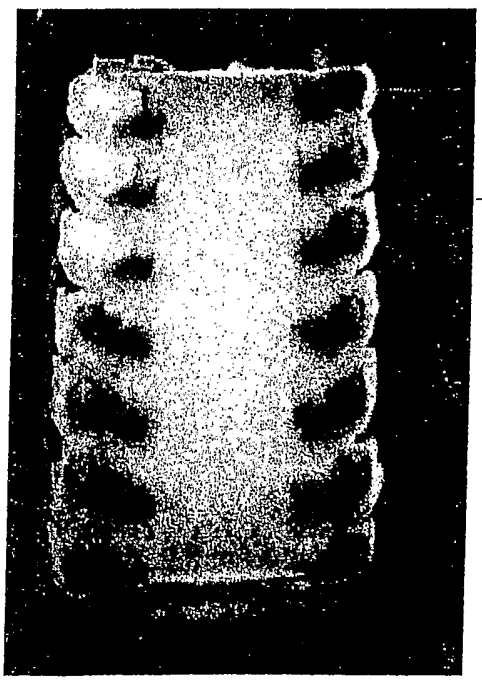


FIG. 20D

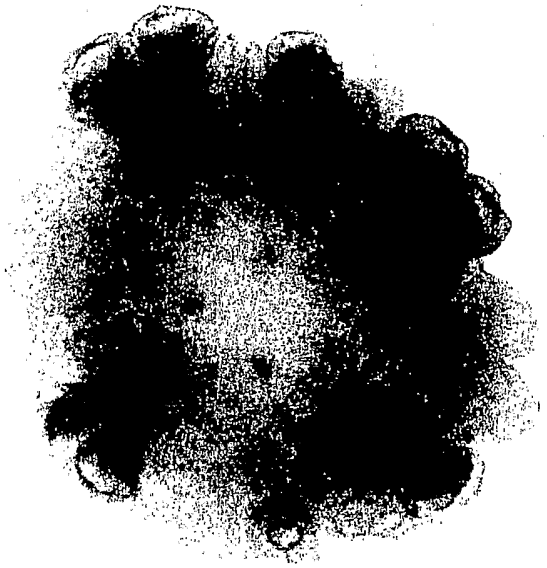


FIG. 20E

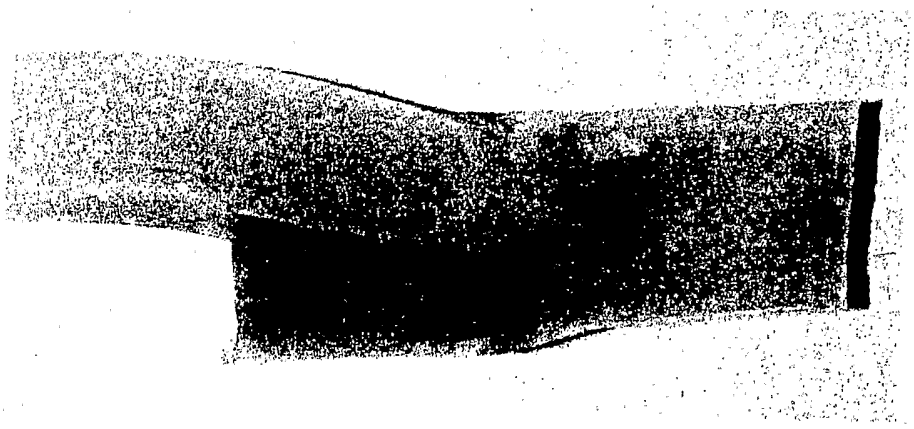


FIG. 21D

FIG. 21A

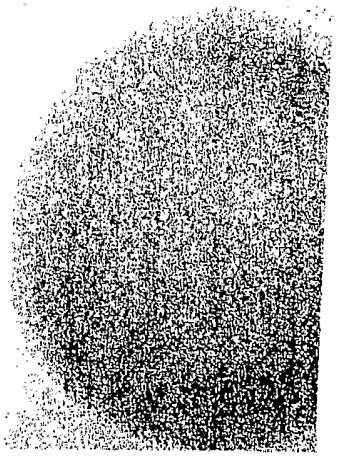


FIG. 21B



FIG. 21C



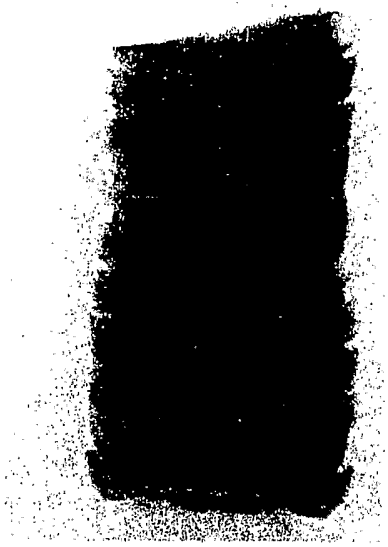


FIG. 22B



FIG. 22D



FIG. 22A

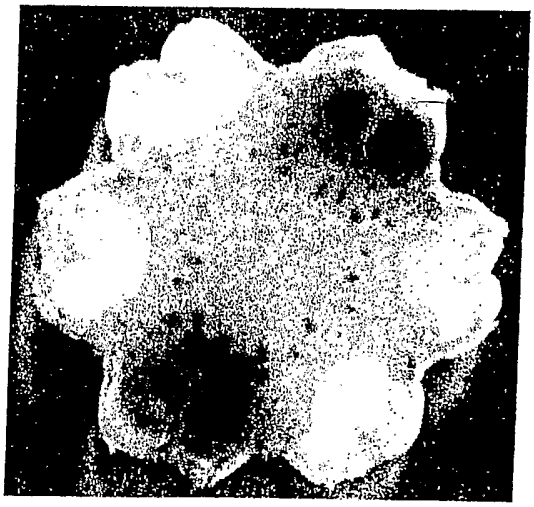


FIG. 22C

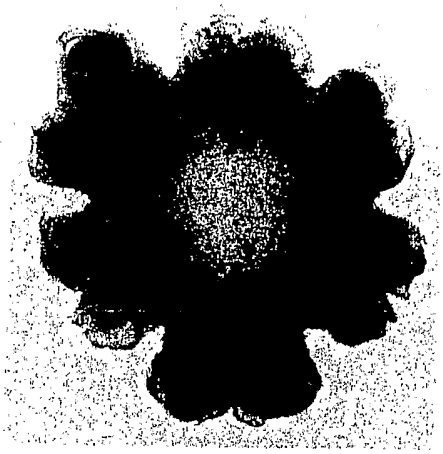


FIG. 23A

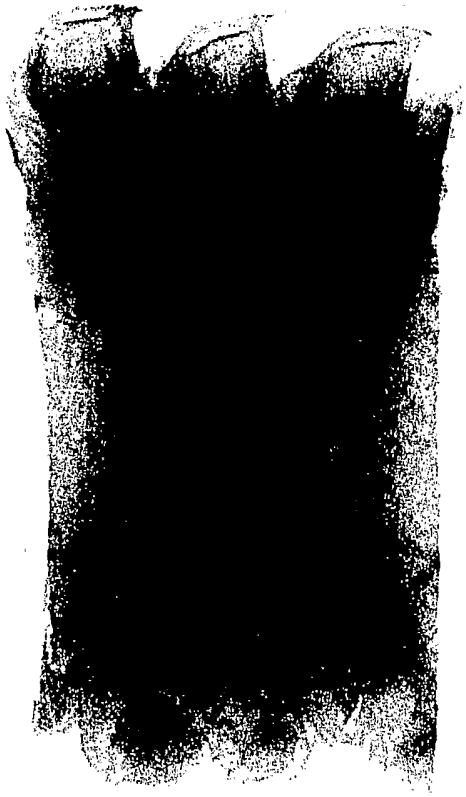


FIG. 23C

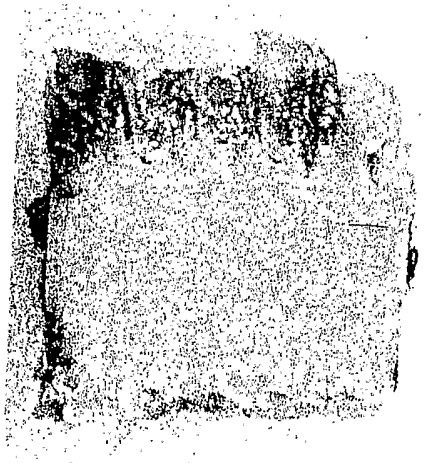


FIG. 23B

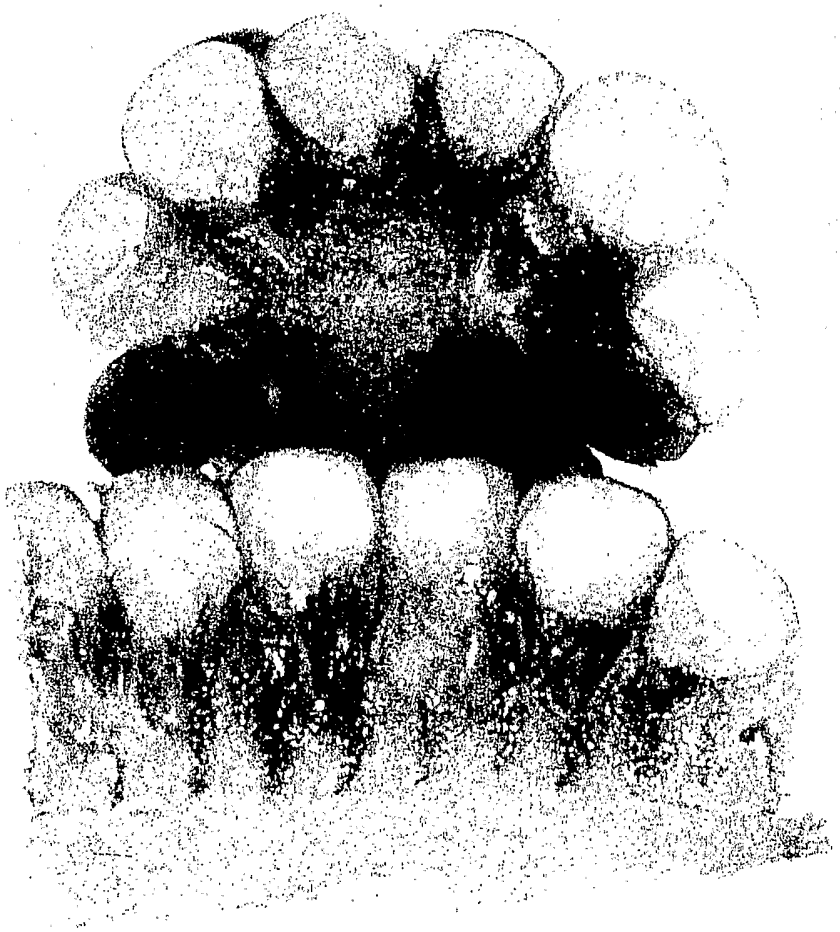


FIG. 24



FIG. 25A

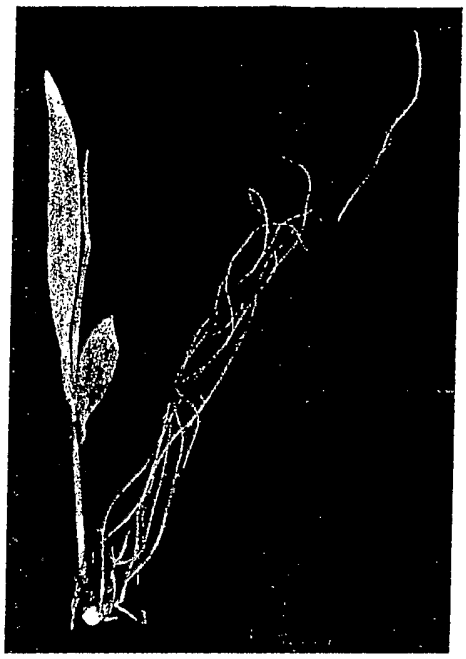


FIG. 25B

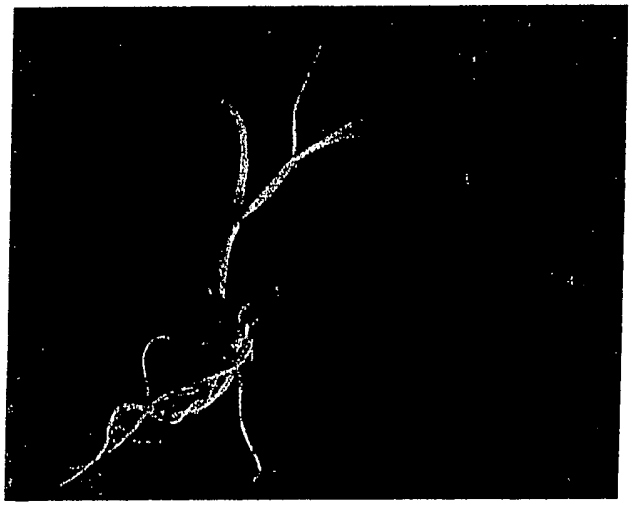


FIG. 25C

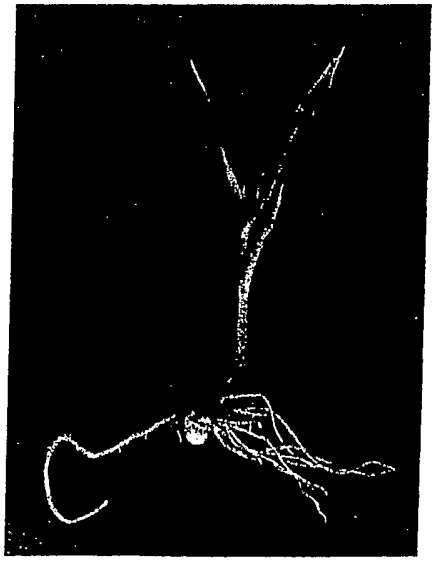


FIG. 25D

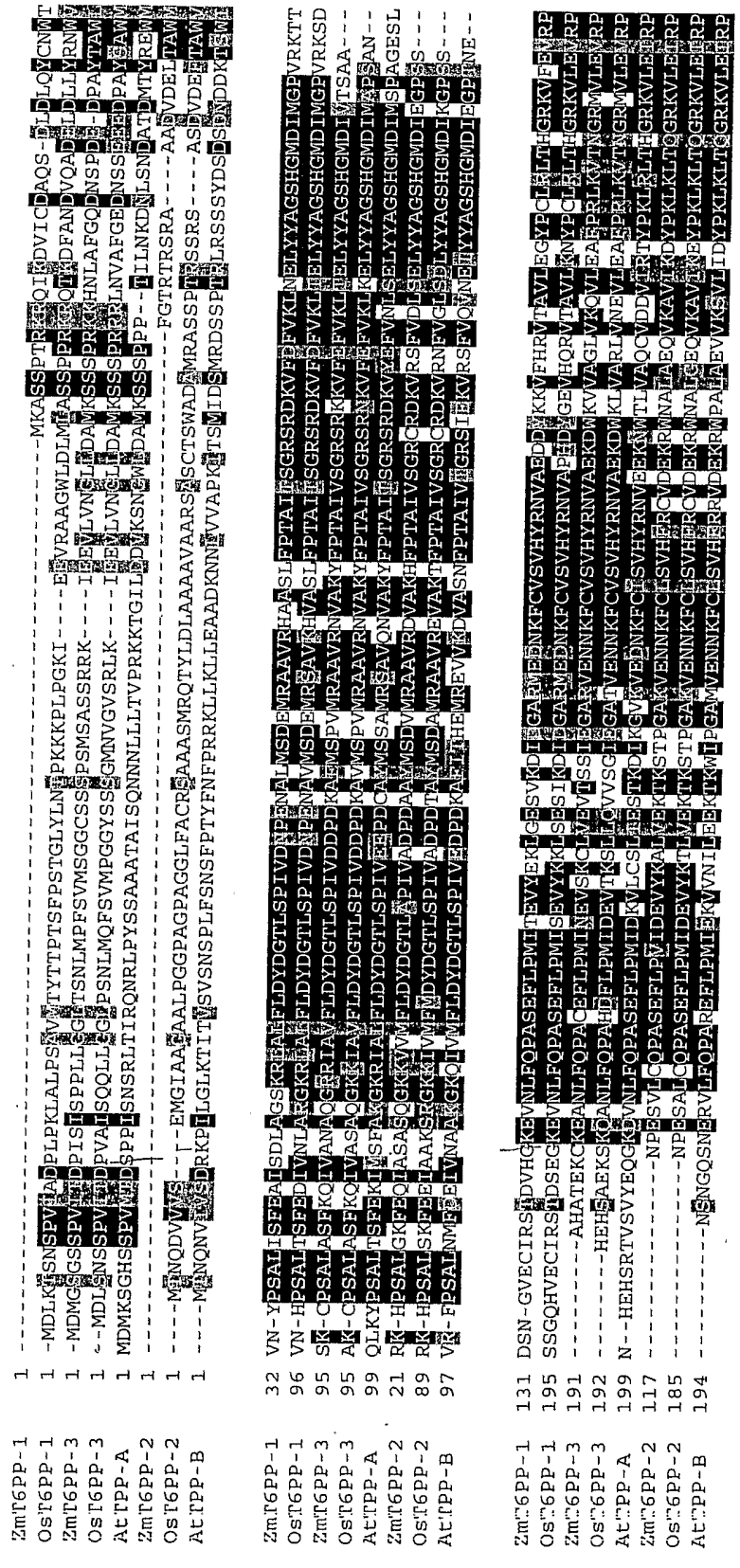
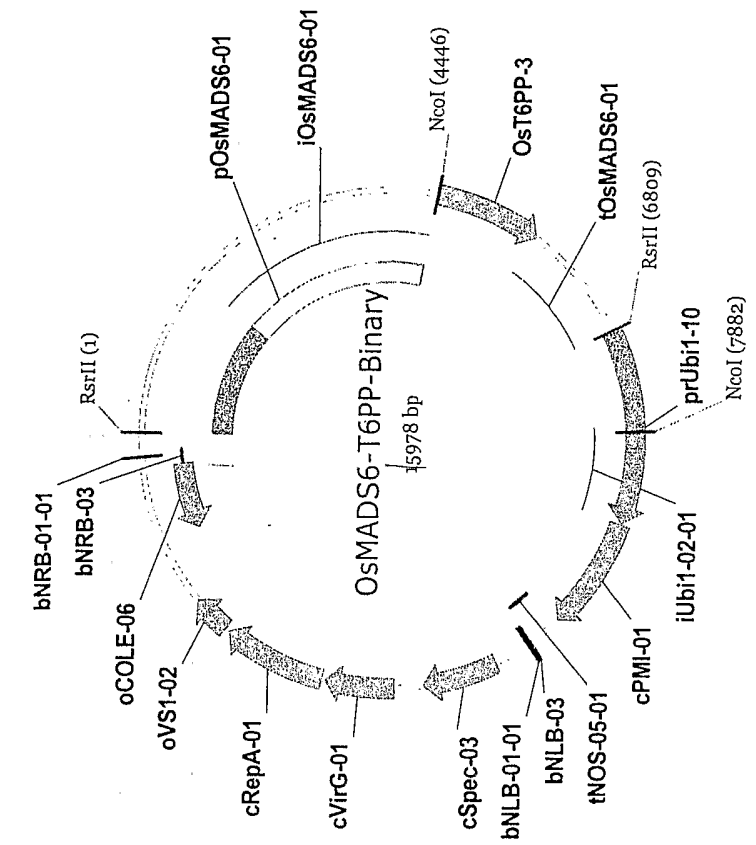


Fig 26


B
OsMADS6-T6PP-Binary
15978 bp
pOsMADS6-01
iOsMADS6-01
NcoI (4446)
OsT6PP-3
tOsMADS6-01
RsrII (6809)
prUbi1-10
NcoI (7882)
iUbi1-02-01
cPMI-01
tNOS-05-01
bNLB-01-01
bNLB-03
cSpec-03
cVirG-01
cRepA-01
oVS1-02
oCOLE-06
bNRB-03
bNRB-01-01
RsrII (1)

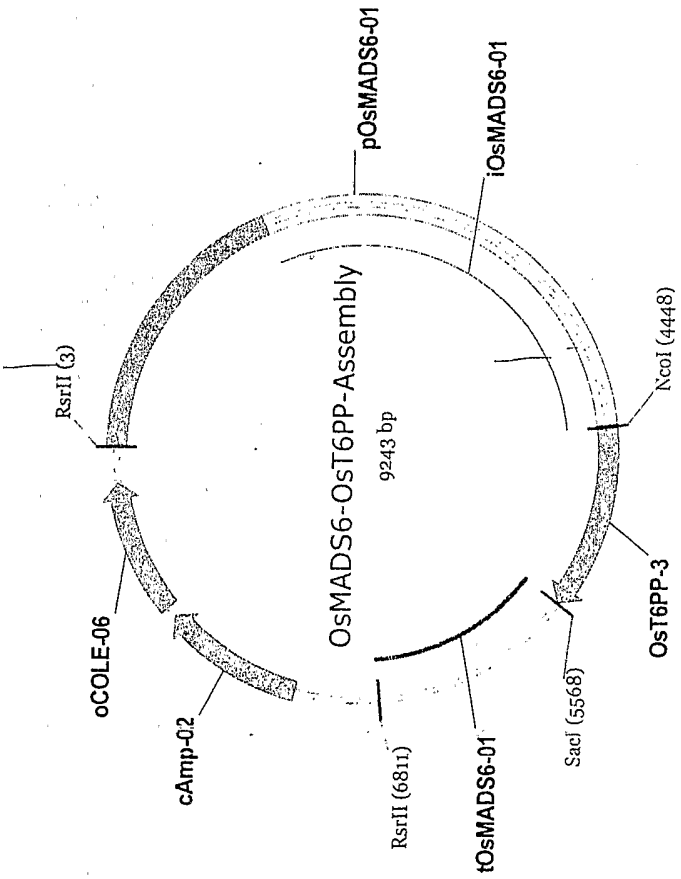

A
OsMADS6-OsT6PP-Assembly
9243 bp
pOsMADS6-01
iOsMADS6-01
NcoI (4448)
OsT6PP-3
SacI (5568)
tOsMADS6-01
RsrII (6811)
cAmp-02
oCOLE-06
RsrII (3)

FIG 27

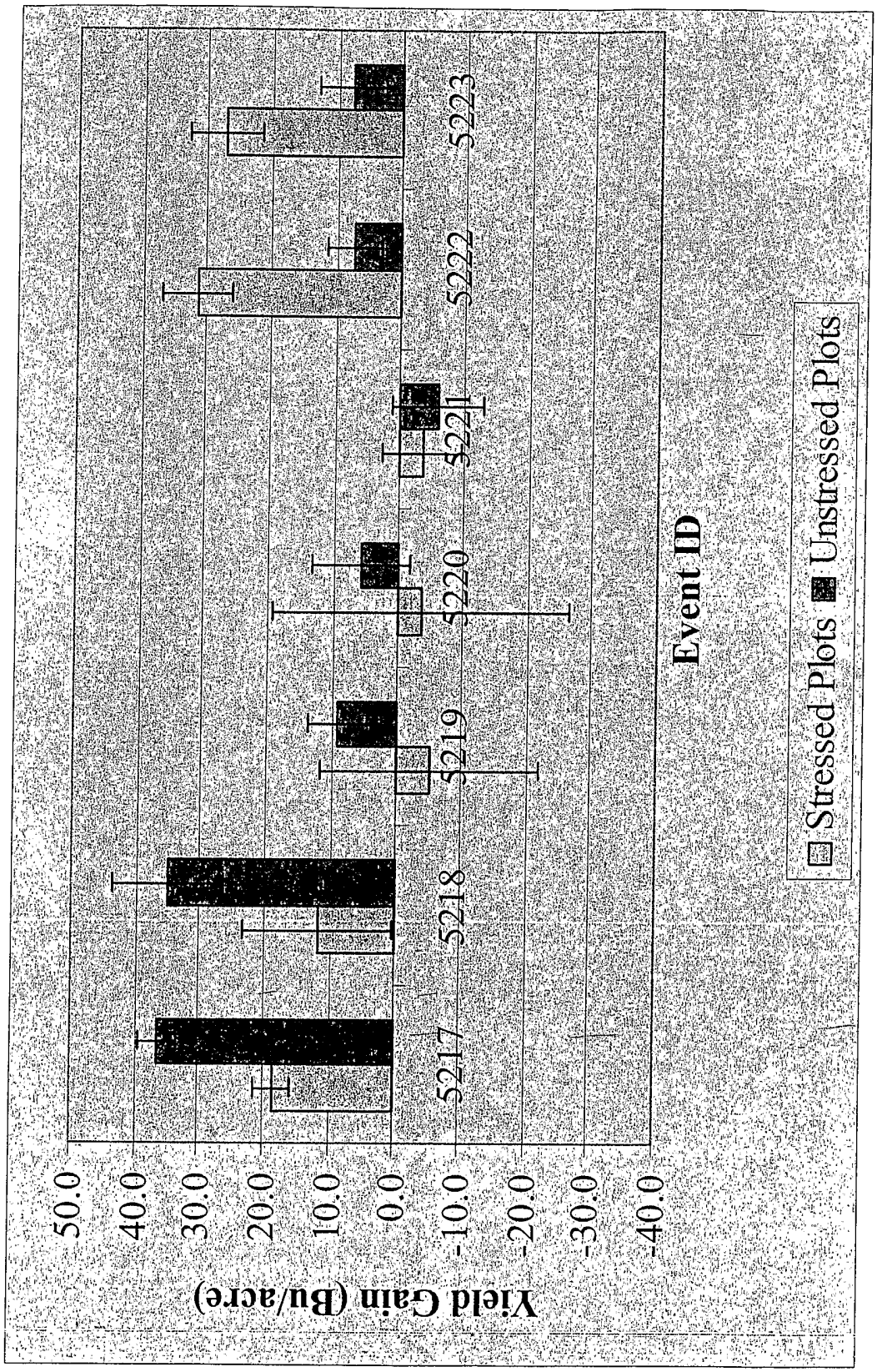

Yield Gain (Bu/acre)
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
-30.0
-40.0
5217
5218
5219
5220
5221
5222
5223
Event ID
Stressed Plots
Unstressed Plots

Fig 28