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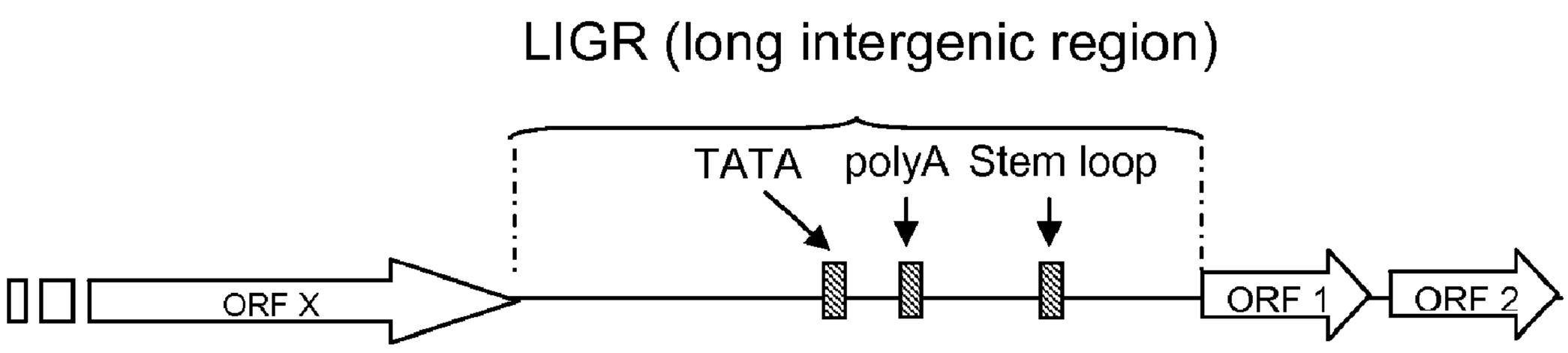
(54) Title: VIRAL PROMOTER, TRUNCATIONS THEREOF, AND METHODS OF USE

Figure 1



(57) Abrégé/Abstract:
The present disclosure provides compositions and methods for regulating expression of heterologous nucleotide sequences in a plant. Compositions include a novel nucleotide sequence for a promoter. A method for expressing a heterologous nucleotide sequence in a plant using the promoter sequence disclosed herein is provided. The method comprises stably incorporating into the genome of a plant cell a nucleotide sequence operably linked to the promoter of the present invention and regenerating a stably transformed plant that expresses the nucleotide sequence.

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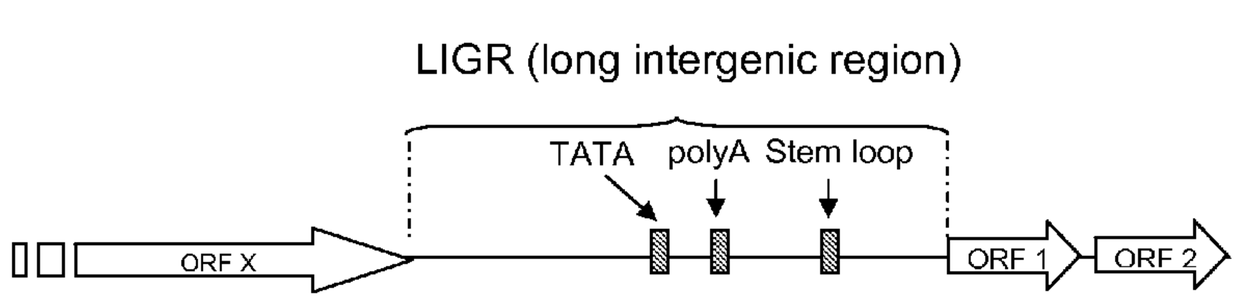
(54) **Title**: VIRAL PROMOTER, TRUNCATIONS THEREOF, AND METHODS OF USE

Figure 1



(57) **Abstract**: The present disclosure provides compositions and methods for regulating expression of heterologous nucleotide sequences in a plant. Compositions include a novel nucleotide sequence for a promoter. A method for expressing a heterologous nucleotide sequence in a plant using the promoter sequence disclosed herein is provided. The method comprises stably incorporating into the genome of a plant cell a nucleotide sequence operably linked to the promoter of the present invention and regenerating a stably transformed plant that expresses the nucleotide sequence.

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VIRAL PROMOTER, TRUNCATIONS THEREOF, AND METHODS OF USE

BACKGROUND OF THE INVENTION

Recent advances in plant genetic engineering have enabled the engineering of plants having improved characteristics or traits, such as disease resistance, insect resistance, herbicide resistance, enhanced stability or shelf-life of the ultimate consumer product obtained from the plants and improvement of the nutritional quality of the edible portions of the plant. Thus, one or more desired genes from a source different than the plant, but engineered to impart different or improved characteristics or qualities, can be incorporated into the plant's genome. One or more new genes can then be expressed in the plant cell to exhibit the desired phenotype such as a new trait or characteristic.

The proper regulatory signals must be present and be in the proper location with respect to the gene in order to obtain expression of the newly inserted gene in the plant cell. These regulatory signals may include a promoter region, a 5' non-translated leader sequence and a 3' transcription termination/polyadenylation sequence.

A promoter is a DNA sequence that directs cellular machinery of a plant to produce RNA from the contiguous coding sequence downstream (3') of the promoter. The promoter region influences the rate, developmental stage, and cell type in which the RNA transcript of the gene is made. The RNA transcript is processed to produce messenger RNA (mRNA) which serves as a template for translation of the RNA sequence into the amino acid sequence of the encoded polypeptide. The 5' non-translated leader sequence is a region of the mRNA upstream of the protein coding region that may play a role in initiation and translation of the mRNA. The 3' transcription termination/polyadenylation signal is a non-translated region downstream of the protein coding region that functions in the plant cells to cause termination of the RNA transcript and the addition of polyadenylate nucleotides to the 3' end of the RNA.

Expression of heterologous DNA sequences in a plant host is dependent upon the presence of an operably linked promoter that is functional within the plant host. The type of promoter sequence chosen is based on when and where within the organism expression of the heterologous DNA is desired. Where expression

in specific tissues or organs is desired, tissue-preferred promoters may be used. Where gene expression in response to a stimulus is desired, inducible promoters are the regulatory element of choice. In contrast, where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized.

An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed. The inducer can be a chemical agent, such as a metabolite, growth regulator, herbicide or phenolic compound, or a physiological stress directly imposed upon the plant such as cold, heat, salt, toxins. In the case of fighting plant pests, it is also desirable to have a promoter which is induced by plant pathogens, including plant insect pests, nematodes or disease agents such as a bacterium, virus or fungus. Contact with the pathogen will induce activation of transcription, such that a pathogen-fighting protein will be produced at a time when it will be effective in defending the plant. A pathogen-induced promoter may also be used to detect contact with a pathogen, for example by expression of a detectable marker, so that the need for application of pesticides can be assessed. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating, or by exposure to the operative pathogen.

A constitutive promoter is a promoter that directs expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of some constitutive promoters that are widely used for inducing the expression of heterologous genes in transgenic plants include the nopaline synthase (NOS) gene promoter, from *Agrobacterium tumefaciens*, (U.S. Patent No. 5,034,322), the cauliflower mosaic virus (CaMv) 35S and 19S promoters (U.S. Patent No. 5,352,605), those derived from any of the several actin genes, which are known to be expressed in most cells types (U.S. Patent No. 6,002,068), and the ubiquitin promoter (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689), which is a gene product known to accumulate in many cell types.

Additional regulatory sequences upstream and/or downstream from the core promoter sequence may be included in expression constructs of

transformation vectors to bring about varying levels of expression of heterologous nucleotide sequences in a transgenic plant. Genetically altering plants through the use of genetic engineering techniques to produce plants with useful traits thus requires the availability of a variety of promoters.

In order to maximize the commercial application of transgenic plant technology, it is important to direct the expression of the introduced DNA in a site-specific manner. For example, it is desirable to produce toxic defensive compounds in tissues subject to pathogen attack, but not in tissues that are to be harvested and eaten by consumers. By site-directing the synthesis or storage of desirable proteins or compounds, plants can be manipulated as factories, or production systems, for a tremendous variety of compounds with commercial utility. Cell-specific promoters provide the ability to direct the synthesis of compounds, spatially and temporally, to highly specialized tissues or organs, such as roots, leaves, vascular tissues, embryos, seeds, or flowers.

Alternatively, it might be desirable to inhibit expression of a native DNA sequence within a plant's tissues to achieve a desired phenotype. In this case, such inhibition might be accomplished with transformation of the plant using siRNA or antisense technology.

Since the patterns of expression of a chimeric gene introduced into a plant are controlled using promoters, there is an ongoing interest in the isolation and identification of novel promoters which are capable of controlling gene expression.

SUMMARY OF THE INVENTION

Compositions and methods for regulating gene expression in a plant are provided. Compositions comprise novel nucleotide sequences for a promoter that initiates transcription in a constitutive manner, while also driving very high levels of expression in selected tissues. More specifically, a transcriptional initiation region isolated from a banana streak virus (Mysore) (BSV(MYS))isolate is provided. Further embodiments of the invention comprise the nucleotide sequences set forth in SEQ ID NOs:1-3 and the promoter sequence deposited as Patent Deposit No. NRRL B-50311 with the Agricultural Research Service (ARS) Culture Collection, housed in the Microbial Genomics and Bioprocessing Research Unit of the National Center for Agricultural Utilization Research (NCAUR), under the

Budapest Treaty provisions. Variations of the full length promoter were also created and tested, including a truncated version and a repeated (or duplicated) version of the promoter. The compositions of the embodiments further comprise nucleotide sequences having at least 70% sequence identity to the sequences set forth in SEQ ID NOs:1-3, and which drive expression of an operably linked nucleotide sequence. Also included are nucleotide sequences that hybridize under stringent conditions to either the sequences set forth in SEQ ID NO: 1-3. or to the promoter sequence deposited in bacterial hosts as Patent Deposit No. NRRL B-50311, or their complements.

Compositions also include DNA constructs comprising a promoter of the embodiments operably linked to a heterologous nucleotide sequence of interest wherein said promoter is capable of driving expression of said nucleotide sequence in a plant cell and said promoter comprises the nucleotide sequences of the embodiments. The embodiments further provide expression vectors, and plants or plant cells having stably incorporated into their genomes a DNA construct mentioned above. Additionally, compositions include transgenic seed of such plants.

Methods of the embodiments comprise a means for selectively expressing a nucleotide sequence in a plant, comprising transforming a plant cell with a DNA construct, and regenerating a transformed plant from said plant cell, said DNA construct comprising a promoter and a heterologous nucleotide sequence operably linked to said promoter, wherein said promoter initiates transcription of said nucleotide sequence in a plant cell. In this manner, the promoter sequences are useful for controlling the expression of operably linked coding sequences.

Downstream from and under the transcriptional initiation regulation of the promoter will be a sequence of interest that will provide for modification of the phenotype of the plant. Such modification includes modulating the production of an endogenous product, as to amount, relative distribution, or the like, or production of an exogenous expression product to provide for a novel function or product in the plant. For example, a heterologous nucleotide sequence that encodes a gene product that confers pathogen, herbicide, salt, cold, drought, or insect resistance is encompassed.

In a further aspect, disclosed methods relate to a method for modulating expression in selected tissues of a stably transformed plant comprising the steps of (a) transforming a plant cell with a DNA construct comprising the promoter of the embodiments operably linked to at least one nucleotide sequence; (b) growing the plant cell under plant growing conditions and (c) regenerating a stably transformed plant from the plant cell wherein expression of the nucleotide sequence alters the phenotype of the plant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the general structure of the Caulimoviridae long intergenic region. The 1184 base pair sequence referred to herein as the BSV(MYS) full-length promoter was identified from this kind of region. The positions of the TATA box, polyA signal, stem loop and ORF regions are indicated.

Figure 2 is a schematic diagram of the full length 1184 bp promoter sequence as well as the 521 base pair truncation that was developed from the full length promoter sequence, and the duplicate promoter that was created. FL= full-length; TR= truncated; Dup= duplication of region upstream of putative TATA box.

DETAILED DESCRIPTION OF THE INVENTION

The compositions of the embodiments comprise novel nucleotide sequences for promoters, particularly a promoter obtained from a BSV (MYS) isolate. The embodiments provide for isolated nucleic acid molecules comprising the nucleotide sequences set forth in SEQ ID NOs:1-3 and the promoter sequence deposited in bacterial hosts as Patent Deposit No. NRRL B-50311 on July 30, 2009 and fragments, variants, and complements thereof.

A deposit of the BSV(MYS) promoter was made on July 30, 2009 with the Agricultural Research Service (ARS) Culture Collection, housed in the Microbial Genomics and Bioprocessing Research Unit of the National Center for Agricultural Utilization Research (NCAUR), under the Budapest Treaty provisions. The deposit was given the following accession number: NRRL B-50311. The address of NCAUR is 1815 N. University Street, Peoria, IL, 61604. This deposit will be maintained under the terms of the Budapest Treaty on the International

Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112. The deposit will irrevocably and without restriction or condition be available to the public upon issuance of a patent. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

The promoter sequences of the embodiments are useful for expressing operably linked nucleotide sequences. Particularly, the promoter of the embodiments, when used in conjunction with the maize AdhI intron, drives constitutive expression while also driving expression in selected tissues of the plant. The full length promoter drives expression in root, leaf, tassel, silk, and stalk tissues. In stalks, expression occurs in both nodal and internodal tissue. Expression in pollen was very low. As described further in the Examples section of this disclosure, the expression pattern varies somewhat for expression using the truncated and duplicate promoters.

The sequences of the embodiments find use in the construction of expression vectors for subsequent transformation into plants of interest, as molecular markers, and the like. The BSV(MYS) promoter sequence of the embodiments directs expression of operably linked nucleotide sequences in a constitutive manner. Therefore, the BSV(MYS) promoter sequence finds use in the constitutive expression of an operably linked nucleotide sequence of interest, while also driving expression in selected tissues. The specific method used to obtain the BSV(MYS) promoter of the present embodiments is described in Example 1 appearing in the Examples section of this application.

The embodiments encompass isolated or substantially purified nucleic acid compositions. An “isolated” or “purified” nucleic acid molecule, or biologically active portion thereof, is substantially free of other cellular material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. An “isolated” nucleic acid is essentially free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which

the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived.

The compositions of the embodiments include isolated nucleic acid molecules comprising the promoter nucleotide sequences set forth in SEQ ID NOs:1-3. The term “promoter” is intended to mean a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. It is recognized that having identified the nucleotide sequences for the promoter regions disclosed herein, it is within the state of the art to isolate and identify further regulatory elements in the 5' untranslated region upstream from the particular promoter regions identified herein. Thus, for example, the promoter regions disclosed herein may further comprise upstream regulatory elements such as those responsible for tissue and temporal expression of the coding sequence, enhancers, and the like. See particularly Australian Patent No. AU-A-77751/94 and U.S. Patent Nos. 5,466,785 and 5,635,618. In the same manner, the promoter elements that enable expression in desired tissues, can be identified, isolated, and used with other core promoters. In this aspect of the embodiments, a “core promoter” is intended to mean a promoter without promoter elements.

In the context of this disclosure, the term "regulatory element" also refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which includes sequences which control the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. An example of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter. A promoter comprises a core promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as discussed elsewhere in this application) that modify gene expression. It is to be understood that nucleotide

sequences, located within introns, or 3' of the coding region sequence may also contribute to the regulation of expression of a coding region of interest. Examples of suitable introns include, but are not limited to, the maize IVS6 intron, or the maize actin intron. A regulatory element may also include those elements located downstream (3') to the site of transcription initiation, or within transcribed regions, or both. In the context of this disclosure, a post-transcriptional regulatory element may include elements that are active following transcription initiation, for example translational and transcriptional enhancers, translational and transcriptional repressors, and mRNA stability determinants.

The regulatory elements, or fragments thereof, of the embodiments may be operatively associated with heterologous regulatory elements or promoters in order to modulate the activity of the heterologous regulatory element. Such modulation includes enhancing or repressing transcriptional activity of the heterologous regulatory element, modulating post-transcriptional events, or both enhancing or repressing transcriptional activity of the heterologous regulatory element and modulating post-transcriptional events. For example, one or more regulatory elements, or fragments thereof, of the embodiments may be operatively associated with constitutive, inducible, or tissue preferred promoters or fragments thereof, to modulate the activity of such promoters within desired tissues within plant cells.

Any of the promoter sequences of the embodiments, when assembled within a DNA construct such that the promoter is operably linked to a nucleotide sequence of interest, enables expression of the nucleotide sequence in the cells of a plant stably transformed with this DNA construct. The term “operably linked” is intended to mean that the transcription or translation of the heterologous nucleotide sequence is under the influence of the promoter sequence. “Operably linked” is also intended to mean the joining of two nucleotide sequences such that the coding sequence of each DNA fragment remain in the proper reading frame. In this manner, the nucleotide sequences for the promoters of the embodiments are provided in DNA constructs along with the nucleotide sequence of interest, typically a heterologous nucleotide sequence, for expression in the plant of interest. The term “heterologous nucleotide sequence” is intended to mean a sequence that is not naturally operably linked with the promoter sequence. While

this nucleotide sequence is heterologous to the promoter sequence, it may be homologous, or native; or heterologous, or foreign, to the plant host.

It is recognized that the promoters of the embodiments thereof may be used with their native coding sequences to increase or decrease expression, thereby resulting in a change in phenotype of the transformed plant.

Modifications of the isolated promoter sequences of the embodiments can provide for a range of expression of the heterologous nucleotide sequence. Thus, they may be modified to be weak promoters or strong promoters. Generally, a "weak promoter" is intended to mean a promoter that drives expression of a coding sequence at a low level. A "low level" of expression is intended to mean expression at levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

Fragments and variants of the disclosed promoter sequences are also encompassed. A “fragment” is intended to mean a portion of the promoter sequence. Fragments of a promoter sequence may retain biological activity and hence encompass fragments capable of driving constitutive expression of an operably linked nucleotide sequence. Thus, for example, less than the entire promoter sequence disclosed herein may be utilized to drive expression of an operably linked nucleotide sequence of interest, such as a nucleotide sequence encoding a heterologous protein. It is within skill in the art to determine whether such fragments decrease expression levels or alter the nature of expression, i.e., constitutive or inducible expression. Alternatively, fragments of a promoter nucleotide sequence that are useful as hybridization probes, such as described below, generally do not retain this regulatory activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length of the nucleotide sequences disclosed herein.

Thus, a fragment of the BSV(MYS) promoter nucleotide sequence may be a biologically active portion of the BSV(MYS) promoter or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of the BSV(MYS) promoter can be prepared

by isolating a portion of one of the BSV(MYS) promoter nucleotide sequences and assessing the activity of that portion of the BSV(MYS) promoter. Nucleic acid molecules that are fragments of a promoter nucleotide sequence comprise at least 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1100 or up to the number of nucleotides present in the full-length promoter nucleotide sequence disclosed herein, e.g. 1184 nucleotides for SEQ ID NO:1. For example, a specific fragment of the BSV(MYS) promoter which retains promoter activity is disclosed in the application as SEQ ID NO: 2. The truncation of the promoter is 521 bp (SEQ ID NO 2) in length.

The nucleotides of such fragments will usually comprise the TATA recognition sequence of the particular promoter sequence. Such fragments may be obtained by use of restriction enzymes to cleave the naturally occurring promoter nucleotide sequence disclosed herein; by synthesizing a nucleotide sequence from the naturally occurring sequence of the promoter DNA sequence; or may be obtained through the use of PCR technology. See particularly, Mullis *et al.* (1987) *Methods Enzymol. 155*:335-350, and Erlich, ed. (1989) *PCR Technology* (Stockton Press, New York). Variants of these promoter fragments, such as those resulting from site-directed mutagenesis and a procedure such as DNA "shuffling", are also encompassed by the compositions.

An "analogue" of the regulatory elements of the embodiments includes any substitution, deletion, or addition to the sequence of a regulatory element provided that said analogue maintains at least one regulatory property associated with the activity of the regulatory element of the embodiments. Such properties include directing organ or tissue preference, or a combination thereof, or temporal activity, or developmental activity, or a combination thereof.

The term “variants” is intended to mean sequences having substantial similarity with a promoter sequence disclosed herein. For nucleotide sequences, naturally occurring variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis. Generally, variants of a particular nucleotide sequence will have at least 40%, 50%, 60%, 65%, 70%,

generally at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, to 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters. Biologically active variants are also encompassed. Biologically active variants include, for example, the native promoter sequence having one or more nucleotide substitutions, deletions, or insertions. Variants can also include promoters that are comprised of repeated units of the full length promoter of the embodiments, or from selected portions of the full length promoter, linked together into a single synthetic promoter. Promoter activity may be measured by using techniques such as Northern blot analysis, reporter activity measurements taken from transcriptional fusions, and the like. See, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), hereinafter "Sambrook," herein incorporated by reference. Alternatively, levels of a reporter gene such as green fluorescent protein (GFP) or the like produced under the control of a promoter fragment or variant can be measured. See, for example, U.S. Patent No. 6,072,050, herein incorporated by reference.

Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* *82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein.

Variant promoter nucleotide sequences also encompass sequences derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different promoter sequences can be manipulated to create a new promoter possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri et al. (1997) *Nature Biotech.* 15:436-438; Moore et al. (1997) *J. Mol. Biol.* 272:336-347; Zhang et al. (1997) *Proc. Natl. Acad. Sci.*

USA 94:4504-4509; Crameri et al. (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the embodiments can be used to isolate corresponding sequences from other organisms, particularly other plants, for example, other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequence set forth herein. Sequences isolated based on their sequence identity to the entire BSV(MYS) promoter sequence set forth herein or to fragments thereof are encompassed. The promoter regions of the embodiments may be isolated from any plant, including, but not limited to corn (*Zea mays*), *Brassica* (*Brassica napus, Brassica rapa ssp.*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea spp.*), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus spp.*), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa spp.*), avocado (*Persea americana*), fig (*Ficus casica),* guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), oats, barley, vegetables, ornamentals, and conifers. Plants include corn, soybean, sunflower, safflower, *Brassica* or canola, wheat, barley, rye, alfalfa, and sorghum.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook, *supra*. See also Innis *et al*., eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the BSV(MYS) promoter sequences of the embodiments. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook, *supra*.

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, often less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to

60 °C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 to 65 °C for at least 30 minutes. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the thermal melting point (T_m) can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem*. *138*:267-284: T_m = 81.5 °C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1 °C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the T_m for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the T_m; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the T_m; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the T_m. Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current*

Protocols in Molecular Biology, Chapter 2 (Greene Publishing and Wiley-Interscience, New York), hereinafter "Ausubel". See also Sambrook *supra*.

Thus, isolated sequences that have promoter activity and which hybridize under stringent conditions to the BSV(MYS) promoter sequences disclosed herein, or to fragments thereof, are encompassed.

In general, sequences that have promoter activity and hybridize to the promoter sequences disclosed herein will be at least 40% to 50% homologous, about 60% to 70% homologous, and even about 80%, 85%, 90%, 95% to 98% homologous or more with the disclosed sequences. That is, the sequence similarity of sequences may range, sharing at least about 40% to 50%, about 60% to 70%, and even about 80%, 85%, 90%, 95% to 98% sequence similarity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller

(1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al*. (1981) *Adv. Appl. Math. 2*:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 87:*2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0); the ALIGN PLUS program (Version 3.0, copyright 1997): and GAP, BESTFIT, BLAST, FASTA, agnd TFASTA in the Wisconsin Genetics Software Package of Genetics Computer Group, Version 10 (available from Accelrys, 9685 Scranton Road, San Diego, CA, 92121, USA). The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al*. (1988) *Gene 73*:237-244 (1988); Higgins *et al*. (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res*. *16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth*. *Mol*. *Biol*. *24*:307-331. The ALIGN and the ALIGN PLUS programs are based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the embodiments. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the embodiments. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al*. (1997) *Nucleic Acids Res*. *25*:3389. Alternatively, PSI-

BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al*. (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See the web site for the National Center for Biotechnology Information on the world wide web. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the GAP program with default parameters, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP.

The GAP program uses the algorithm of Needleman and Wunsch (1970) *supra*, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two

sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, at least 80%, 90%, or 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine

corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, 70%, 80%, 90%, or 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5 °C lower than the T_m for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1 °C to about 20 °C lower than the T_m, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

The BSV(MYS) promoter sequence disclosed herein, as well as variants and fragments thereof, are useful for genetic engineering of plants, e.g. for the production of a transformed or transgenic plant, to express a phenotype of interest. As used herein, the terms "transformed plant" and "transgenic plant" refer to a plant that comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome of a transgenic or transformed plant such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct. It is to be understood that as used herein the term "transgenic" includes any cell, cell line, callus, tissue, plant part, or plant the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as

random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

A transgenic “event” is produced by transformation of plant cells with a heterologous DNA construct, including a nucleic acid DNA construct that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. The term “event” also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of same. Parts of transgenic plants are to be understood within the scope of the embodiments to comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, ovules, leaves, or roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention, and therefore consisting at least in part of transgenic cells.

As used herein, the term "plant cell" includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the embodiments is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

The promoter sequences and methods disclosed herein are useful in regulating expression of any heterologous nucleotide sequence in a host plant. Thus, the heterologous nucleotide sequence operably linked to the promoters disclosed herein may be a structural gene encoding a protein of interest. Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for

transformation will change accordingly. General categories of genes of interest for the embodiments include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding proteins conferring resistance to abiotic stress, such as drought, temperature, salinity, and toxins such as pesticides and herbicides, or to biotic stress, such as attacks by fungi, viruses, bacteria, insects, and nematodes, and development of diseases associated with these organisms. Various changes in phenotype are of interest including modifying expression of a gene in a specific plant tissue, altering a plant's pathogen or insect defense mechanism, increasing the plant's tolerance to herbicides, altering tissue development to respond to environmental stress, and the like. The results can be achieved by providing expression of heterologous or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes, transporters, or cofactors, or affecting nutrients uptake in the plant. These changes result in a change in phenotype of the transformed plant.

It is recognized that any gene of interest can be operably linked to the promoter sequences disclosed herein and expressed in plant tissues.

A DNA construct comprising one of these genes of interest can be used with transformation techniques, such as those described below, to create disease or insect resistance in susceptible plant phenotypes or to enhance disease or insect resistance in resistant plant phenotypes. Accordingly, this disclosure encompasses methods that are directed to protecting plants against fungal pathogens, bacteria, viruses, nematodes, insects, and the like. By "disease resistance" or "insect resistance" is intended that the plants avoid the harmful symptoms that are the outcome of the plant-pathogen interactions.

Disease resistance and insect resistance genes such as lysozymes, cecropins, maganins, or thionins for antibacterial protection, or the pathogenesis-related (PR) proteins such as glucanases and chitinases for anti-fungal protection, or *Bacillus thuringiensis* endotoxins, protease inhibitors, collagenases, lectins, and

glycosidases for controlling nematodes or insects are all examples of useful gene products.

Pathogens of the embodiments include, but are not limited to, viruses or viroids, bacteria, insects, nematodes, fungi, and the like. Viruses include tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc. Nematodes include parasitic nematodes such as root knot, cyst, and lesion nematodes, etc.

Genes encoding disease resistance traits include detoxification genes, such as against fumonisin (U.S. Patent No. 5,792,931) avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science 266*:789; Martin *et al.* (1993) *Science 262*:1432; Mindrinos *et al.* (1994) *Cell 78*:1089); and the like.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European corn borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Pat. Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; and Geiser et al. (1986) *Gene* 48:109); lectins (Van Damme et al. (1994) *Plant Mol. Biol.* 24:825); and the like.

Herbicide resistance traits may be introduced into plants by genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (*e.g.*, the acetolactate synthase (ALS) gene containing mutations leading to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or BASTA® (glufosinate) (*e.g.*, the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide BASTA®, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

Glyphosate resistance is imparted by mutant 5-enolpyruvl-3-phosphikimate synthase (EPSP) and *aroA* genes. See, for example, U.S. Patent No. 4,940,835, which discloses the nucleotide sequence of a form of EPSPS which can confer glyphosate resistance. U.S. Patent No. 5,627,061 also describes genes encoding EPSPS enzymes. See also U.S. Patent Nos. 6,248,876; 6,040,497; 5,804,425; 5,633,435; 5,145,783; 4,971,908; 5,312,910; 5,188,642; 4,940,835; 5,866,775;

6,225,114; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; RE 36,449; RE 37,287; and 5,491,288; and international publications WO 97/04103; WO 97/04114; WO 00/66746; WO 01/66704; WO 00/66747 and WO 00/66748, which are incorporated herein by reference for this purpose. Glyphosate resistance is also imparted to plants that express a gene that encodes a glyphosate oxido-reductase enzyme as described more fully in U.S. Patent Nos. 5,776,760 and 5,463,175, which are incorporated herein by reference for this purpose. In addition glyphosate resistance can be imparted to plants by the over-expression of genes encoding glyphosate N-acetyltransferase. See, for example, U.S. Patent Application Serial Nos. 10/004,357; and 10/427,692.

Sterility genes can also be encoded in a DNA construct and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in U.S. Patent No. 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-ketothiolase, PHBase (polyhydroxyburyrate synthase), and acetoacetyl-CoA reductase (see Schubert et al. (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs).

Agronomically important traits that affect quality of grain, such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, levels of cellulose, starch, and protein content can be genetically altered using the methods of the embodiments. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and modifying starch. Hordothionin protein modifications in corn are described in U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802 and 5,703,049; herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Patent No. 5,850,016, and the chymotrypsin inhibitor from

barley, Williamson *et al*. (1987) *Eur. J. Biochem.* 165:99-106, the disclosures of which are herein incorporated by reference.

Exogenous products include plant enzymes and products as well as those from other sources including prokaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like.

Examples of other applicable genes and their associated phenotype include the gene that encodes viral coat protein and/or RNA, or other viral or plant genes that confer viral resistance; genes that confer fungal resistance; genes that confer insect resistance; genes that promote yield improvement; and genes that provide for resistance to stress, such as dehydration resulting from heat and salinity, toxic metal or trace elements, or the like.

"RNAi" refers to a series of related techniques to reduce the expression of genes (See for example U.S. Patent No. 6,506,559). Older techniques referred to by other names are now thought to rely on the same mechanism, but are given different names in the literature. These include "antisense inhibition," the production of antisense RNA transcripts capable of suppressing the expression of the target protein, and "co-suppression" or "sense-suppression," which refer to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference). Such techniques rely on the use of constructs resulting in the accumulation of double stranded RNA with one strand complementary to the target gene to be silenced. The BSV(MYS) promoter sequence of the embodiments, and its related biologically active fragments or variants disclosed herein, may be used to drive expression of constructs that will result in RNA interference including microRNAs and siRNAs.

The heterologous nucleotide sequence operably linked to the BSV(MYS) promoter and related promoter sequences disclosed herein may be an antisense sequence for a targeted gene. The terminology "antisense DNA nucleotide sequence" is intended to mean a sequence that is in inverse orientation to the 5′-to-3′ normal orientation of that nucleotide sequence. When delivered into a plant cell, expression of the antisense DNA sequence prevents normal expression of the DNA nucleotide sequence for the targeted gene. The antisense nucleotide sequence encodes an RNA transcript that is complementary to and capable of

hybridizing to the endogenous messenger RNA (mRNA) produced by transcription of the DNA nucleotide sequence for the targeted gene. In this case, production of the native protein encoded by the targeted gene is inhibited to achieve a desired phenotypic response. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having at least 70%, 80%, or 85% or more sequence identity to the corresponding antisense sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used. Thus, the promoter sequences disclosed herein may be operably linked to antisense DNA sequences to reduce or inhibit expression of a native protein in selected plant tissues.

In one embodiment, DNA constructs will comprise a transcriptional initiation region comprising one of the promoter nucleotide sequences disclosed herein, or variants or fragments thereof, operably linked to a heterologous nucleotide sequence whose expression is to be controlled by the promoter of the embodiments. Such a DNA construct is provided with a plurality of restriction sites for insertion of the nucleotide sequence to be under the transcriptional regulation of the regulatory regions. The DNA construct may additionally contain selectable marker genes.

The DNA construct will include in the 5'-3' direction of transcription, a transcriptional initiation region (i.e., a promoter of the embodiments), translational initiation region, a heterologous nucleotide sequence of interest, a translational termination region and, optionally, a transcriptional termination region functional in the host organism. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotide of the embodiments may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the polynucleotide of the embodiments may be heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention.

For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

The optionally included termination region may be native with the transcriptional initiation region, may be native with the operably linked polynucleotide of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the polynucleotide of interest, the host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639. In particular embodiments, the potato protease inhibitor II gene (PinII) terminator is used. See, for example, Keil *et al*. (1986) *Nucl. Acids Res*. 14:5641-5650; and An *et al*. (1989) *Plant Cell* 1:115-122, herein incorporated by reference in their entirety.

The DNA construct comprising a promoter sequence of the embodiments operably linked to a heterologous nucleotide sequence may also contain at least one additional nucleotide sequence for a gene to be cotransformed into the organism. Alternatively, the additional sequence(s) can be provided on another DNA construct.

Where appropriate, the heterologous nucleotide sequence whose expression is to be under the control of the promoter sequence of the embodiments and any additional nucleotide sequence(s) may be optimized for increased expression in the transformed plant. That is, these nucleotide sequences can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred nucleotide sequences. See, for example, U.S. Patent Nos. 5,380,831 and

5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the heterologous nucleotide sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The DNA constructs may additionally contain 5′ leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5′ noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Nat. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986) *Virology 154*:9-20); MDMV leader (Maize Dwarf Mosaic Virus); human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) *Molecular Biology of RNA*, pages 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also Della-Cioppa *et al.* (1987) *Plant Physiology 84*:965-968. Other methods known to enhance translation and/or mRNA stability can also be utilized, for example, introns, such as the maize Ubiquitin intron (Christensen and Quail (1996) *Transgenic Res.* 5:213-218; Christensen *et al.* (1992) *Plant Molecular Biology* 18:675-689) or the maize Adhl intron (Kyozuka *et al.* (1991) *Mol. Gen. Genet.* 228:40-48; Kyozuka *et al.* (1990) *Maydica* 35:353-357), and the like.

The DNA constructs of the embodiments can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure

translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the regulatory element selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. It is recognized that to increase transcription levels enhancers may be utilized in combination with the promoter regions of the disclosure. Enhancers are known in the art and include the SV40 enhancer region, the 35S enhancer element, and the like.

In preparing the DNA construct, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites. Restriction sites may be added or removed, superfluous DNA may be removed, or other modifications of the like may be made to the sequences of the embodiments. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, re-substitutions, for example, transitions and transversions, may be involved.

Reporter genes or selectable marker genes may be included in the DNA constructs. Examples of suitable reporter genes known in the art can be found in, for example, Jefferson *et al.* (1991) in *Plant Molecular Biology Manual*, ed. Gelvin *et al.* (Kluwer Academic Publishers), pp. 1-33; DeWet *et al.* (1987) *Mol. Cell. Biol. 7*:725-737; Goff *et al.* (1990) *EMBO J. 9*:2517-2522; Kain *et al.* (1995) *BioTechniques 19*:650-655; and Chiu *et al.* (1996) *Current Biology 6*:325-330.

Selectable marker genes for selection of transformed cells or tissues can include genes that confer antibiotic resistance or resistance to herbicides. Examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to chloramphenicol (Herrera Estrella *et al.* (1983) *EMBO J. 2*:987-992); methotrexate (Herrera Estrella *et al.* (1983) *Nature 303*:209-213; Meijer *et al.* (1991) *Plant Mol. Biol. 16*:807-820); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol. 5*:103-108; Zhijian *et al.* (1995) *Plant Science 108*:219-227); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet. 210*:86-91); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res. 5*:131-137);

bleomycin (Hille *et al.* (1990) *Plant Mol. Biol. 7*:171-176); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Biol. 15*:127-136); bromoxynil (Stalker *et al.* (1988) *Science 242*:419-423); glyphosate (Shaw *et al.* (1986) *Science 233*:478-481); phosphinothricin (DeBlock *et al.* (1987) *EMBO J. 6*:2513-2518).

Other genes that could serve utility in the recovery of transgenic events but might not be required in the final product would include, but are not limited to, examples such as GUS (b-glucuronidase; Jefferson (1987) *Plant Mol. Biol. Rep. 5*:387), GFP (green florescence protein; Chalfie *et al.* (1994) *Science 263*:802), luciferase (Riggs *et al.* (1987) *Nucleic Acids Res. 15*(19):8115 and Luehrsen *et al.* (1992) *Methods Enzymol. 216*:397-414), and the maize genes encoding for anthocyanin production (Ludwig *et al.* (1990) *Science 247*:449).

The nucleic acid molecules of the embodiments are useful in methods directed to expressing a nucleotide sequence in a plant. This may be accomplished by transforming a plant cell of interest with a DNA construct comprising a promoter identified herein, operably linked to a heterologous nucleotide sequence, and regenerating a stably transformed plant from said plant cell. The methods of the embodiments are also directed to selectively expressing a nucleotide sequence in a plant tissue. Those methods comprise transforming a plant cell with a DNA construct comprising a promoter identified herein that initiates transcription in a plant cell, operably linked to a heterologous nucleotide sequence, and regenerating a transformed plant from said plant cell.

The DNA construct comprising the particular promoter sequence of the embodiments operably linked to a nucleotide sequence of interest can be used to transform any plant. In this manner, genetically modified, i.e. transgenic or transformed, plants, plant cells, plant tissue, seed, root, and the like can be obtained.

Plant species suitable for the embodiments include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean

(*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the embodiments include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow cedar (C*hamaecyparis nootkatensis*). Plants of the embodiments may be crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.) This disclosure is particularly suitable for any member of the monocot plant family including, but not limited to, maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, yams, onion, banana, coconut, and dates.

As used herein, “vector” refers to a DNA molecule such as a plasmid, cosmid, or bacterial phage for introducing a nucleotide construct, for example, a

DNA construct, into a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, hygromycin resistance, or ampicillin resistance.

The methods of the embodiments involve introducing a nucleotide construct into a plant. The term "introducing" is used herein to mean presenting to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the embodiments do not depend on a particular method for introducing a nucleotide construct to a plant, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

By "stable transformation" is intended that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleotide construct introduced into a plant does not integrate into the genome of the plant.

The nucleotide constructs of the embodiments may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the embodiments within a viral DNA or RNA molecule. Methods for introducing nucleotide constructs into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367, and 5,316,931; herein incorporated by reference.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant

genome include microinjection (Crossway *et al*. (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al*. (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,981,840 and 5,563,055), direct gene transfer (Paszkowski *et al*. (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent Nos. 4,945,050; 5,879,918; 5,886,244; 5,932,782; Tomes *et al*. (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al*. (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al*. (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al*. (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al*. (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al*. (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al*. (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al*. (1990) *Biotechnology 8*:736-740 (rice); Klein *et al*. (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al*. (1988) *Biotechnology 6*:559-563 (maize); U.S. Patent Nos. 5,240,855; 5,322,783 and 5,324,646; Klein *et al*. (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al*. (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al*. (1984) *Nature (London) 311*:763-764; U.S. Patent No. 5,736,369 (cereals); Bytebier *et al*. (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al*. (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al*. (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al*. (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al*. (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al*. (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al*. (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al*. (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two

or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. Thus as used herein, “transformed seeds” refers to seeds that contain the nucleotide construct stably integrated into the plant genome.

There are a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated. The regeneration, development and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, (1988) In.: Methods for Plant Molecular Biology, (Eds.), Academic Press, Inc., San Diego, CA). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. The regenerated plants are generally self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the embodiments containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

The embodiments provide compositions for screening compounds that modulate expression within selected tissues of embryos and plants. The vectors, cells, and plants can be used for screening candidate molecules for agonists and antagonists of the BSV(MYS) promoter. For example, a reporter gene can be operably linked to a BSV(MYS) promoter and expressed as a transgene in a plant. Compounds to be tested are added and reporter gene expression is measured to determine the effect on promoter activity.

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

EXPERIMENTAL

The embodiments are further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. Techniques in molecular biology were typically performed as described in Ausubel or Sambrook, *supra*. It should be understood that these Examples, while indicating certain embodiments, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the characteristics of the embodiments, and without departing from the spirit and scope thereof, can make various changes and modifications to the embodiments to adapt them to various usages and conditions. Thus, various modifications of the embodiments in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

Example 1: Banana Streak Virus (Mysore) promoter sequences

The promoter of SEQ ID NO: 1 was obtained through a search of GenBank Genomes for viral genomes that had been sequenced and belonged to the Caulimoviridae virus family. The search was initiated based on the well-known Cauliflower Mosaic Virus 35S (CaMV35S) promoter. It drives constitutive expression of heterologous genes in most tissues of most plants. Other promoters from this virus family, such as the Figwort Mosaic Virus 34S promoter also direct constitutive-like expression in plants. Therefore, additional promoters derived from the Caulimoviridae virus family also may drive constitutive expression in plants. The structure of the Caulimoviridae genome is fairly conserved (See Figure 1). The region of the genome found in what is called the Long Intergenic Region (LIGR) generally contains the regulatory sequences necessary for promoter function in plants.

The BSV(Mysore) genome has an LIGR, so this region was targeted for functional promoter analysis. Two sequences containing the LIGR were selected to be tested in plants. The longest sequence consists of 1184 bp and has a putative TATA box 208 bp from the 3' end of SEQ1 (Figure 2). A putative polyadenylation signal is approximately 131 bp from the 3' end of the sequence

and about 44 bp from a putative transcription start site. The transcription start site is predicted based on results from mapping the BSV(AY) (See U.S. Provisional Application No. 61/184,158) and BSV(AV) (See U.S. Provisional Application No. 61/184,043) promoters. An 11 bp region containing the transcription start site is identical between all 3 promoters. The entire 1184 bp sequence is referred to as the BSV(MYS) full-length promoter, BSV(MYS) FL.

The second sequence is a truncated version of the full-length promoter (See Figure 2). Termed the BSV(MYS) TR promoter, it is 521 bp in length and consists of the 3' end of the full-length promoter. By deleting 663 bp of the 5' end of the promoter, the expression pattern directed in plants may be altered relative to the full-length and thereby provide insight to important regulatory elements in the promoter.

Duplicating promoter regions can also alter expression pattern and even enhance expression directed by a promoter. Duplicating the upstream region of the CaMV35S promoter has been shown to increase expression by approximately tenfold (Kay, R. *et al.*, (1987) 236: 1299-1302). To see what effect duplication might have on the BSV(MYS) TR promoter, 290 bp of the truncated promoter was placed upstream of the 521 bp sequence, creating a promoter that had repeating 290 bp segments upstream of the putative TATA box (BSV(MYS) Dup; See Figure 2). All 3 promoter sequences were synthetically made for cloning into expression vectors.

Example 2: Expression analysis using a reporter gene

The BSV(MYS) FL, BSV(MYS) TR, and BSV(MYS) Dup promoters were operably linked to B-glucuronidase (GUS) gene, with and without the Adh1 intron 1, in an expression vector, to test whether the synthetic DNA fragments would direct expression. The Adh1 intron was included for the purpose of increased expression as it has been shown that in cereal plant cells the expression of transgenes is enhanced by the presence of an intron in gene constructs (See Callis *et al.* (1987) Genes and Development 1: 1183-1200; Kyozuka et al. (1990) Maydica 35:353-357).

The *Ubi-1* promoter from maize (using its own intron) was used as a positive control in the analysis of the BSV(MYS) promoters. It, too, was operably linked to the B-glucuronidase (GUS) gene so that it could be used to compare the

expression pattern and expression levels of the 3 BSV(MYS) promoters. The *Ubi-1* promoter is a strong constitutive promoter in nearly all tissues of maize.

Stable transformed plants were created using Agrobacterium protocols (detailed in Example 3). Ten plants were regenerated for each promoter and promoter x intron combination. The plants were grown under greenhouse conditions until they reached a growth stage ranging from V4 to V6. Vegetative growth stages are determined by the number of collared leaves on the plant. Therefore, a plant at V6 stage has 6 fully collared leaves. Leaf and root tissue were sampled from each plant at this stage. The plants were then allowed to grow to early R1 stage, a point just prior to pollen shed, where silk and stalk (node and internode) and tassel tissue were collected. Finally, pollen was collected when the plants started shedding. Combinations of histochemical staining, quantitative fluorometric assays and qRT-PCR were used to look at expression pattern and expression levels directed by the 3 promoters.

The results of the analysis are highlighted in Tables 1 and 2. Overall, the BSV(MYS) FL promoter performed well in most tissues (Table 1). GUS expression was detected at levels that were equivalent to or below levels directed by the *Ubi-1* maize promoter. The exception was stalk tissue where expression levels exceed levels directed by the *Ubi-1* promoter by 1.5 fold on average. The addition of the Adh1 intron benefited expression mostly in roots (Table 2). The BSV(MYS) FL promoter modestly exceeded *Ubi-1* root expression levels by 1.0 fold.

The BSV(MYS) TR promoter performed at levels that were generally less than the full-length promoter (Table 1). However, expression levels recovered with the addition of the Adh1 intron. In comparison to the BSV(MYS) FL:Adh1 combination, expression levels were higher in stalk, silks and tassels. Expression levels in stalk node relative to the *Ubi-1* promoter were approximately 3.6 fold higher on average. In stalk internode, the expression levels were 1.8 fold higher than *Ubi-1*.

The BSV(MYS) Dup promoter performed similarly to the full-length promoter in most tissues (Table 1). But like the truncated promoter, it performed better with the Adh1 intron present (Table 2). Average expression levels were essentially equivalent to the truncated promoter in root, stalk, tassels and silks. Interestingly, expression in leaves was not influenced. In fact, expression was

mostly constant among all 3 of the BSV(MYS) promoters, with and without the Adh1 intron. The same can be said for pollen, where expression was consistently low.

Table 1: Plant Expression Results for the BSV(MYS) Promoter (no Adh1 Intron 1)

	V5-V6		R1-R2			
	Leaf	**Root**	**Stalk**	**Tassel**	**Silk**	**Pollen**
BSV(MYS) FL	1	3	4	1	0.5	0.1
BSV(MYS) TR	1	2	3	0.5	0.1	0.1
BSV(MYS) Dup	1	3	3	2	0.5	0.1
Ubi-1	2	3	3	3	2	3
untransformed (negative control)	0	0	0	0	0	0

Data expressed on a 0-6 scale with the maize *Ubi-1* promoter representing a median value.

Table 2: Plant Expression Results for the BSV(MYS) Promoter (with Adh1 intron 1)

	V5-V6		R1-R2			
	Leaf	**Root**	**Stalk**	**Tassel**	**Silk**	**Pollen**
BSV(MYS) FL	1	4	4	1	1	0.1
BSV(MYS) TR	1	4	5	2	2	0.1
BSV(MYS) Dup	1	4	5	2	2	0.1
Ubi-1	2	3	3	3	2	3
untransformed (negative control)	0	0	0	0	0	0

Data expressed on a 0-6 scale with the maize *Ubi-1* promoter representing a median value.

Example 3: *Agrobacterium*-Mediated Transformation of Maize and Regeneration of Transgenic Plants

For *Agrobacterium*-mediated transformation of maize with a promoter sequence of the invention, the method of Zhao was employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos were isolated from maize and the embryos contacted with a suspension of *Agrobacterium* under

conditions whereby the bacteria were capable of transferring the promoter sequence of the invention to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos were immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos were co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos were cultured on solid medium following the infection step. Following the co-cultivation period an optional "resting" step was performed. In this resting step, the embryos were incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos were cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos were cultured on medium containing a selective agent and growing transformed callus was recovered (step 4: the selection step). The immature embryos were cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus was then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium were cultured on solid medium to regenerate the plants.

Example 4: Transformation of Maize by Particle Bombardment and Regeneration of Transgenic Plants

Immature maize embryos from greenhouse donor plants are bombarded with a DNA molecule containing a promoter of the embodiments operably linked to a gene of interest. A selectable marker is provided in the same transformation vector, or alternatively, the selectable marker gene is provided on a separate DNA molecule. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are husked and surface sterilized in 30% Clorox™ bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5 cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising a promoter sequence of the embodiments is made. The vector additionally contains a PAT selectable marker gene driven by a CAMV35S promoter and includes a CAMV35S terminator. Optionally, the selectable marker can reside on a separate plasmid. A DNA molecule comprising a promoter sequence of the embodiments as well as a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 μL prepared tungsten particles in water

10 μL (1 μg) DNA in Tris EDTA buffer (1 μg total DNA)

100 μL 2.5 M $CaCl_2$

10 μL 0.1 M spermidine

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

Particle Gun Treatment

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

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/L Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant

regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for expression by assays known in the art, such as, for example, immunoassays and western blotting with an antibody that binds to the protein of interest.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000x SIGMA-1511), 0.5 mg/L thiamine HCl, 120.0 g/L sucrose, 1.0 mg/L 2,4-D, and 2.88 g/L L-proline (brought to volume with dI H_20 following adjustment to pH 5.8 with KOH); 2.0 g/L Gelrite™ (added after bringing to volume with dI H_20); and 8.5 mg/L silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000x SIGMA-1511), 0.5 mg/L thiamine HCl, 30.0 g/L sucrose, and 2.0 mg/L 2,4-D (brought to volume with dI H_20 following adjustment to pH 5.8 with KOH); 3.0 g/L Gelrite™ (added after bringing to volume with dI H_20); and 0.85 mg/L silver nitrate and 3.0 mg/L Bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/L thiamine HCl, 0.10 g/L pyridoxine HCl, and 0.40 g/L Glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/L myo-inositol, 0.5 mg/L zeatin, 60 g/L sucrose, and 1.0 mL/L of 0.1 mM abscisic acid (brought to volume with polished dI H_20 after adjusting to pH 5.6); 3.0 g/L Gelrite™ (added after bringing to volume with dI H_20); and 1.0 mg/L indoleacetic acid and 3.0 mg/L Bialaphos (added after sterilizing the medium and

cooling to 60 ºC). Hormone-free medium (272V) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g/L nicotinic acid, 0.02 g/L thiamine HCl, 0.10 g/L pyridoxine HCl, and 0.40 g/L Glycine brought to volume with polished dI H_20), 0.1 g/L myo-inositol, and 40.0 g/L sucrose (brought to volume with polished dI H_20 after adjusting pH to 5.6); and 6 g/L Bacto-agar (added after bringing to volume with polished dI H_20), sterilized and cooled to 60 ºC.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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

THAT WHICH IS CLAIMED:

1. An isolated nucleic acid molecule comprising a nucleotide sequence that initiates transcription in the plant cell selected from the group consisting of:

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:2; and

b) a nucleotide sequence comprising the promoter sequence comprised in Patent Deposit No. NRRL B-50311.

2. The isolated nucleic acid molecule of claim 1, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 1.

3. The isolated nucleic acid molecule of claim 1, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 3.

4. A DNA construct comprising a nucleotide sequence of claim 1 operably linked to a heterologous nucleotide sequence of interest.

5. A vector comprising the DNA construct of claim 4.

6. A plant cell having stably incorporated into its genome the DNA construct of claim 4.

7. The plant cell of claim 6, wherein said plant cell is from a monocot.

8. The plant cell of claim 7, wherein said monocot is maize.

9. The plant cell of claim 6, wherein said plant cell is from a dicot.

10. A plant having stably incorporated into its genome the DNA construct of claim 4.

11. The plant of claim 10, wherein said plant is a monocot.

12. The plant of claim 11, wherein said monocot is maize.

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

14. A transgenic seed of the plant of claim 10, wherein the seed comprises the DNA construct.

15. The plant of claim 10, wherein the heterologous nucleotide sequence of interest encodes a gene product that confers herbicide, salt, cold, drought, pathogen, or insect resistance.

16. A method for expressing a nucleotide sequence in a plant, said method comprising introducing into a plant a DNA construct, said DNA construct comprising a promoter and operably linked to said promoter a heterologous nucleotide sequence of interest, wherein said promoter comprises a nucleotide sequence selected from the group consisting of:

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:2; and

a) a nucleotide sequence comprising the promoter sequence comprised in Patent Deposit No. NRRL B-50311.

17. The method of claim 16, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 1.

18. The method of claim 16, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 3.

19. The method of claim 16, wherein said plant is maize, and wherein said heterologous nucleotide sequence of interest is selectively expressed in the leaf, root, stalk, silk, and tassel tissues.

20. The method of claim 16, wherein said plant is a dicot.

21. The method of claim 16, wherein said plant is a monocot.

22. The method of claim 22, wherein said monocot is maize.

23. The method of claim 16, wherein the heterologous nucleotide sequence encodes a gene product that confers herbicide, salt, cold, drought, pathogen, or insect resistance.

24. A method for expressing a nucleotide sequence in a plant cell, said method comprising introducing into a plant cell a DNA construct comprising a promoter operably linked to a heterologous nucleotide sequence of interest, wherein said promoter comprises a nucleotide sequence selected from the group consisting of:

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:2; and;

b) a nucleotide sequence comprising the promoter sequence comprised in Patent Deposit No. NRRL B-50311.

25. The method of claim 24, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 1.

26. The method of claim 24, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 3.

27. The method of claim 24, wherein said plant cell is from a monocot.

28. The method of claim 27, wherein said monocot is maize.

29. The method of claim 24, wherein said plant cell is from a dicot.

30. The method of claim 24, wherein the heterologous nucleotide sequence encodes a gene product that confers herbicide, salt, cold, drought, pathogen, or insect resistance.

31. A method for selectively expressing a nucleotide sequence in maize leaf, root, stalk, silk, and tassel tissues, said method comprising introducing into a plant cell a DNA construct, and regenerating a transformed plant from said plant cell, said DNA construct comprising a promoter and a heterologous nucleotide

sequence operably linked to said promoter, wherein said promoter comprises a nucleotide sequence selected from the group consisting of:

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:2; and;

b) a nucleotide sequence comprising the promoter sequence comprised in Patent Deposit No. NRRL B-50311.

32. The method of claim 31, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 1.

33. The method of claim 31, wherein said nucleotide sequence is the sequence set forth in SEQ ID NO: 3.

34. The method of claim 31, wherein expression of said heterologous nucleotide sequence alters the phenotype of said maize plant.

35. The method of claim 31, wherein the heterologous nucleotide sequence encodes a gene product that confers herbicide, salt, cold, drought, pathogen, or insect resistance.

Figure 1

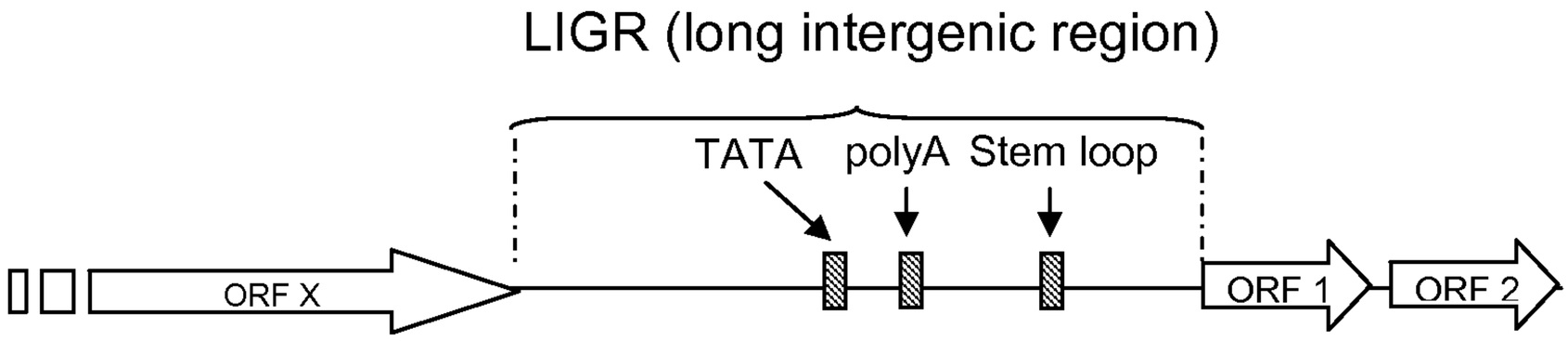

LIGR (long intergenic region)
TATA
polyA
Stem loop
ORF X
ORF 1
ORF 2

Figure 2

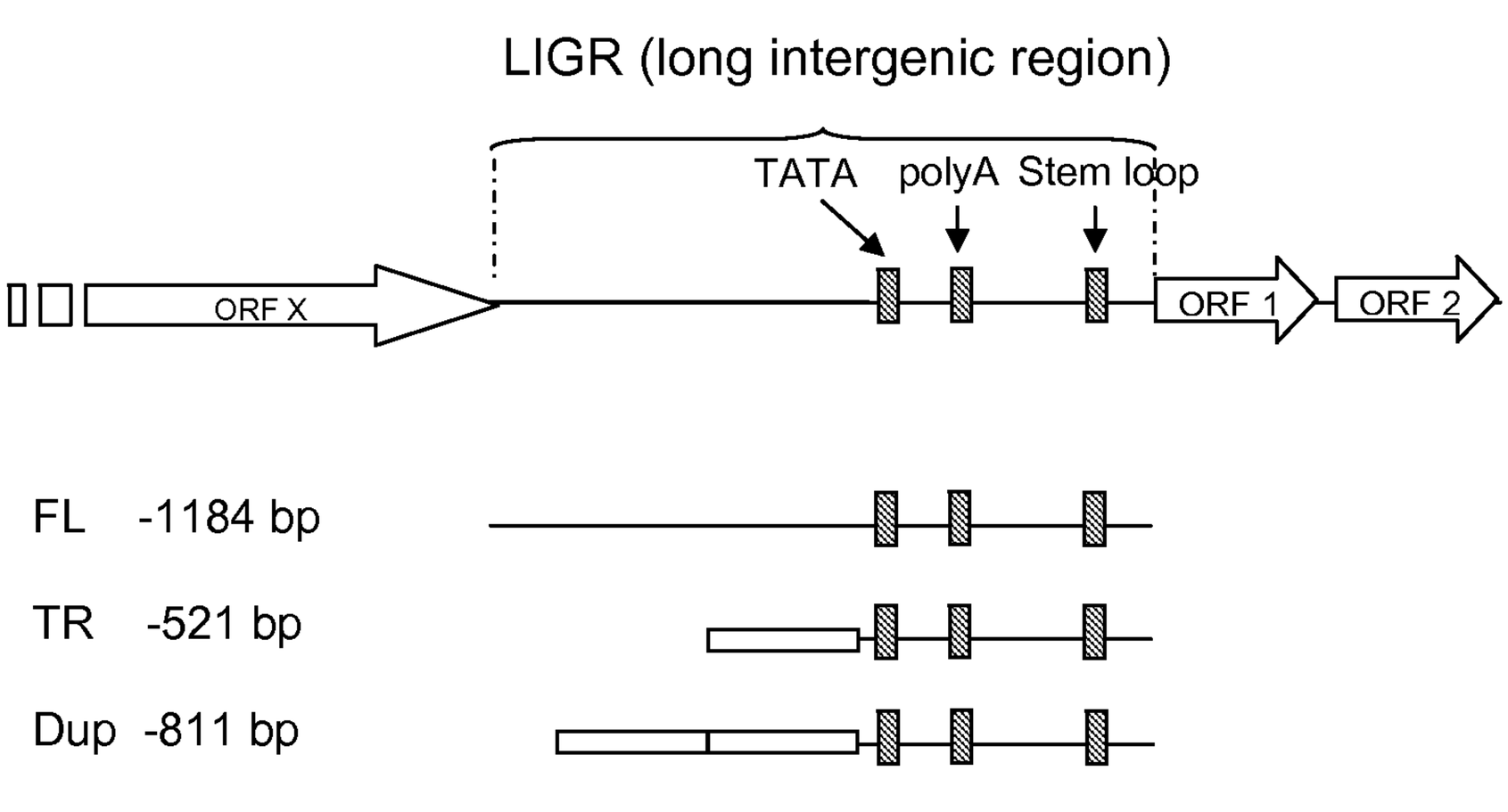
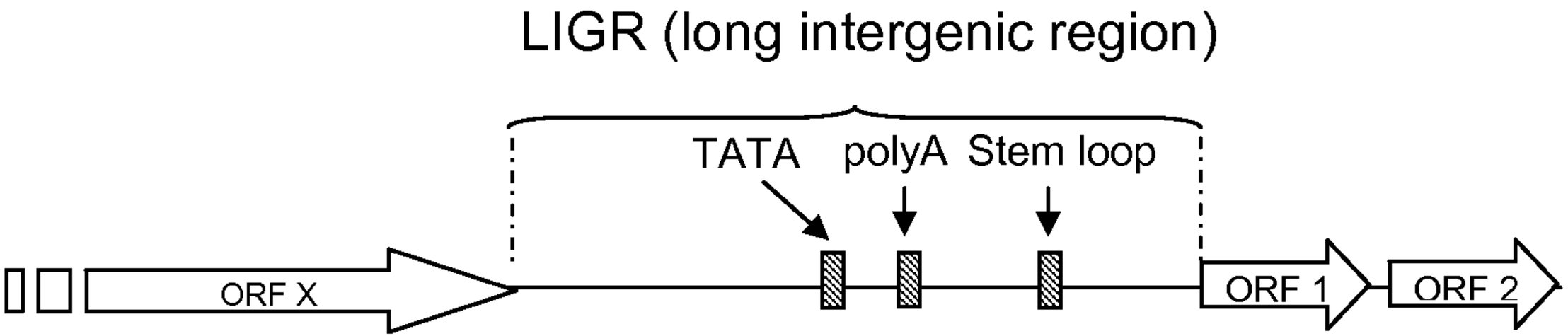

LIGR (long intergenic region)
TATA
polyA
Stem loop
ORF X
ORF 1
ORF 2
FL -1184 bp
TR -521 bp
Dup -811 bp

Figure 1


LIGR (long intergenic region)
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ORF X
ORF 1
ORF 2