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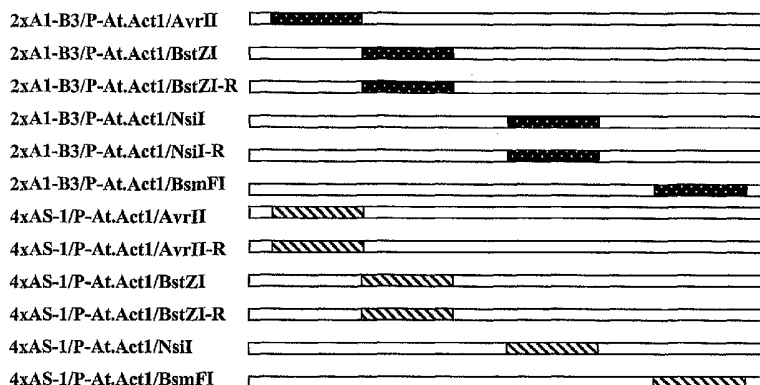
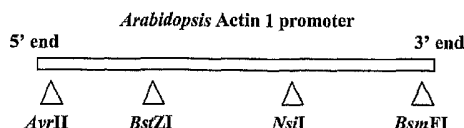
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(54) Title: CHIMERIC PROMOTERS FOR USE IN PLANTS



(57) Abstract: The present invention provides polynucleotide molecules useful for expressing transgenes in plants. The present invention also provides expression constructs containing the polynucleotide molecules useful for expressing transgenes in plants. The present invention also provides transgenic plants and seeds containing the polynucleotide molecules useful for expressing transgenes in plants.



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CHIMERIC PROMOTERS FOR USE IN PLANTS

This application claims the benefit of US application 60/537,793 filed January 20, 2004, incorporated herein by reference in its entirety.

INCORPORATION OF SEQUENCE LISTING

Two copies of the sequence listing (Seq. Listing Copy 1 and Seq. Listing Copy 2) and a computer-readable form of the sequence listing, all on CD-ROMs, each containing the file named pa_01117.rpt, which is 61,440 bytes (measured in MS-DOS) and was created on January 18, 2005 all of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to the field of plant molecular biology and plant genetic engineering and polynucleotide molecules useful for modulating gene expression in plants.

BACKGROUND

One of the goals of plant genetic engineering is to produce plants with agronomically desirable characteristics or traits. The proper expression of a desirable transgene in a transgenic plant is one way to achieve this goal. Promoters are non-coding polynucleotide molecules which play an integral role in the overall expression of genes in living cells. Isolated promoters that function in plants are useful for modifying plant phenotypes through the methods of genetic engineering.

Many constitutive promoters are available and are useful for providing good overall gene expression. For example, constitutive promoters such as P-FMV, the promoter from the 35S transcript of the Figwort mosaic virus, (U.S. Patent No. 6,051,753); P-CaMV 35S, the promoter from the 35S RNA transcript of the Cauliflower mosaic virus, (U.S. Patent No. 5,530,196); P-Rice Actin 1, the promoter from the actin 1 gene of *Oryza sativa*, (U.S. Patent No. 5,641,876); and P-NOS, the promoter from the nopaline synthase gene of *Agrobacterium tumefaciens* are known to provide some level of gene expression in most or all of the tissues of a plant during most or all of the plant's lifespan. Alternately, many promoters are available with more specific

expression patterns such as tissue specificity, temporal specificity, or developmental specificity. These promoters are useful for the targeted expression of a transgene in plants.

Optimal expression of a transgene is useful for producing plants with agronomically desirable characteristics or traits. Such optimal expression often requires a promoter having a specific expression pattern which may not be readily available in known promoters. One example of such a specific expression pattern is a high level of transgene expression in both vegetative and reproductive tissues. The present invention solves this problem by producing novel chimeric promoters containing elements from known promoters. These novel chimeric promoters can then be tested in plants to determine whether the desired expression pattern is indeed achieved.

SUMMARY

In one embodiment the invention provides novel chimeric promoters provided as SEQ ID NO: 9-35 comprising a caulimovirus promoter enhancer fused with a plant actin gene promoter and useful for modulating gene expression in plants. In another embodiment the invention provides constructs comprising the novel chimeric promoter and useful for modulating gene expression in plants. In another embodiment the invention provides a transgenic plant comprising the novel chimeric promoter and the seed of the transgenic plant. In another embodiment the invention provides a method of inhibiting weed growth in a field of transgenic glyphosate tolerant crop plants comprising planting the transgenic plants transformed with an expression cassette comprising the novel chimeric promoter operably linked to a DNA molecule encoding a glyphosate tolerance gene and applying glyphosate to the field at an application rate that inhibits the growth of weeds.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents a section of the CaMV 35S promoter with enhancer domains marked. Also diagrammatically represented are five enhancer domains constructed for use in creating chimeric actin promoters.

Figure 2 represents the native rice actin 1 promoter and novel chimeric promoters made by fusing the rice actin 1 promoter and selected CaMV 35S promoter enhancer domains.

Figure 3 represents the native *Arabidopsis* actin 1 promoter and novel chimeric promoters made by fusing the *Arabidopsis* actin 1 promoter and selected CaMV 35S promoter enhancer domains.

DETAILED DESCRIPTION

5 The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

The invention disclosed herein provides novel combinations of polynucleotide molecules
10 for use in constructing novel chimeric promoters. The design, construction, and use of chimeric or hybrid promoters comprising one or more of the enhancer domains of a caulimovirus 35S promoter and a plant actin gene promoter is one object of this invention. The novel chimeric promoter sequences thereof of SEQ ID NO: 9-35, are capable of transcribing operably linked DNA sequences in multiple tissues and therefore can selectively regulate expression of
15 transgenes in multiple tissues.

As used herein, the term “polynucleotide molecule” refers to the single- or double-stranded DNA or RNA of genomic or synthetic origin, *i.e.*, a polymer of deoxyribonucleotide or ribonucleotide bases, respectively, read from the 5’ (upstream) end to the 3’ (downstream) end.

As used herein, the term “polynucleotide sequence” refers to the sequence of a
20 polynucleotide molecule. The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used.

As used herein, the term “gene regulatory activity” refers to the ability to affect transcription or translation of an operably linked transcribable polynucleotide molecule. An isolated polynucleotide molecule having gene regulatory activity may provide temporal or
25 spatial expression or modulate levels and rates of expression of the operably linked transcribable polynucleotide molecule. An isolated polynucleotide molecule having gene regulatory activity may comprise a promoter, intron, leader, or 3’ transcription termination region.

As used herein, the term “gene expression” refers to the transcription of a DNA molecule into a transcribed RNA molecule. Gene expression may be described as related to temporal,

spatial, developmental, or morphological qualities as well as quantitative or qualitative indications.

As used herein, the term “regulatory element” refers to a polynucleotide molecule that may affect the transcription or translation of an operably linked transcribable polynucleotide molecule. Regulatory elements such as promoters, leaders, introns, and transcription termination regions are non-coding polynucleotide molecules having gene regulatory activity which play an integral part in the overall expression of genes in living cells. Isolated regulatory elements that function in plants are therefore useful for modifying plant phenotypes through the methods of genetic engineering.

As used herein, the term “promoter” refers to a polynucleotide molecule that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) to initiate transcription. A plant promoter is a native or non-native promoter that is functional in plant cells. A promoter can be used as a 5' regulatory element for modulating expression of an operably linked transcribable polynucleotide molecule. Promoters may be defined by their temporal, spatial, or developmental expression pattern.

As used herein, the term “enhancer domain” refers to a cis-acting transcriptional regulatory element, a.k.a. cis-element, which confers an aspect of the overall control of gene expression. An enhancer domain may function to bind transcription factors, trans-acting protein factors that regulate transcription. Some enhancer domains bind more than one transcription factor, and transcription factors may interact with different affinities with more than one enhancer domain. Enhancer domains can be identified by a number of techniques, including deletion analysis, *i.e.*, deleting one or more nucleotides from the 5' end or internal to a promoter; DNA binding protein analysis using DNase I footprinting, methylation interference, electrophoresis mobility-shift assays, *in vivo* genomic footprinting by ligation-mediated PCR, and other conventional assays; or by DNA sequence similarity analysis with known cis-element motifs by conventional DNA sequence comparison methods. The fine structure of an enhancer domain can be further studied by mutagenesis (or substitution) of one or more nucleotides or by other conventional methods. Enhancer domains can be obtained by chemical synthesis or by isolation from promoters that include such elements, and they can be synthesized with additional flanking nucleotides that contain useful restriction enzyme sites to facilitate subsequence manipulation. Thus, the design, construction, and use of enhancer domains according to the

methods disclosed herein for modulating the expression of operably linked transcribable polynucleotide molecules are encompassed by the present invention

As used herein, the term “chimeric” refers to the product of the fusion of portions of two or more different polynucleotide molecules. As used herein, the term “chimeric promoter” refers to a promoter produced through the manipulation of known promoters or other polynucleotide molecules. Such chimeric promoters may combine enhancer domains that can confer or modulate gene expression from one or more promoters, for example, by fusing a heterologous enhancer domain from a first promoter to a second promoter with its own partial or complete regulatory elements. The novel chimeric promoters of the present invention desirably contain at least one enhancer domain fused to a plant actin promoter. Thus, the design, construction, and use of chimeric promoters according to the methods disclosed herein for modulating the expression of operably linked transcribable polynucleotide molecules are encompassed by the present invention.

As used herein, the term “percent sequence identity” refers to the percentage of identical nucleotides in a linear polynucleotide sequence of a reference (“query”) polynucleotide molecule (or its complementary strand) as compared to a test (“subject”) polynucleotide molecule (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and preferably by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG® Wisconsin Package® (Accelrys Inc., Burlington, MA). An “identity fraction” for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, *i.e.*, the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more polynucleotide sequences may be to a full-length polynucleotide sequence or a portion thereof, or to a longer polynucleotide sequence.

As used herein, the term “substantial percent sequence identity” refers to a percent sequence identity of at least about 80% sequence identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity. Thus, one embodiment of the invention is a polynucleotide molecule that has at least about 80% sequence identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity with a polynucleotide sequence described herein.

Polynucleotide molecules that are capable of regulating transcription of operably linked transcribable polynucleotide molecules and have a substantial percent sequence identity to the polynucleotide sequences of the promoters provided herein are encompassed within the scope of this invention.

Promoter Isolation and Modification Methods

Any number of methods well known to those skilled in the art can be used to isolate fragments of a promoter disclosed herein. For example, PCR (polymerase chain reaction) technology can be used to amplify flanking regions from a genomic library of a plant using publicly available sequence information. A number of methods are known to those of skill in the art to amplify unknown polynucleotide molecules adjacent to a core region of known polynucleotide sequence. Methods include but are not limited to inverse PCR (IPCR), vectorette PCR, Y-shaped PCR, and genome walking approaches. Polynucleotide fragments can also be obtained by other techniques such as by directly synthesizing the fragment by chemical means, as is commonly practiced by using an automated oligonucleotide synthesizer. For the present invention, the polynucleotide molecules were isolated by designing PCR primers based on available sequence information.

Novel chimeric promoters can be designed or engineered by a number of methods. For example, a chimeric promoter may be produced by fusing an enhancer domain from a first promoter to a second promoter. The resultant chimeric promoter may have novel expression properties relative to the first or second promoters. Novel chimeric promoters can be constructed such that the enhancer domain from a first promoter is fused at the 5' end, at the 3' end, or at any position internal to the second promoter. The location of the enhancer domain fusion relative to the second promoter may cause the resultant chimeric promoter to have novel expression properties relative to a fusion made at a different location.

Those of skill in the art are familiar with the standard resource materials that describe specific conditions and procedures for the construction, manipulation, and isolation of macromolecules (e.g., polynucleotide molecules, plasmids, etc.), as well as the generation of recombinant organisms and the screening and isolation of polynucleotide molecules.

5 **Constructs**

As used herein, the term “construct” refers to any recombinant polynucleotide molecule such as a plasmid, cosmid, virus, autonomously replicating polynucleotide molecule, phage, or linear or circular single-stranded or double-stranded DNA or RNA polynucleotide molecule, derived from any source, capable of genomic integration or autonomous replication, comprising
10 a polynucleotide molecule where one or more transcribable polynucleotide molecule has been operably linked.

As used herein, the term “operably linked” refers to a first polynucleotide molecule, such as a promoter, connected with a second transcribable polynucleotide molecule, such as a gene of interest, where the polynucleotide molecules are so arranged that the first polynucleotide
15 molecule affects the function of the second polynucleotide molecule. The two polynucleotide molecules may be part of a single contiguous polynucleotide molecule and may be adjacent. For example, a promoter is operably linked to a gene of interest if the promoter regulates or mediates transcription of the gene of interest in a cell.

As used herein, the term “transcribable polynucleotide molecule” refers to any
20 polynucleotide molecule capable of being transcribed into a RNA molecule. Methods are known for introducing constructs into a cell in such a manner that the transcribable polynucleotide molecule is transcribed into a functional mRNA molecule that is translated and therefore expressed as a protein product. Constructs may also be constructed to be capable of expressing antisense RNA molecules, in order to inhibit translation of a specific RNA molecule of interest.
25 For the practice of the present invention, conventional compositions and methods for preparing and using constructs and host cells are well known to one skilled in the art, see for example, *Molecular Cloning: A Laboratory Manual, 3rd edition Volumes 1, 2, and 3* (2000). J.F. Sambrook, D.W. Russell, and N. Irwin, Cold Spring Harbor Laboratory Press.

Constructs of the present invention would typically contain a promoter operably linked to
30 a transcribable polynucleotide molecule operably linked to a 3' transcription termination

polynucleotide molecule. In addition, constructs may include but are not limited to additional regulatory polynucleotide molecules from the 3'-untranslated region (3' UTR) of plant genes (*e.g.*, a 3' UTR to increase mRNA stability of the mRNA, such as the PI-II termination region of potato or the octopine or nopaline synthase 3' termination regions). Constructs may include but are not limited to the 5' untranslated regions (5' UTR) of an mRNA polynucleotide molecule which can play an important role in translation initiation and can also be a genetic component in a plant expression construct. For example, non-translated 5' leader polynucleotide molecules derived from heat shock protein genes have been demonstrated to enhance gene expression in plants (see for example, U.S. Patent No. 5,659,122 and U.S. Patent No. 5,362,865, all of which are incorporated herein by reference). These additional upstream and downstream regulatory polynucleotide molecules may be derived from a source that is native or heterologous with respect to the other elements present on the construct.

Thus, one embodiment of the invention is a promoter such as provided in SEQ ID NO: 9-35, operably linked to a transcribable polynucleotide molecule so as to direct transcription of said transcribable polynucleotide molecule at a desired level or in a desired tissue or developmental pattern upon introduction of said construct into a plant cell. In one embodiment, the transcribable polynucleotide molecule comprises a protein-coding region of a gene, and the promoter provides for transcription of a functional mRNA molecule that is translated and expressed as a protein product. In another embodiment, the transcribable polynucleotide molecule comprises an antisense region of a gene, and the regulatory element affects the transcription of an antisense RNA molecule or other similar inhibitory RNA in order to inhibit expression of a specific RNA molecule of interest in a target host cell.

Exemplary transcribable polynucleotide molecules for incorporation into constructs of the present invention include, for example, polynucleotide molecules or genes from a species other than the target species or genes that originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. The type of polynucleotide molecule can include but is not limited to a polynucleotide molecule that is already present in the plant cell, a polynucleotide molecule from another plant, a polynucleotide molecule from a different organism, or a polynucleotide molecule generated externally, such as a polynucleotide molecule containing an

antisense message of a gene, or a polynucleotide molecule encoding an artificial, synthetic, or otherwise modified version of a transgene.

The regulatory elements of the present invention can be incorporated into a construct using marker genes as described and tested in transient or stable plant analyses to provide an indication of the regulatory element's gene expression pattern in stable transgenic plants. As used herein the term "marker gene" refers to any transcribable polynucleotide molecule whose expression can be screened for or scored in some way. Marker genes for use in the practice of the present invention include, but are not limited to transcribable polynucleotide molecules encoding β -glucuronidase (GUS described in U.S. Patent No. 5,599,670, which is incorporated herein by reference) and green fluorescent protein (GFP described in U.S. Patent No. 5,491,084 and U.S. Patent No. 6,146,826, both of which are incorporated herein by reference), proteins that confer antibiotic resistance, or proteins that confer herbicide tolerance. Useful antibiotic resistance markers, including those encoding proteins conferring resistance to kanamycin (*nptII*), hygromycin B (*aph IV*), streptomycin or spectinomycin (*aad*, *spec/strep*) and gentamycin (*aac3* and *aacC4*) are known in the art. Herbicides for which transgenic plant tolerance has been demonstrated and the method of the present invention can be applied, include but are not limited to: glyphosate, glufosinate, sulfonylureas, imidazolinones, bromoxynil, delapone, cyclohezanedione, protoporphyrionogen oxidase inhibitors, and isoxasflutole herbicides. Polynucleotide molecules encoding proteins involved in herbicide tolerance are known in the art, and include, but are not limited to a polynucleotide molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS described in U.S. Patent No. 5,627,061, U.S. Patent No. 5,633,435, U.S. Patent No. 6,040,497 and in U.S. Patent No. 5,094,945 for glyphosate tolerance, all of which are incorporated herein by reference); polynucleotides encoding a glyphosate oxidoreductase and a glyphosate-N-acetyl transferase (GOX described in U.S. Patent 5,463,175 and GAT described in U.S. Patent publication 20030083480, both of which are incorporated herein by reference); a polynucleotide molecule encoding bromoxynil nitrilase (*Bxn* described in U.S. Patent No. 4,810,648 for Bromoxynil tolerance, which is incorporated herein by reference); a polynucleotide molecule encoding phytoene desaturase (*crtI*) described in Misawa et al, (1993) *Plant J.* 4:833-840 and Misawa et al, (1994) *Plant J.* 6:481-489 for norflurazon tolerance; a polynucleotide molecule encoding acetohydroxyacid synthase (AHAS, *aka* ALS) described in Sathasiivan et al. (1990) *Nucl. Acids Res.* 18:2188-2193 for tolerance to sulfonylurea herbicides;

a polynucleotide molecule encoding a dicamba-degrading oxygenase enzyme (described in U.S. Patent Publications US20030135879 and US20030115626, for dicamba tolerance, all of which are incorporated herein by reference); and the *bar* gene described in DeBlock, *et al.* (1987) *EMBO J.* 6:2513-2519 for glufosinate and bialaphos tolerance. The regulatory elements of the present invention can express transcribable polynucleotide molecules that encode for phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hydroxyphenyl pyruvate dehydrogenase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase, anthranilate synthase, glyphosate oxidoreductase and glyphosate-N-acetyl transferase.

Thus, in one embodiment of the invention, a polynucleotide molecule of the present invention as shown in SEQ ID NO: 9-35 is incorporated into a DNA construct such that a polynucleotide molecule of the present invention is operably linked to a transcribable polynucleotide molecule that provides for a selectable, screenable, or scorable marker. The constructs containing the regulatory elements operably linked to a marker gene may be delivered to the tissues and the tissues analyzed by the appropriate mechanism, depending on the marker. The quantitative or qualitative analyses are used as a tool to evaluate the potential expression profile of a regulatory element when operatively linked to a gene of agronomic interest in stable plants. Any marker gene can be used in a transient assay. Methods of testing for marker gene expression in transient assays are known to those of skill in the art. Transient expression of marker genes has been reported using a variety of plants, tissues, and DNA delivery systems. For example, types of transient analyses can include but are not limited to direct gene delivery via electroporation or particle bombardment of tissues in any transient plant assay using any plant species of interest. Such transient systems would include but are not limited to electroporation of protoplasts from a variety of tissue sources or particle bombardment of specific tissues of interest. The present invention encompasses the use of any transient expression system to evaluate regulatory elements operably linked to any transcribable polynucleotide molecule, including but not limited to marker genes or genes of agronomic interest. Examples of plant tissues envisioned to test in transients via an appropriate delivery system would include but are not limited to leaf base tissues, callus, cotyledons, roots, endosperm, embryos, floral tissue, pollen, and epidermal tissue.

In one embodiment of the invention, a polynucleotide molecule as shown in SEQ ID NO: 9-35 is incorporated into a construct such that a polynucleotide molecule of the present invention is operably linked to a transcribable polynucleotide molecule that is a gene of agronomic interest. As used herein, the term "gene of agronomic interest" refers to a transcribable polynucleotide molecule that includes but is not limited to a gene that provides a desirable characteristic associated with plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance. The expression of a gene of agronomic interest is desirable in order to confer an agronomically important trait. A gene of agronomic interest that provides a beneficial agronomic trait to crop plants may be, for example, including, but not limited to genetic elements comprising herbicide resistance (U.S. Patents 6,803,501; 6,448,476; 6,248,876; 6,225,114; 6,107,549; 5,866,775; 5,804,425; 5,633,435; 5,463,175; and U.S. Patent Publications US20030135879 and US20030115626), increased yield (U.S. Patents USRE38,446; 6,716,474; 6,663,906; 6,476,295; 6,441,277; 6,423,828; 6,399,330; 6,372,211; 6,235,971; 6,222,098; 5,716,837), insect control (U.S. Patents 6,809,078; 6,713,063; 6,686,452; 6,657,046; 6,645,497; 6,642,030; 6,639,054; 6,620,988; 6,593,293; 6,555,655; 6,538,109; 6,537,756; 6,521,442; 6,501,009; 6,468,523; 6,326,351; 6,313,378; 6,284,949; 6,281,016; 6,248,536; 6,242,241; 6,221,649; 6,177,615; 6,156,573; 6,153,814; 6,110,464; 6,093,695; 6,063,756; 6,063,597; 6,023,013; 5,959,091; 5,942,664; 5,942,658; 5,880,275; 5,763,245; 5,763,241), fungal disease resistance (U.S. Patents 6,653,280; 6,573,361; 6,506,962; 6,316,407; 6,215,048; 5,516,671; 5,773,696; 6,121,436; 6,316,407; 6,506,962), virus resistance (U.S. Patents 6,617,496; 6,608,241; 6,015,940; 6,013,864; 5,850,023; 5,304,730), nematode resistance (U.S. Patent 6,228,992), bacterial disease resistance (U.S. Patent 5,516,671), plant growth and development (U.S. Patents 6,723,897; 6,518,488), starch production (U.S. Patents 6,538,181; 6,538,179; 6,538,178; 5,750,876; 6,476,295), modified oils production (U.S. Patents 6,444,876; 6,426,447; 6,380,462), high oil production (U.S. Patents 6,495,739; 5,608,149; 6,483,008; 6,476,295), modified fatty acid content (U.S. Patents 6,828,475; 6,822,141; 6,770,465; 6,706,950; 6,660,849; 6,596,538; 6,589,767; 6,537,750; 6,489,461; 6,459,018), high protein production (U.S. Patent 6,380,466), fruit ripening (U.S. Patent 5,512,466), enhanced animal and human nutrition (U.S. Patents 6,723,837; 6,653,530; 6,5412,59; 5,985,605; 6,171,640), biopolymers (U.S. Patents USRE37,543; 6,228,623; 5,958,745 and U.S. Patent Publication No. US20030028917),

environmental stress resistance (U.S. Patent 6,072,103), pharmaceutical peptides and secretable peptides (U.S. Patents 6,812,379; 6,774,283; 6,140,075; 6,080,560), improved processing traits (U.S. Patent 6,476,295), improved digestibility (U.S. Patent 6,531,648) low raffinose (U.S. Patent 6,166,292), industrial enzyme production (U.S. Patent 5,543,576), improved flavor (U.S. Patent 6,011,199), nitrogen fixation (U.S. Patent 5,229,114), hybrid seed production (U.S. Patent 5,689,041), fiber production (U.S. Patent 6,576,818; 6,271,443; 5,981,834; 5,869,720) and biofuel production (U.S. Patent 5,998,700); the genetic elements, methods, and transgenes described in the patents listed above are incorporated herein by reference.

Alternatively, a transcribable polynucleotide molecule can effect the above mentioned plant characteristic or phenotypes by encoding a RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via antisense, inhibitory RNA (RNAi), or cosuppression-mediated mechanisms. The RNA could also be a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave a desired endogenous mRNA product. Thus, any transcribable polynucleotide molecule that encodes a transcribed RNA molecule that affects a phenotype or morphology change of interest may be useful for the practice of the present invention.

The constructs of the present invention are generally double Ti plasmid border DNA constructs that have the right border (RB or AGRtu.RB) and left border (LB or AGRtu.LB) regions of the Ti plasmid isolated from *Agrobacterium tumefaciens* comprising a T-DNA, which along with transfer molecules provided by the *Agrobacterium* cells, permit the integration of the T-DNA into the genome of a plant cell. The constructs may also contain the plasmid backbone DNA segments that provide replication function and antibiotic selection in bacterial cells, for example, an *Escherichia coli* origin of replication such as *ori322*, a broad host range origin of replication such as *oriV* or *oriRi*, and a coding region for a selectable marker such as Spec/Strp that encodes for Tn7 aminoglycoside adenyltransferase (*aadA*) conferring resistance to spectinomycin or streptomycin, or a gentamicin (Gm, Gent) selectable marker gene. For plant transformation, the host bacterial strain is often *Agrobacterium tumefaciens* ABI, C58, or LBA4404, however, other strains known to those skilled in the art of plant transformation can function in the present invention.

Transformed Plants And Plant Cells

As used herein, the term “transformed” refers to a cell, tissue, organ, or organism into which has been introduced a foreign polynucleotide molecule, such as a construct. The introduced polynucleotide molecule may be integrated into the genomic DNA of the recipient cell, tissue, organ, or organism such that the introduced polynucleotide molecule is inherited by subsequent progeny. A “transgenic” or “transformed” cell or organism also includes progeny of the cell or organism and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a foreign polynucleotide molecule. A plant transformation construct containing a promoter of the present invention may be introduced into plants by any plant transformation method. Methods and materials for transforming plants by introducing a plant expression construct into a plant genome in the practice of this invention can include any of the well-known and demonstrated methods including electroporation (as illustrated in U.S. Patent No. 5,384,253; microprojectile bombardment as illustrated in U.S. Patent Nos. 5,015,580; U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 6,160,208; U.S. Patent No. 6,399,861; and U.S. Patent No. 6,403,865; *Agrobacterium*-mediated transformation as illustrated in U.S. Patent No. 5,824,877; U.S. Patent No. 5,591,616; U.S. Patent No. 5,981,840; and U.S. Patent No. 6,384,301; and protoplast transformation as illustrated in U.S. Patent No. 5,508,184, all of which are incorporated herein by reference.

Methods for specifically transforming dicots are well known to those skilled in the art. Transformation and plant regeneration using these methods have been described for a number of crops including, but not limited to, cotton (*Gossypium hirsutum*), soybean (*Glycine max*), peanut (*Arachis hypogaea*), and members of the genus *Brassica*.

Methods for transforming monocots are well known to those skilled in the art. Transformation and plant regeneration using these methods have been described for a number of crops including, but not limited to, barley (*Hordeum vulgare*); maize (*Zea mays*); oats (*Avena sativa*); orchard grass (*Dactylis glomerata*); rice (*Oryza sativa*, including indica and japonica varieties); sorghum (*Sorghum bicolor*); sugar cane (*Saccharum sp*); tall fescue (*Festuca arundinacea*); turfgrass species (e.g. species: *Agrostis stolonifera*, *Poa pratensis*, *Stenotaphrum secundatum*); wheat (*Triticum aestivum*), and alfalfa (*Medicago sativa*). It is apparent to those of

skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

The transformed plants are analyzed for the presence of the genes of interest and the expression level and/or profile conferred by the promoters of the present invention. Those of skill in the art are aware of the numerous methods available for the analysis of transformed plants. For example, methods for plant analysis include, but are not limited to Southern blots or northern blots, PCR-based approaches, biochemical analyses, phenotypic screening methods, field evaluations, and immunodiagnostic assays.

The seeds of this invention can be harvested from fertile transgenic plants and be used to grow progeny generations of transformed plants of this invention including hybrid plant lines comprising the construct of this invention and expressing a gene of agronomic interest.

The present invention also provides for parts of the plants of the present invention. Plant parts, without limitation, include seed, endosperm, ovule and pollen. In a particularly preferred embodiment of the present invention, the plant part is a seed.

Still yet another aspect of the invention is a method of inhibiting weed growth in a field of transgenic crop plants comprising first planting the transgenic plants transformed with an expression cassette comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 9-35 operably linked to a transcribable polynucleotide molecule encoding a glyphosate tolerance gene and then applying glyphosate to the field at an application rate that inhibits the growth of weeds, wherein the growth and yield of the transgenic crop plant is not substantially affected by the glyphosate application. The glyphosate application rate is the effective rate necessary to control weeds in a particular glyphosate tolerant crop; these rates may range from 8 ounces/acre to 256 ounces/acre, preferably 16 ounces/acre to 128 ounces/acre, and more preferably 32 ounces/acre to 96 ounces/acre. The glyphosate is applied at least once during the growth of the glyphosate tolerant crop and may be applied 2, 3, or 4 times during the growth of the crop or more as necessary to control weeds in the field.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are

disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

Examples

5 Example 1: Construction of chimeric promoters.

Novel chimeric promoters are constructed by fusing at least one enhancer domain from a Caulimovirus promoter with a plant actin gene promoter. A brief description of the sequences referred to herein is provided in Table 1 below.

Table 1: Brief Listing of the SEQ ID NO

SEQ ID NO	Sequence Name	Brief Description
1	P-CaMV.35S	35S promoter sequence from the Cauliflower mosaic virus
2	4xB3	P-CaMV.35S Enhancer Domain -- four tandem copies of the B3 domain (-208 to -155) as described in U.S. Patent No. 5,097,025
3	4xAS-1	P-CaMV.35S Enhancer Domain -- four tandem copies of the "activation sequence" (-83 to -62) as described in U.S. Patent No. 5,097,025
4	2xB1-B2	P-CaMV.35S Enhancer Domain -- two tandem copies of the B1-B2 domain (-148 to -90) as described in U.S. Patent No. 5,097,025
5	2xA1-B3	P-CaMV.35S Enhancer Domain -- two tandem copies of the A1-B3 domain (-208 to -46) as described in U.S. Patent No. 5,097,025
6	2xB1-B5	P-CaMV.35S Enhancer Domain -- two tandem copies of the B1-B5 domain (-343 to -90) as described in U.S. Patent No. 5,097,025
7	P-Os.Act1	Rice actin 1 promoter
8	P-At.Act1	<i>Arabidopsis</i> Actin 1 promoter
9	P-4xB3/P-Os.Act1-1	Chimeric promoter -- 4xB3 fused to the rice Act1 promoter at the -848 nt position
10	P-4xB3/P-Os.Act1-2	Chimeric promoter -- 4xB3 fused to the rice Act1 promoter at the -462 nt position
11	P-4xB3/P-Os.Act1-3	Chimeric promoter -- 4xB3 fused to the rice Act1 promoter at the -80 nt position
12	P-4xAS-1/P-Os.Act1-1	Chimeric promoter -- 4xAS-1 fused to the rice Act1 promoter at the -848 nt position

SEQ ID NO	Sequence Name	Brief Description
13	P-4xAS-1/P-Os.Act1-2	Chimeric promoter -- 4xAS-1 fused to the rice Act1 promoter at the -462 nt position
14	P-4xAS-1/P-Os.Act1-3	Chimeric promoter -- 4xAS-1 fused to the rice Act1 promoter at the -80 nt position
15	P-2xB1-B2/P-Os.Act1-1	Chimeric promoter -- 2xB1-B2 fused to the rice Act1 promoter at the -848 nt position
16	P-2xB1-B2/P-Os.Act1-2	Chimeric promoter -- 2xB1-B2 fused to the rice Act1 promoter at the -462 nt position
17	P-2xB1-B2/P-Os.Act1-3	Chimeric promoter -- 2xB1-B2 fused to the rice Act1 promoter at the -80 nt position
18	P-2xA1-B3/P-Os.Act1-1	Chimeric promoter -- 2xA1-B3 fused to the rice Act1 promoter at the -848 nt position
19	P-2xA1-B3/P-Os.Act1-2	Chimeric promoter -- 2xA1-B3 fused to the rice Act1 promoter at the -462 nt position
20	P-2xA1-B3/P-Os.Act1-3	Chimeric promoter -- 2xA1-B3 fused to the rice Act1 promoter at the -80 nt position
21	P-2xB1-B5/P-Os.Act1-1	Chimeric promoter -- 2xB1-B5 fused to the rice Act1 promoter at the -848 nt position
22	P-2xB1-B5/P-Os.Act1-2	Chimeric promoter -- 2xB1-B5 fused to the rice Act1 promoter at the -462 nt position
23	P-2xB1-B5/P-Os.Act1-3	Chimeric promoter -- 2xB1-B5 fused to the rice Act1 promoter at the -80 nt position
24	P-2xA1-B3/At.Act1/ArvII	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the ArvII position
25	P-2xA1-B3/At.Act1/BstZI	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the BstZI position
26	P-2xA1-B3/At.Act1/BstZI-R	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the BstZI position in reverse orientation
27	P-2xA1-B3/At.Act1/NsiI	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the NsiI position
28	P-2xA1-B3/At.Act1/NsiI-R	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the NsiI position in reverse orientation
29	P-2xA1-B3/At.Act1/BsmFI	Chimeric promoter -- 2xA1-B3 fused to the <i>Arabidopsis</i> Act1 promoter at the BsmFI position

SEQ ID NO	Sequence Name	Brief Description
30	P-4xAS-1/At.Act1/ArvII	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the ArvII position
31	P-4xAS-1/At.Act1/ArvII-R	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the ArvII position in reverse orientation
32	P-4xAS-1/At.Act1/BstZI	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the BstZI position
33	P-4xAS-1/At.Act1/BstZI-R	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the BstZI position in reverse orientation
34	P-4xAS-1/At.Act1/NsiI	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the NsiI position
35	P-4xAS-1/At.Act1/BsmFI	Chimeric promoter -- 4xAS-1 fused to the <i>Arabidopsis</i> Act1 promoter at the BsmFI position

The Caulimovirus promoter can be any promoter from a virus in the Caulimovirus family, including but not limited to promoters from Cauliflower mosaic virus (CaMV) such as the promoter from the 35S RNA transcript of the Cauliflower mosaic virus (P-CaMV.35S) (SEQ ID NO: 1) (U.S. Patent No. 5,530,196) and promoters from Figwort mosaic virus (FMV) such as the promoter from the 35S transcript of the Figwort mosaic virus, (U.S. Patent No. 6,051,753), all of which are incorporated herein by reference. Promoter enhancer domains constructed as multimers of enhancer domains from the P-CaMV.35S promoter include but are not limited to the 4xB3 domain (SEQ ID NO: 2), 4xAS-1 (SEQ ID NO: 3), 2xB1-B2 domain (SEQ ID NO: 4), 2xA1-B3 domain (SEQ ID NO: 5), and 2xB1-B5 domain (SEQ ID NO: 6). See Figure 1. The monomers comprising the multimers listed above as well as other enhancer domains from P-CaMV.35S are described in U.S. Patent No. 5,097,025, incorporated herein by reference.

The plant actin gene promoter can be any promoter from a plant actin gene, including but not limited to actin promoters from *Oryza sativa* such as the rice actin 1 promoter (P-Os.Act1) (SEQ ID NO: 7) (U.S. Patent No. 5,641,876), actin promoters from *Arabidopsis thaliana* such as the *Arabidopsis* Actin 1 promoter (P-At.Act1) (SEQ ID NO: 8), and actin promoters from *Zea mays* such as the Actin-2 promoter (U.S. Patent No. 6,670,467), all of which are incorporated herein by reference.

The fusion of at least one enhancer domain with a plant actin gene promoter may be to any region of the plant actin gene promoter including but not limited to the 5' end of the plant actin gene promoter, the 3' end of the plant actin gene promoter, or any region internal to the plant actin gene promoter. The enhancer domain may be in either the reverse or the forward orientation.

Enhancer domains derived from P-CaMV.35S were inserted in three locations in the rice actin 1 (relative to rice Actin1 transcription initiation site): -848 nt (HindIII site), -462 nt (EcoRI site), and -80 nt (FseI site). See Figure 2. Construction of these chimeric promoters is described in detail below.

The P-4xB3/P-Os.Act1 chimeric promoters were created by fusing the four tandem copies CaMV 35s B3 enhancer sequence to the rice Act1 promoter (P-Os.Act1). Chimeras were made by fusing the enhancer region at the -848 nt position of P-Os.Act1 (SEQ ID NO: 9), at the -462 nt position of P-Os.Act1 (SEQ ID NO: 10) and at the -80 nt position of P-Os.Act1 (SEQ ID NO: 11).

The P-4xAS-1/P-Os.Act1 chimeric promoter was created by fusing four tandem copies of the CaMV 35s AS-1 enhancer sequence to the rice Act1 promoter (P-Os.Act1). Chimeras were made by fusing the enhancer region at the -848 nt position of P-Os.Act1 (SEQ ID NO: 12), at the -462 nt position of P-Os.Act1 (SEQ ID NO: 13) and at the -80 nt position of P-Os.Act1 (SEQ ID NO: 14).

The P-2xB1-B2/P-Os.Act1 chimeric promoter was created by fusing two tandem copies of the CaMV 35s B1-B2 enhancer sequence to the rice Act1 promoter (P-Os.Act1). Chimeras were made by fusing the enhancer region at the -848 nt position of P-Os.Act1 (SEQ ID NO: 15), at the -462 nt position of P-Os.Act1 (SEQ ID NO: 16) and at the -80 nt position of P-Os.Act1 (SEQ ID NO: 17).

The P-2xA1-B3/P-Os.Act1 chimeric promoters were created by fusing two tandem copies of the CaMV 35s A1-B3 enhancer sequence to the rice Act1 promoter (P-Os.Act1). Chimeras were made by fusing the enhancer region at the -848 nt position of P-Os.Act1 (SEQ ID NO: 18), at the -462 nt position of P-Os.Act1 (SEQ ID NO: 19), and at the -80 nt position of P-Os.Act1 (SEQ ID NO: 20).

The P-2xB1-B5/P-Os.Act1 chimeric promoter was created by fusing two tandem copies of the CaMV 35s B1-B5 enhancer sequence to the rice Act1 promoter (P-Os.Act1). Chimeras

were made by fusing the enhancer region at the -848 nt position of P-Os.Act1 (SEQ ID NO: 21), at the -462 nt position of P-Os.Act1 (SEQ ID NO: 22) and at the -80 nt position of P-Os.Act1 (SEQ ID NO: 23).

Enhancer domains derived from P-CaMV.35S were inserted in four locations in the *Arabidopsis* actin 1 promoter (P-At.Act1) (with the P-At.Act1 transcription initiation site designated as +1). Four unique restriction sites in P-At.Act1 were used as insertion sites: AvrII, BstZI, NsiI, BsmFI. Construction of these chimeric promoters is described in detail below.

The 2xA1B3 and 4xAS-1 fragments were isolated by restriction enzyme digest. Fragments ends were then made blunt by treatment with T4 DNA polymerase. Vectors containing the P-At.Act1 promoter were cut with one of the four unique enzymes and then the fragment ends were made blunt. Each of the two enhancer domains was fused into each of the four blunted restriction enzyme sites. Chimeric promoters were selected with inserts in both the reverse and forward orientations. See Figure 3.

Selected chimeric promoters were subcloned into a binary vector to operably link the chimeric promoter with a reporter gene such as the GUS reporter gene (β -glucuronidase gene) or the CP4 gene (for glyphosate tolerance). These vectors were used for plant transformation and subsequent promoter characterization. Transgene expression levels of the chimeric promoters were compared with the transgene expression levels of control constructs.

Example 2: Promoter Characterization in Transient Systems

Selected chimeric promoters were used for transient transformation for reporter expression analysis. Transient systems used included tobacco protoplasts, corn protoplasts, wheat ovary, wheat anther, and barley microspores. Cells were harvested and protein extracted for GUS activity analysis. Methods for measuring GUS activity are well known to those skilled in the art, see for instance *Using the Gus Gene as a Reporter of Gene Expression* (1992) edited by Sean R. Gallagher, Academic Press, Inc., San Diego.

Monocot Analysis

Constructs containing chimeric P-Os.Act1 promoters were used to transform corn protoplasts and assay for GUS/LUX activity relative to the P-Os.Act1 promoter and the P-CaMV.e35S promoter. Data are provided in Table 2.

Table 2: Transient Analysis in Corn Protoplasts

Chimeric Promoter	SEQ ID NO	Construct	Relative activity
P-Os.Act1	7	pMON25455	1.0
P-2xB1-B5/P-Os.Act1-1	21	pMON38303	1.6
P-2xB1-B5/P-Os.Act1-2	22	pMON38304	1.1
P-2xB1-B5/P-Os.Act1-3	23	pMON38305	3.6
P-2xA1-B3/P-Os.Act1-1	18	pMON38310	4.5
P-2xA1-B3/P-Os.Act1-2	19	pMON38312	4.6
P-2xA1-B3/P-Os.Act1-3	20	pMON38314	11.0
P-2xB1-B2/P-Os.Act1-1	15	pMON38309	1.1
P-2xB1-B2/P-Os.Act1-2	16	pMON38311	1.5
P-2xB1-B2/P-Os.Act1-3	17	pMON38313	2.2
P-4xAS-1/P-Os.Act1-1	12	pMON38300	1.8
P-4xAS-1/P-Os.Act1-2	13	pMON38301	1.3
P-4xAS-1/P-Os.Act1-3	14	pMON38302	8.9
P-4xB3/P-Os.Act1-1	9	pMON38306	1.8
P-4xB3/P-Os.Act1-2	10	pMON38307	3.3
P-4xB3/P-Os.Act1-3	11	pMON38308	5.4
P-CaMV.e35S	1	pMON25456	5.1

All the chimeric promoters tested were found to have higher activity than the native P-Os.Act1 promoter when tested in corn protoplasts. The three chimeric promoters P-2xA1-B3/P-Os.Act1-3 (SEQ ID NO: 20), P-4xAS-1/P-Os.Act1-3 (SEQ ID NO: 14), and P-4xB3/P-Os.Act1-3 (SEQ ID NO: 11) also showed increased activity when compared to the CaMV e35S promoter. Two factors contributed the enhanced activity in the chimeric promoter, namely the enhancer domain selected and the fusion location of the enhancer domain. Every enhancer domain tested appeared to be more powerful when the fusion location was closer to the 3' end of the promoter, and attenuated when the fusion location was at the 5' end of the promoter. Among the enhancer domains tested, the 2xA1-B3 enhancer was found to be the strongest enhancer domain in corn protoplasts. The 4xAS-1 enhancer was found to convey desirable strength to the chimeric promoter. The 4xB3 enhancer was found to be most effective when fused closer to the transcription start site.

One limitation of expression analysis in the corn protoplast system is that it only represents vegetative tissue. An increased expression level in the corn protoplast system is not

necessarily indicative of performance in reproductive tissue. A few selected chimeric promoters were therefore further tested for GUS activity (pmol/min in 10ul) in wheat anther, wheat ovary, barley microspore, and corn pollen transient assay systems for comparison with corn leaf protoplast data. Data are provided in Table 3 below. In comparison to P-Os.Act1 promoter which is known to express well in reproductive tissue, these chimeric promoters did not show any decrease of activity in reproductive tissue.

Table 3: Transient Analysis of Selected Chimeric Promoters in Reproductive Tissues

Promoter	SEQ ID NO	Construct	Wheat Anther	Wheat Ovary	Barley Microspore	Corn Pollen
P-Os.Act1	7	pMON25455	1.0	1.0	1.0	1.0
P-CaMV.E35S	1	pMON25456	3.3	0.5	0.8	0.03
P-4xAS-1/P-Os.Act1-3	14	pMON38302	16.4	1.2	2.0	2.2
P-4xB3/P-Os.Act1-2	10	pMON38307	1.5	2.1	-	6.0
P-4xB3/P-Os.Act1-3	11	pMON38308	6.4	2.5	-	0.6
P-2xA1-B3/P-Os.Act1-1	18	pMON38310	3.3	1.3	2.0	1.4
P-2xA1-B3/P-Os.Act1-2	19	pMON38312	7.6	3.3	2.4	0.5
P-2xA1-B3/P-Os.Act1-3	20	pMON38314	13.2	3.9	7.8	0.05

Dicot Analysis

Constructs containing chimeric P-At.Act1 promoters were used to transform tobacco protoplasts and assay for GUS activity (nM MUG/ μ g total protein). Data are provided in Table 4 below.

Table 4: Transient Analysis in Tobacco Protoplasts

Chimeric Promoter	SEQ ID NO	Construct	GUS Activity
P-At.Act1	8	pMON54945	10
P-2xA1-B3/At.Act1/ArvII	24	pMON59394	18
P-2xA1-B3/At.Act1/BstZI	25	pMON59392	20
P-2xA1-B3/At.Act1/BstZI-R	26	pMON59392-R	16
P-2xA1-B3/At.Act1/NsiI	27	pMON59386	23
P-2xA1-B3/At.Act1/NsiI-R	28	pMON59386-R	12
P-2xA1-B3/At.Act1/BsmFI	29	pMON59384	65
P-4xAS-1/At.Act1/ArvII	30	pMON59393	19

P-4xAS-1/At.Act1/ArvII-R	31	pMON59393-R	15
P-4xAS-1/At.Act1/BstZI	32	pMON59391	16
P-4xAS-1/At.Act1/BstZI-R	33	pMON59391-R	21
P-4xAS-1/At.Act1/NsiI	34	pMON59385	150
P-4xAS-1/At.Act1/BsmFI	35	pMON59383	179
P-e35S	1	pMON26180	365

Both the fusion location and choice of enhancer domain was found to produce a significant effect on promoter activity in tobacco protoplasts. The two promoters with the highest GUS activity were the P-4xAS-1/At.Act1/NsiI (SEQ ID NO: 34) and P-4xAS-1/At.Act1/BsmFI (SEQ ID NO: 35). For constructs comprising the 2xAlB3 enhancer, the highest activity of GUS was shown with the enhancer fused at the BsmFI position. The 2xAl-B3 chimeric promoters were generally less active in all positions when compared to the 4xAS-1 chimeric promoters. Reverse orientation of the enhancer domain did not change significantly the activity of the chimeric promoter.

Example 3: Characterization of Chimeric Promoters in Transgenic Corn Plants

Selected chimeric promoters were used for stable corn plant transformation for reporter expression analysis. Plants were transformed using agrobacterium-mediated methods.

In order to have a direct side-by-side comparison of GUS activity in the cytoplasm and CP4 expression in plastids, and to minimize variations in sampling and environmental factors, a few selected chimeric promoter constructs were built. Each test construct comprised the test promoter driving the GUS reporter gene (beta-glucuronidase coding sequence from *E. coli*) and the test promoter driving the CP4 gene (bacterial strain CP4 *aroA* gene encoding class II EPSPS enzyme) in a linear array. Four test constructs, pMON46172 (P-2xAl-B3/P-Os.Act1-3, SEQ ID NO: 20); pMON46173 (P-4xAS-1/P-Os.Act1-3, SEQ ID NO: 14); pMON46174 (P-4xB3/P-Os.Act1-2, SEQ ID NO: 10); and pMON46175 (P-2xAl-B3/P-Os.Act1-1, SEQ ID NO: 18), and the reference construct, pMON46170 (P-CaMV.E35S driving GUS and P-Os.Act1 driving CP4), were used to transform corn. Transformed corn plants were selected in glyphosate containing medium. Three R0 plants were generated for each transformation event. The first plant was sprayed with the equivalent of 64 ounce/acre of Roundup® Ultra, the second plant was sprayed with the equivalent of 96 ounce/acre of Roundup® Ultra. The third plant from each

event was left as unsprayed control. Ten to fourteen days after Roundup® application each plant was rated for % chlorosis and % malformation. At mid-pollen shed, each R0 plant was rated for male fertility. Positive R0 plants were pollinated with LH198 pollen grains to produce F1 seed.

Transgenic events with single or lowest copy numbers of cassette based on CP4 copy number estimation by Taqman were selected for F1 corn analysis. A total of 5 events from each construct with triplicate plants in each data point were used. The plants were grown in greenhouse from F1 seeds, and selected for positive segregates via glyphosate spray at 16 oz / acre when plants reach V-2 stage. Leaf tissue was taken at V-4 stage, and V-4 again at V-8/V-9 stage. Also at V-8/ V-9 stage, tissue was collected from V-8 leaf, root tip, and immature tassel ranging from 0.5cm-3cm. Pollen was collected when shedding. Embryo and endosperm were harvested at 12 days after pollination. Several positive embryos were pooled from GUS positive individual kernels. Samples were extracted and used for both GUS quantitative analysis and CP4 ELISA. The P-2xA1-B3/P-Os.Act1-3 and P-4xAS-1/P-Os.Act1-3 chimeric promoters provided GUS gene expression which was as good as or better than that provided by P-CaMV.E35S in most of the tissues analyzed. The P-4xB3/P-Os.Act1-2 and P-2xA1-B3/P-Os.Act1-1 chimeric promoters provided high levels of expression in pollen with moderate or low levels of expression in other tissues as compared to that provided by P-CaMV.E35S. The Data are provided for GUS activity (pmole/min/mg protein) as mean and standard error measurements in Table 5 below.

Table 5: F1 Transgenic Corn GUS Activity (pmole/min/mg protein)

Tissue/Stage	pmon46170 P-CaMV.E35S		pmon46172 P-2xA1-B3/P- Os.Act1-3		pmon46173 P-4xAS-1/P- Os.Act1-3		pmon46174 P-4xB3/P- Os.Act1-2		pmon46175 P-2xA1-B3/P- Os.Act1-1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Young V4	6.54	0.29	12.28	3.00	5.11	0.75	1.02	0.35	5.70	0.60
Aged V4	2.82	0.85	6.47	1.43	6.78	1.59	0.10	0.02	4.14	1.09
V8	0.67	0.20	1.27	0.51	0.64	0.12	0.17	0.05	0.13	0.01
Tassel	0.57	0.19	0.49	0.25	0.50	0.23	0.19	0.07	0.13	0.07
Root Tip	0.38	0.04	0.84	0.10	1.11	0.43	0.19	0.04	0.97	0.38
Pollen	1.44	0.11	2.27	0.26	2.87	0.52	15.26	2.84	13.76	2.05
Embryo	2.19	0.22	2.35	0.68	5.68	1.64	1.42	0.18	2.53	0.65
Endosperm	1.73	0.15	9.21	4.35	6.18	1.59	1.40	0.51	2.09	0.37

All four chimeric promoters provided CP4 gene expression which was as good as or better than that provided by P-Os.Act1 in all of the tissues analyzed with the exception of pollen. Expression levels in pollen for chimeric promoter constructs were approximately 20% to 73% that of expression levels in pollen for P-Os.Act1 constructs. Data are provided for CP4 expression levels (μg CP4 protein/g total protein) as mean and standard error measurements in Table 6 below.

Table 6: F1 Transgenic Corn CP4 Expression levels (μg CP4 protein/g total protein)

Tissue/Stage	pmon46170 and P-Os.Act1		pmon46172 P-2xA1-B3/P- Os.Act1-3		pmon46173 P-4xAS-1/P- Os.Act1-3		pmon46174 P-4xB3/P- Os.Act1-2		pmon46175 P-2xA1-B3/P- Os.Act1-1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Young V4	31	3	35	2	408	122	280	66	952	540
Aged V4	20	2	2187	656	1802	411	524	257	688	298
V8	17	2	1055	298	870	272	311	35	284	110
Tassel	225	27	1334	515	1002	178	408	31	1072	303
Root Tip	89	8	745	185	765	173	184	22	279	68
Pollen	797	97	165	13	217	33	370	16	582	74
Embryo	241	54	623	69	1101	187	305	69	566	84
Endosperm	229	21	792	212	889	229	237	36	375	46

The F1 progenies were also used for field tests. Three F1 populations derived from three R0 plants of each event were tested. Commercial Roundup Ready[®] corn lines GA21 and NK603 were used as positive controls. Three Roundup[®] rates were used in this test: 0, 96, and 128 oz/A. Roundup[®] was applied at V4 leaf stage. Data obtained were % transformation efficiency (TE), number of single copy events per total events generated, Leaf CP4 levels (μg CP4/g fresh weight tissue), Pollen CP4 levels (μg CP4/g fresh weight tissue), percent chlorosis (CHL), percent malformation (MAL) at 10-14 DAT (days after treatment), and male fertility score (MFR) measured 1-5 with 5 the highest. Data represent the average score collected across the events generated for each construct and only tasseled plants were included for some measurements. Plants transformed with the P-4xB3/P-Os.Act1-2 or the P-4xAS-1/P-Os.Act1-3 chimeric promoter constructs had chlorosis scores equivalent to the reference construct

(pMON46170). P-4xAS-1/P-Os.Act1-3 had a malformation score lower than that of the reference construct. All chimeric promoters tested had male fertility rates comparable to that of plants transformed with the reference construct. Data are provided in Table 7 below.

Table 7: Roundup® Tolerance in Transgenic Corn Field Tests

Promoter	Construct	% TE	Single Copy	Leaf CP4	Pollen CP4	Pollen GUS	% CHL	% MAL	MFR
P-CaMV.E35S and P-Os.Act1	pMON46170	12	7/15	22	1471	4.4	1	1	5
P-2xA1-B3/P- Os.Act1-3	pMON46172	10	5/15	116	313	5.8	7	4	5
P-4xAS-1/P- Os.Act1-3	pMON46173	16	9/15	319	475	9.7	1	0	5
P-4xB3/P- Os.Act1-2	pMON46174	24	8/15	78	674	24.3	1	3	5
P-2xA1-B3/P- Os.Act1-1	pMON46175	-	1/15	77	767	34.1	2	3	5

5

Example 4: Characterization of Chimeric Promoters in Transgenic Dicots

Several constructs were evaluated in transgenic *Arabidopsis* plants for GUS expression. GUS expression in leaf and flower tissue was measured as pM MUG/ µg total protein and results were averaged for all the events produced for construct. The CaMV e35S promoter (P-CaMV.e35S) and Arabidopsis actin 1 promoter (P-At.Act1) were used as controls. Data are provided in Table 8 below

10

Table 8: Transgenic *Arabidopsis* analysis with chimeric At-Act1 promoters

Promoter	SEQ ID NO	Construct	Flower	Leaf
P-At.Act1	8	pMON59382	139	62
P-CaMV.e35S	1	pMON59381	374	176
P-2xA1-B3/At.Act1/BstZI	25	pMON59378	0	24
P-4xAS-1/At.Act1/BstZI	32	pMON59377	12	47
P-4xAS-1/At.Act1/ArvII	30	pMON59379	58	18
P-4xAS-1/At.Act1/NsiI	34	pMON59375	46	36
P-4xAS-1/At.Act1/BstZI-R	33	pMON59371	66	21
P-2xA1-B3/At.Act1/NsiI	27	pMON59376	113	56

P-2xA1-B3/At.Act1/BstZI-R	26	pMON59372	149	114
P-4xAS-1/At.Act1/BsmFI	35	pMON59373	29	256
P-2xA1-B3/At.Act1/ArvII	24	pMON59380	88	199
P-2xA1-B3/At.Act1/BsmFI	29	pMON59374	153	193

GUS expression analysis in *Arabidopsis* showed that P-2xA1-B3/At.Act1/BstZI-R (SEQ ID NO: 26) and P-2xA1-B3/At.Act1/BsmFI (SEQ ID NO: 29) provided transgene expression in flowers comparable to that of the P-At.Act1 promoter. The P-2xA1-B3/At.Act1/BstZI-R (SEQ ID NO: 26), P-4xAS-1/At.Act1/BsmFI (SEQ ID NO: 35), P-2xA1-B3/At.Act1/ArvII (SEQ ID NO: 24), and P-2xA1-B3/At.Act1/BsmFI (SEQ ID NO: 29) promoters provided transgene expression in leaves higher than or comparable to the P-At.Act1 and P-CaMV.e35S.

Example 4: Insect Control Analysis in Corn

Two constructs (pMON38858 and pMON38859) were used to generate transgenic corn plants. Transformations were performed using *Agrobacterium* mediated methods. Both constructs contained the 2xA1-B3/P-Os.Act1-3 promoter (SEQ ID NO: 20) operably linked to a nucleotide sequence encoding a Cry2Ab insecticidal protein (U.S. Patent No. 6,489,542). Several transgenic corn plant lines produced from each construct were analyzed for Cry2Ab protein levels. The 2xA1-B3/P-Os.Act1-3 promoter was found to express the Cry2Ab protein at high levels in corn leaf tissue when compared with standard controls.

Example 5: Glyphosate Tolerance Analysis in Wheat

Three chimeric promoters were tested in transgenic wheat plants. Transgenic wheat plants were generated from each of the single cassette constructs pMON43646 (P-4xAS-1/P-Os.Act1-3, SEQ ID NO: 14), pMON43647 (P-2xA1-B3/P-Os.Act1-2, SEQ ID NO: 19), and pMON43648 (P-2xA1-B3/P-Os.Act1-3, SEQ ID NO: 20). Transformations were performed with *Agrobacterium* in immature Bobwhite embryos. All three constructs contain a single copy of the CP4 EPSPS gene for glyphosate tolerance. The distinguishing element in each construct is the promoter. Events from each single cassette construct were analyzed for vegetative and reproductive tolerance to glyphosate equivalent to the double cassette lead event 33391 (U.S. Patent Publication US20020062503) generated from pMON30139 which contains two copies of the CP4 EPSPS gene driven by the P-e35S and P-Os.Act1 promoters, respectively. Plants were

analyzed for glyphosate tolerance, phenotype, copy number, molecular profile, and genome location. Results are provided below.

R0 plants were spray tested for vegetative and reproductive tolerance with 64oz/A Roundup® Ultra (1.68kg/ha acid equivalents of glyphosate) prior to jointing. Plants with vegetative damage were discarded. Fertility was estimated by counting the number of seeds in 20 florets from the central portion of the head and reported as % fertility. Data are provided in Table 9 below.

Table 9: Glyphosate Tolerance in Transgenic R0 Wheat Plants

Promoter	Construct	R0 Events	# Events with Vegetative Tolerance (% of total)	# Events with \geq 80% Fertility (% with Veg Tol)
P-Os.Act1	pMON30167	63	4 (6%)	2 (50%)
P-CaMV.e35S and P-Os.Act1	pMON30139	150	104 (69%)	24 (23%)
P-4xAS-1/P-Os.Act1-3	pMON43646	87	65 (75%)	13 (20%)
P-2xA1-B3/P-Os.Act1-2	pMON43647	83	61 (73%)	16 (26%)
P-2xA1-B3/P-Os.Act1-3	pMON43648	69	51 (74%)	12 (24%)

Data on the vegetative and reproductive glyphosate tolerance of R0 wheat plants transformed with a single cassette vector containing CP4 driven by the rice actin promoter (pMON30167) and a double cassette vector containing two CP4 genes driven by P-CaMV.e35s and P-Os.Act1 are also provided in Table 15. Wheat transformation experiments that used the P-CaMV.e35S cassette alone resulted in plants that were vegetatively tolerant but had low fertility. For example plants generated with pMON42411, comprising only the P-CaMV.e35S promoter driving the CP4 gene, produced only 1 of 37 vegetatively tolerant plants with a fertility of \geq 80%. This data indicates that the e35S promoter is responsible for vegetative expression of CP4 while the rice actin promoter is responsible for reproductive expression of CP4. Results for the single cassette vector containing the three chimeric promoters (pMON43646, pMON43647, and pMON43648) were similar to those for the double cassette vector pMON30139. 73-75% of the total events generated using these three chimeric promoter constructs had vegetative tolerance, and 20-26% of those events had \geq 80% fertility. Thus, using one of the three chimeric promoters to drive a single copy of the CP4 EPSPS gene produced similar results in R0 tests as double cassette lead event.

In addition to glyphosate tolerance, transformation efficiencies (TE), leaf CP4 levels ($\mu\text{gCP4/mg}$ total protein from R0 wheat plants), and meristem CP4 levels ($\mu\text{gCP4/mg}$ total protein from R0 wheat plants) were measured in R0 plants. Plants transformed with pMON30139 (the double cassette construct) and pMON30159 (P-ScBV see U.S. Patent No. 6,489,462) were used as controls. Data are provided in Table 10 below.

Table 10: CP4 Expression in Transgenic R0 Wheat Plants

Promoter	Construct	TE	Leaf CP4	Meristem CP4
P-CaMV.e35S and P-Os.Act1	pMON30139	4%	2.23 (0.93%)	4.54 (1.48%)
P-ScBV	pMON30159	4.50%	6.99 (1.43%)	9.7 (1.71%)
P-4xAS-1/P-Os.Act1-3	pMON43646	3.30%	2.13 (.99%)	3.72 (1.55%)
P-2xA1-B3/P-Os.Act1-2	pMON43647	2.90%	1.41 (1%)	1.66 (1.23%)
P-2xA1-B3/P-Os.Act1-3	pMON43648	2.20%	4.62 (0.77%)	5.79 (1.31%)

R1 seeds were then collected from the R0 plants and advanced to R1 testing. R1 plants were simultaneously tested for glyphosate tolerance and copy number. The transgene copy number for each event was determined by Southern blot and/or TaqMan quantitative PCR analysis. CP4 gene sequences were used as probes for both assays. A good correlation was observed between single copy calls on Southern blots and by TaqMan analysis. Single copy events, with good tolerance to high doses of glyphosate, were advanced to the R2 generation.

Seeds from selected wheat lines advanced to the R2 generation were planted in 2" pots and sprayed with 128 oz/A Roundup at the 3 leaf stage. Retained lines were transferred to larger pots and sprayed again with 128 oz/A at the 6 leaf stage. In an attempt to force differentiation among events an extreme pressure test was devised where plants were sprayed with 512 oz/A of Roundup[®]. This was repeated four times between the 3 leaf stage and emergence of the flag leaf. Results for the chimeric promoter lines were compared with results for non-transgenic Bobwhite plants, R4 generation lead event plants (line 33391 generated from pMON30139), and R3 generation plants containing the P-ScBV promoter (line TA_S2520 generated from pMON30159). Yield data (grams) and fertility data (as % of total plants) were collected for each line. Data are provided in Table 11 below.

Table 11: Pressure Test of R2 Wheat Plants

Promoter	Construct	Line	Yield (g)	Fertility (%)
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N/A	N/A	Non-transgenic Bobwhite	23.1	93.5
P-CaMV.e35S and P-Os.Act1	pMON30139	33391	24.1	91.2
P-ScBV	pMON30159	TA_S2520	21.0	87.5
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S7535	22.2	91.5
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S7890	23.0	95.8
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S9215	21.5	95.2
P-4xAS-1/P-Os.Act1-3	pMON43646	TA_S9240	20.6	95.0

From this analysis it is apparent that the use of the P-4xAS-1/P-Os.Act1-3 (SEQ ID NO: 14), P-2xA1-B3/P-Os.Act1-2 (SEQ ID NO: 19), and P-2xA1-B3/P-Os.Act1-3 (SEQ ID NO: 20) promoters in plant CP4 expression cassettes confers glyphosate tolerance to vegetative tissues in wheat plants without negatively impacting fertility. In wheat, cassettes containing the rice actin promoter without these elements produce plants with low vegetative tolerance and are not useful for the production of glyphosate tolerant plants. Therefore one advantage of the chimeric promoters in wheat is that vegetative and reproductive tolerance can be achieved without the use of a double CP4 cassette.

Field trials were performed with R4 generation plants to assess the performance of selected lines. Field trials were done at eight US sites with 4 replications each and treatments of 0, 64, and 128 oz/A Roundup® Ultra equivalent. Each treatment was arranged as a separate, randomized complete block in order to collect equivalence data on unsprayed non-transformed Bobwhite plants. All events had vegetative and reproductive tolerance comparable to the lead event 33391 at doses up to 128 oz/A of Roundup® Ultra. Yield was measured in tons/hectar (T/ha). Data are provided in Table 12 below.

Table 12: Yield Data from Field Trials of R4 Plants (T/ha)

Promoter	Construct	Line	128 oz/A	64 oz/A	No spray
P-CaMV.e35S and P-Os.Act1	pMON30139	33391	3.52	3.58	3.63
P-ScBV	pMON30159	TA_S2520	3.78	3.79	3.69
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S7535	3.72	3.68	3.51
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S7890	3.79	3.72	3.70
P-2xA1-B3/P-Os.Act1-2	pMON43647	TA_S9215	3.59	3.57	3.42
P-4xAS-1/P-Os.Act1-3	pMON43646	TA_S9240	3.58	3.81	3.66

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims. All publications and published patent documents cited in this
5 specification are incorporated herein by reference to the same extent as if each individual publication or patent application is specifically and individually indicated to be incorporated herein by reference.

We claim:

- 1) A chimeric promoter comprising a caulimovirus promoter enhancer fused with a plant actin gene promoter to form said chimeric promoter.
- 2) The chimeric promoter of claim 1 wherein said caulimovirus promoter enhancer
5 comprises a Cauliflower mosaic virus 35S promoter enhancer.
- 3) The chimeric promoter of claim 2 wherein said Cauliflower mosaic virus 35S promoter enhancer comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 2-6.
- 4) The chimeric promoter of claim 1 wherein said plant actin gene promoter comprises an
10 *Arabidopsis* actin gene promoter.
- 5) The chimeric promoter of claim 4 wherein said *Arabidopsis* actin gene promoter is SEQ ID NO: 8.
- 6) The chimeric promoter of claim 1 wherein said plant actin gene promoter comprises a rice actin gene promoter.
- 7) The chimeric promoter of claim 6 wherein said rice actin gene promoter is SEQ ID NO:
15 7.
- 8) The chimeric promoter of claim 1 wherein said chimeric promoter comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 9-35.
- 9) A construct comprising a chimeric promoter comprising a caulimovirus promoter
20 enhancer fused with a plant actin gene promoter to form said chimeric promoter, wherein said chimeric promoter is operably linked to a transcribable polynucleotide molecule operably linked to a 3' transcription termination polynucleotide molecule.
- 10) The construct of claim 9 wherein said chimeric promoter is selected from the group consisting of SEQ ID NO: 9-35.
- 11) The construct of claim 9, wherein said transcribable polynucleotide molecule is a
25 marker gene.
- 12) The construct of claim 9, wherein said transcribable polynucleotide molecule is a gene of agronomic interest.
- 13) The DNA construct of claim 12, wherein said gene of agronomic interest is a herbicide
30 tolerance gene selected from the group consisting of genes that encode for

phosphinothricin acetyltransferase, glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase, hydroxyphenyl pyruvate dehydrogenase, dalapon dehalogenase, bromoxynil resistant nitrilase, anthranilate synthase, glyphosate oxidoreductase, dicamba monooxygenase, and glyphosate-N-acetyl transferase.

- 5 14) A transgenic plant stably transformed with a construct comprising a chimeric promoter comprising a caulimovirus promoter enhancer fused with a plant actin gene promoter to form a chimeric promoter, wherein said chimeric promoter is operably linked to a transcribable polynucleotide molecule operably linked to a 3' transcription termination polynucleotide molecule.
- 10 15) A transgenic plant of claim 14, wherein said transcribable polynucleotide molecule is a gene of agronomic interest.
- 16) A transgenic plant of claim 14, wherein said transcribable polynucleotide molecule is a marker gene.
- 17) A seed of said transgenic plant of claim 14.
- 15 18) A method of inhibiting weed growth in a field of transgenic glyphosate tolerant crop plants comprising:
- 20 i. planting the transgenic plants transformed with an expression cassette comprising an isolated polynucleotide molecule having gene regulatory activity and comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 9-35 and operably linked to a DNA molecule encoding a glyphosate tolerance gene and
- ii. applying glyphosate to the field at an application rate that inhibits the growth of weeds, wherein the growth and yield of the transgenic crop plant is not substantially affected by the glyphosate application.
- 25 19) In the method of claim 18, wherein said glyphosate tolerance gene is selected from the group consisting of a gene encoding for a glyphosate resistant 5-enolpyruvylshikimate-3-phosphate synthase, a glyphosate oxidoreductase, and a glyphosate-N-acetyltransferase.
- 30 20) In the method of claim 18, wherein the transgenic plants are capable of tolerating an application rate up to 256 ounces/acre.

- 21) In the method of claim 18, wherein the transgenic plants are capable of tolerating an application rate ranging from 8 ounces/acre to 128 ounces/acre.
- 22) In the method of claim 18, wherein the transgenic plants are capable of tolerating an application rate ranging from 32 ounces/acre to 96 ounces/acre.
- 5 23) In the method of claim 18, wherein the application of glyphosate is at least once during the growth of the crop.

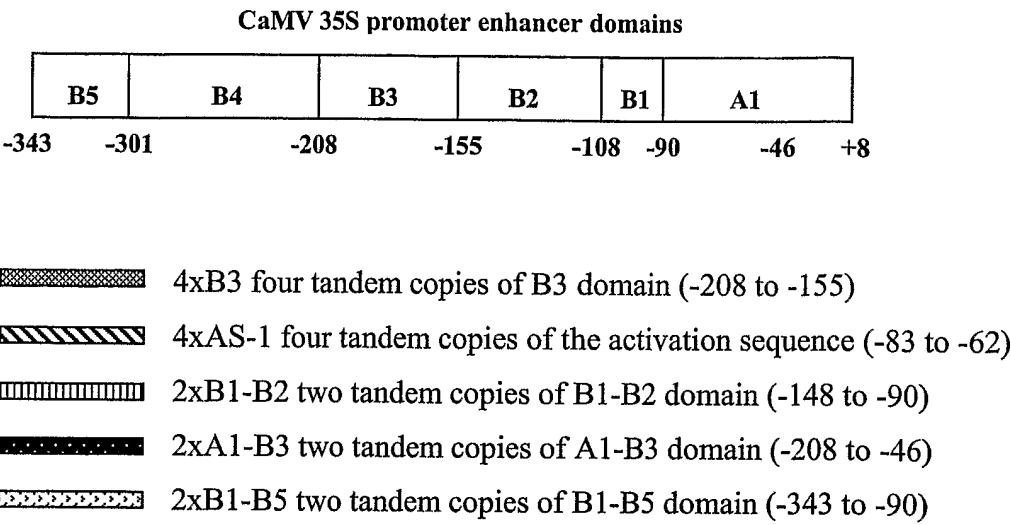


Figure 1

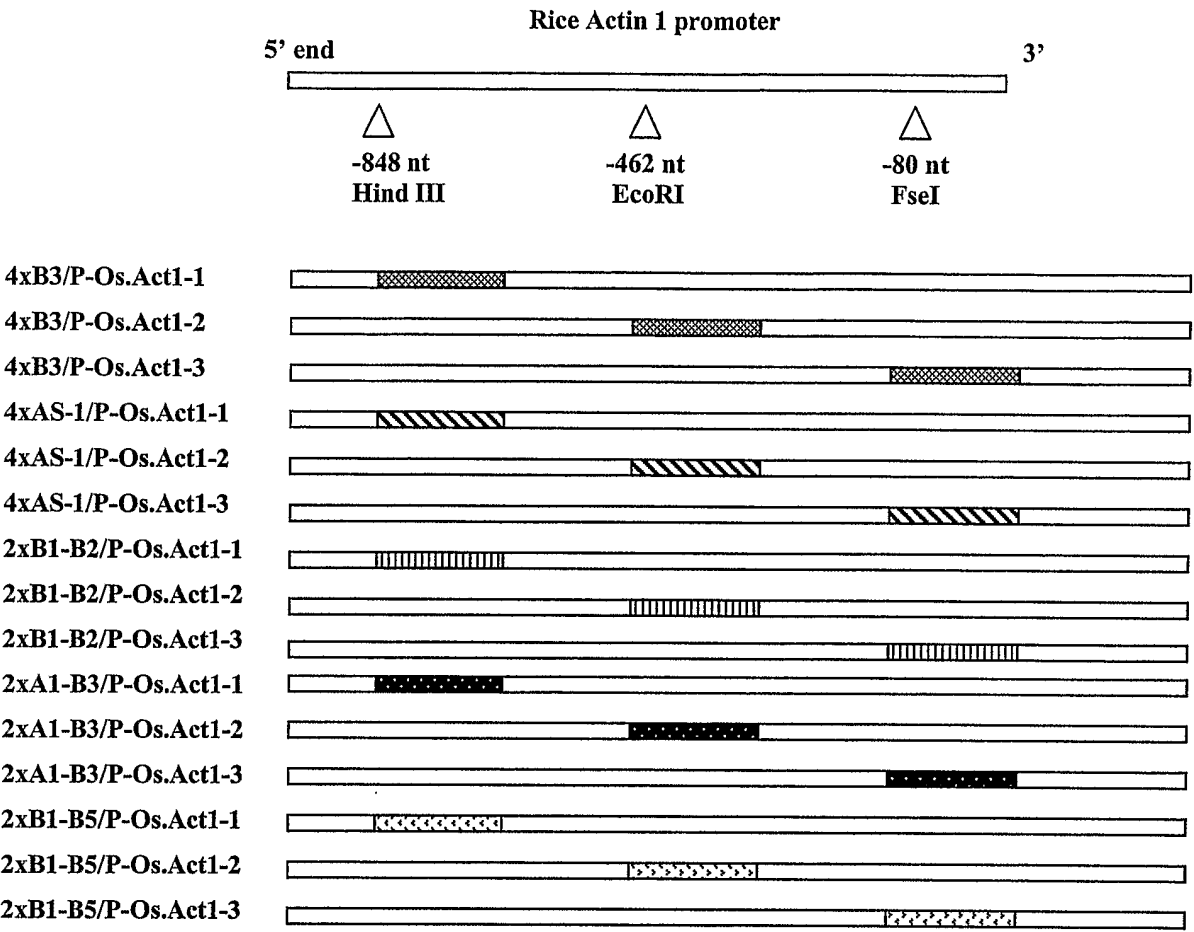


Figure 2

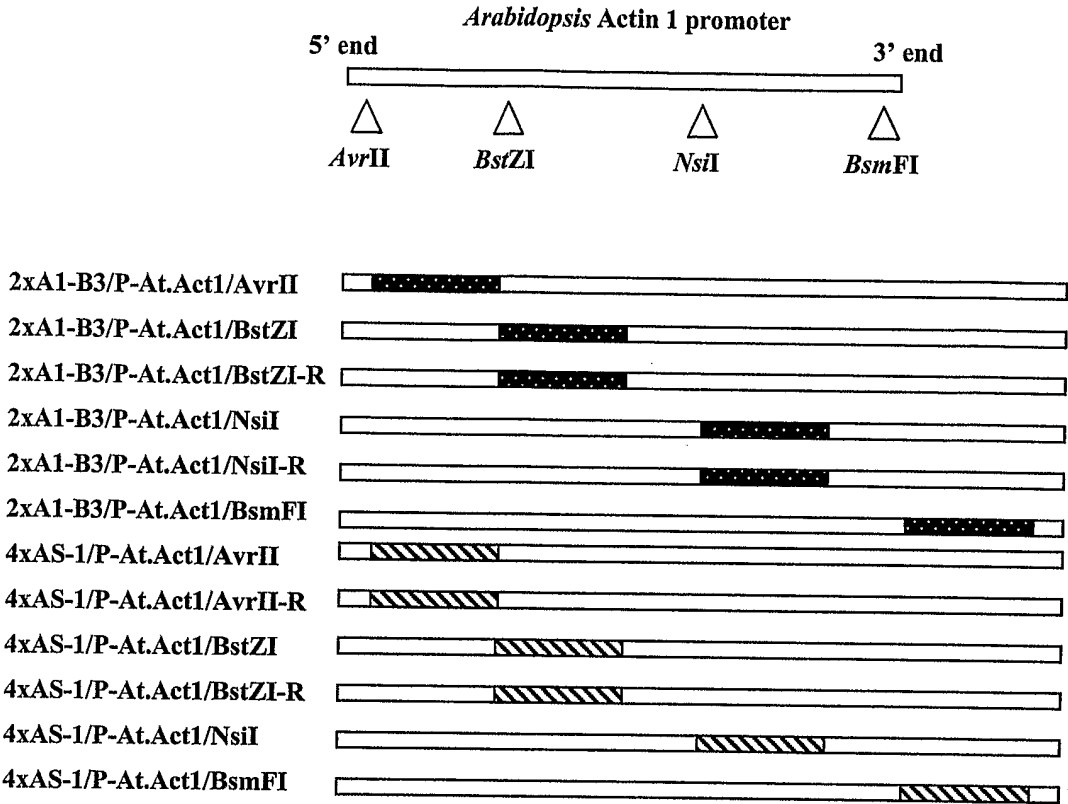


Figure 3