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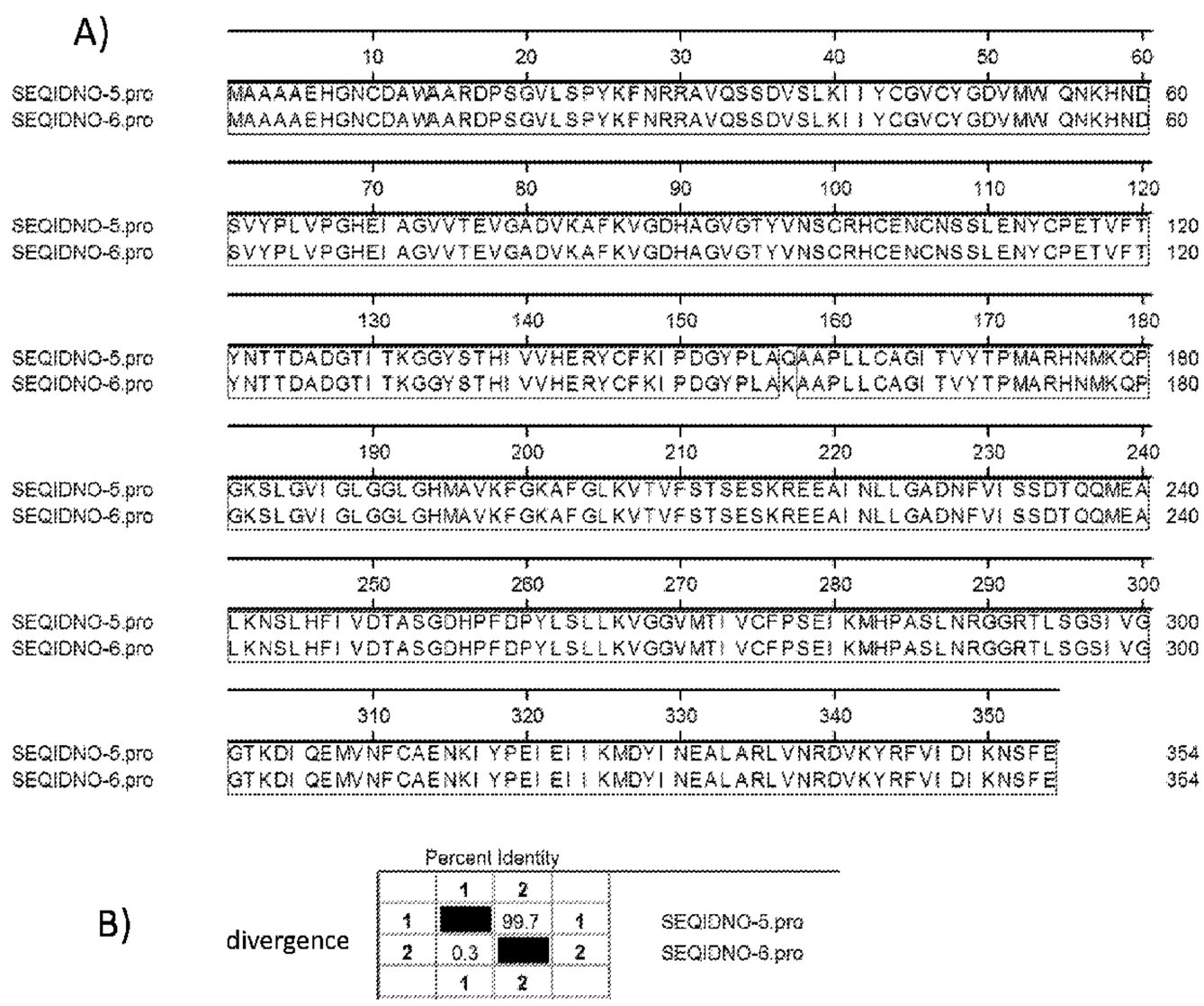
(54) Titre : SEQUENCES DE PROMOTEUR INDUCTIBLE POUR EXPRESSION REGULEE ET PROCEDES D'UTILISATION

(54) Title: INDUCIBLE PROMOTER SEQUENCES FOR REGULATED EXPRESSION AND METHODS OF USE

FIGURE 1



(57) Abrégé/Abstract:
The plant promoter of a CBSU-Anther_Subtraction library (CAS1) gene encoding a mannitol dehydrogenase, and fragments thereof, and their use in promoting the expression of one or more heterologous nucleic acid fragments in an inducible manner in plants are described. These promoter fragments are also useful in creating recombinant DNA constructs comprising nucleic acid sequences encoding a desired gene product operably linked to such promoter fragments which can be utilized to transform plants and bring the expression of the gene product under external chemical and/ or heat control in monocotyledonous and dicotyledonous plants.

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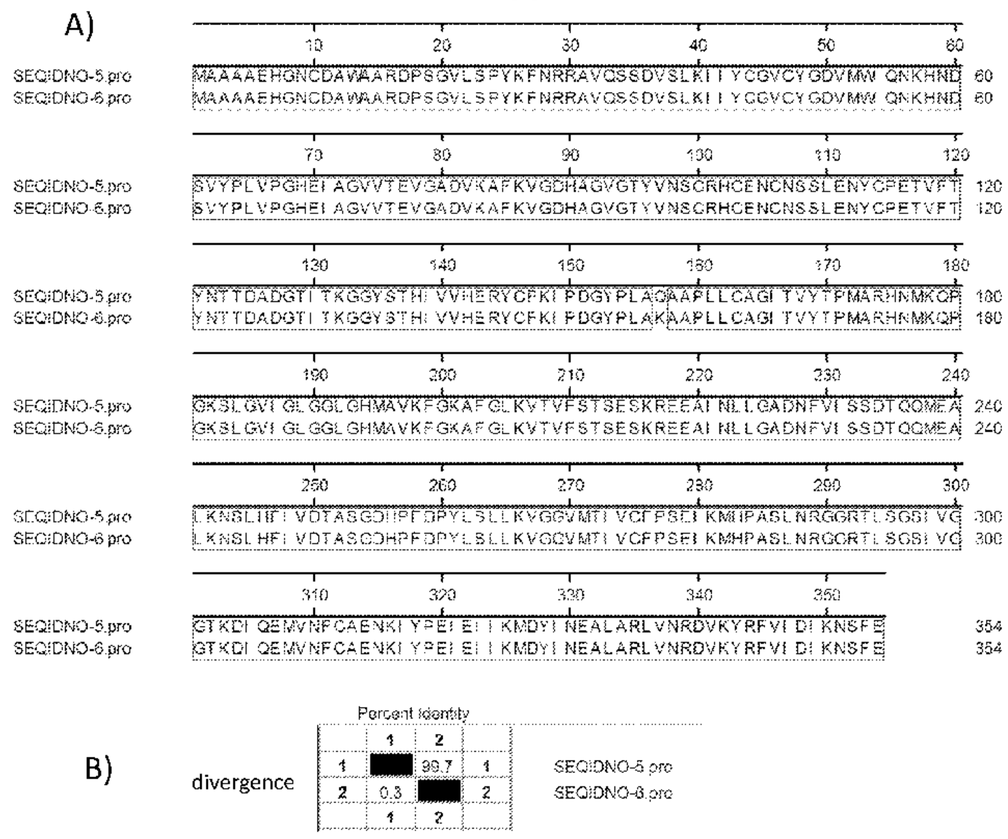
[Continued on next page]

(54) Title: INDUCIBLE PROMOTER SEQUENCES FOR REGULATED EXPRESSION AND METHODS OF USE

FIGURE 1



(57) Abstract: The plant promoter of a CBSU-Anther_Subtraction library (CAS1) gene encoding a mannitol dehydrogenase, and fragments thereof, and their use in promoting the expression of one or more heterologous nucleic acid fragments in an inducible manner in plants are described. These promoter fragments are also useful in creating recombinant DNA constructs comprising nucleic acid sequences encoding a desired gene product operably linked to such promoter fragments which can be utilized to transform plants and bring the expression of the gene product under external chemical and/ or heat control in monocotyledonous and dicotyledonous plants.

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INDUCIBLE PROMOTER SEQUENCES FOR REGULATED EXPRESSION AND METHODS OF USE

This application claims the benefit of U.S. Provisional Application No. 61/648758, filed May, 18, 2012, the entire content of which is herein incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to a plant promoter, and fragments thereof, and their use in altering expression of at least one heterologous nucleic acid sequence in plants in an inducible manner. These promoter fragments are also useful in creating recombinant DNA constructs comprising nucleic acid sequences encoding a desired gene product operably linked to such promoter fragments which can be utilized to transform plants and bring the expression of the gene product under external chemical and/ or stress control in monocotyledonous and dicotyledonous plants.

BACKGROUND OF THE INVENTION

Recent advances in plant genetic engineering have opened new doors to engineer plants to have improved characteristics or traits, such as plant disease resistance, insect resistance, herbicidal resistance, yield improvement, improvement of the nutritional quality of the edible portions of the plant, and enhanced stability or shelf-life of the ultimate consumer product obtained from the plants. Thus, a desired gene (or genes) with the molecular function to impart different or improved characteristics or qualities, can be incorporated properly into the plant's genome. The newly integrated gene (or genes) coding sequence can then be expressed in the plant cell to exhibit the desired new trait or characteristics. It is important that appropriate regulatory signals must be present in proper configurations in order to obtain the expression of the newly inserted gene coding sequence in the plant cell. These regulatory signals typically include a promoter region, a 5' non-translated leader sequence and a 3' transcription termination/polyadenylation sequence.

A promoter is a non-coding genomic DNA sequence, usually upstream (5') to the relevant coding sequence, to which RNA polymerase binds before initiating transcription. This binding aligns the RNA polymerase so that transcription will

initiate at a specific transcription initiation site. The nucleotide sequence of the promoter determines the nature of the enzyme and other related protein factors that attach to it and the rate of RNA synthesis. The RNA 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 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 coding region that functions in the plant cell to cause termination of the RNA synthesis and the addition of polyadenylate nucleotides to the 3' end.

It has been shown that certain promoters are able to direct RNA synthesis at a higher rate than others. These are called “strong promoters”. Certain other promoters have been shown to direct RNA synthesis at higher levels only in particular types of cells or tissues and are often referred to as “tissue specific promoters”, or “tissue-preferred promoters” if the promoters direct RNA synthesis preferably in certain tissues but also in other tissues at reduced levels. Certain promoters are able to direct RNA synthesis at relatively similar levels across all tissues of a plant. These are called “constitutive promoters” or “tissue –independent” promoters. Constitutive promoters can be divided into strong, moderate and weak according to their effectiveness to direct RNA synthesis. In some cases promoters are able to direct RNA synthesis when they are induced by external stimuli such as chemicals, stress, or biotic stimuli. These are called “inducible promoters”.

The ability to externally control the expression of selected genes and thereby their gene products in plant cells and/or field grown plants can provide important agronomic and foodstuff benefits. This control is desirable for the regulation of genes that might be placed into transgenic plants and has many applications including, but not limited to, (1) prolonging or extending the accumulation of desirable nutritional food reserve in seeds, roots, (2) producing and accumulating products in plant tissues at a defined time in the developmental cycle such that these products are convenient for harvest and/or isolation, and (3) initiating the expression a pest-specific toxin at the site of pathogen attack. There is an ongoing interest in the isolation of novel inducible promoters which are capable of controlling the expression

of a chimeric gene or (genes) at certain levels in a plant cell when exposed to external stimuli.

SUMMARY OF THE INVENTION

This invention relates to a plant promoter of a CBSU-Anther_Subtraction library (CAS1) gene encoding a mannitol dehydrogenase, and functional fragments thereof, and their use in promoting the expression of one or more heterologous nucleic acid fragments in an inducible manner in plants. These promoter fragments are also useful in creating recombinant DNA constructs comprising nucleic acid sequences encoding a desired gene product operably linked to such promoter fragments which can be utilized to transform plants and bring the expression of the gene product under external chemical and/ or heat control in monocotyledonous and dicotyledonous plants. One embodiment of the invention concerns an isolated nucleic acid fragment comprising an inducible ZmCAS1 promoter wherein said promoter consists essentially of the nucleotide sequence set forth in SEQ ID NOs: 9 or 10, or said promoter consists essentially of a fragment that is substantially similar and functionally equivalent to the nucleotide sequence set forth in SEQ ID NOs: 9 or 10. The ZmCAS1 promoter can be induced by a chemical or stress treatment. The chemical can be a safener such as, but not limited to, N-(aminocarbonyl)-2-chlorobenzenesulfonamide (2-CBSU). The stress treatment can be a treatment such as, but not limited to, a heat shock treatment of a temperature greater than 26°C.

The invention also concerns a recombinant DNA construct comprising at least one heterologous nucleic acid fragment operably linked to the promoter of the invention.

In another embodiment, this invention concerns a cell, plant, or seed comprising a recombinant expression construct of the present disclosure.

In another embodiment, this invention concerns a plant stably transformed with a recombinant expression construct comprising a plant promoter and a heterologous nucleic acid fragment operably linked to said promoter, wherein said promoter is an inducible promoter and capable of controlling expression of said heterologous nucleic acid fragment in a plant cell, and further wherein said promoter comprises a fragment of SEQ ID NOs: 9 or10.

In another embodiment, this invention concerns a method of expressing a

coding sequence or a functional RNA in a plant cell comprising: a) introducing the recombinant DNA construct of the current disclosure into a plant cell, wherein at least one heterologous sequence comprises a coding sequence or a functional RNA, b) growing the plant cell of step a); c) induction of the inducible promoter by chemical or stress treatment on the plant cell of b); and, d) selecting a plant cell displaying expression of the coding sequence or the functional RNA of the recombinant DNA construct. In another embodiment, this invention concerns a method of expressing a coding sequence or a functional RNA driven by the promoter of the current invention in anther, callus, leaf or root cells.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing that form a part of this application.

Figure 1: Alignment the amino acid sequence encoded by the ZmCAS1cDNA (SEQ ID NO:5) with a maize mannitol dehydrogenase (GI:226528549; SEQ ID NO:6) (A) and percent identity (B).

Figure 2: Northern blot of maize anther RNA of wild-type fertile (F) and sterile (S) maize control plants (-) and maize CBSU treated plants (+). Maize anther RNA was analyzed with probes specific for ZmCAS1, IN2-2, 5126, MS45, ACTIN and UBI gene expression.

Figure 3: Northern blot of maize callus (C), leaf (L) and anther (A) RNAs from wild-type maize tissues and CBSU-treated (+) tissues. Maize RNA was analyzed with probes specific for IN2-2 and ZmCAS1.

Figure 4 shows A) maize callus transformed with PHP16975 comprising the 1.7 kb ZmCAS1 promoter for three different events (1, 2, 3) C= control maintenance media; 10= 10 mg/l CBSU, 100 = 100 mg/l CBSU; B) maize callus transformed with PHP16974 comprising the truncated 1.0 kb ZmCAS1 promoter and induced with either CBSU or heat (37°C); 26C= control callus at 26°C; 26C+CBSU= CBSU treated callus at 26°C; 37C = callus induced by heat treatment of 37°C. Results from seven events (1-7) are shown.

Figure 5 shows maize leaf punches from three (1, 2, 3) maize plants transformed with PHP16975 and induced with CBSU. Leaf punches from plants

regenerated from the 3 bialophos-resistant events were collected pre- (C) and post-watering (S).

Figure 6 shows a Northern blot of maize callus RNA from five (1, 2, 3, 4 and 5) events transformed with PHP16972 and treated with (+) or without (-) CBSU.

Figure 7 shows a Western analysis of leaves from ms45/ms45 maize plants transformed with PHP16973 using antibodies directed against the maize MS45 protein. C= leaves from uninduced control plants, + = leaves from CBSU induced plants. Whole-cell anther extract from a wild-type MS45 plant is shown in Lane 1 and used to identify the mobility of the immunoreactive MS45 protein as indicated by the arrow.

Figure 8 shows a Western analysis of anthers from ms45/ms45 maize plants transformed with PHP16973 using antibodies directed against the maize MS45 protein. C= leaves from uninduced control plants, + = leaves from CBSU induced plants.

Figure 9: Rice events transformed with PHP16974 show GUS expression when driven by the 1.0 kb ZmCAS1 promoter and induced by CBSU.

Figure 10: Rice seedlings transformed with PHP16974 show GUS expression when driven by the 1.0 kb ZmCAS1 promoter and induced by CBSU.

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

The sequence descriptions summarize the Sequence Listing attached hereto. The Sequence Listing contains one letter codes for nucleotide sequence characters and the single and three letter codes for amino acids as defined in the IUPAC-IUB standards described in *Nucleic Acids Research* 13:3021-3030 (1985) and in the *Biochemical Journal* 219(2):345-373 (1984). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. § 1.822.

SEQ ID NO:1 DNA insert comprising the ZmCAS1c-1 cDNA.

SEQ ID NO:2 DNA insert comprising the ZmCAS1c-2 cDNA.

SEQ ID NO:3 A 1354 bp (base pair) SalI-NotI DNA insert comprising the maize ZmCAS1 full length cDNA.

SEQ ID NO:4 the1338 bp maize ZmCAS1 full length cDNA.

SEQ ID NO:5 the amino acid sequence encoded by SEQ ID NO:4

SEQ ID NO:6 the amino acid sequence of a maize mannitol dehydrogenase (GI number 226528549, NP_001147757.1)

SEQ ID NO:7 a 4069 bp DNA fragment comprising the maize B73 ZmCAS1 promoter

SEQ ID NO:8 is the DNA sequence of the oligonucleotide used for mutagenesis to introduce RCAI DNA restriction site.

SEQ ID NO:9 is a 1049 bp truncated form of the maize ZmCAS1 promoter (bp 698-1746 of SEQ ID NO:9) also referred to as the 1.0 kb ZmCAS1 promoter.

SEQ ID NO:10 is 1746 bp maize ZmCAS1 promoter, also referred to as the 1.7 kb ZmCAS1 promoter.

SEQ ID NO: 11 is the nucleotide sequence of PHP16974 comprising the 1.0 kb ZmCAS1 promoter.

SEQ ID NO: 12 is the nucleotide sequence of PHP16975 comprising the 1.7 kb ZmCAS1 promoter.

SEQ ID NO: 13 is the nucleotide sequence of PHP16972 comprising the 1.0 kb ZmCAS1 promoter.

SEQ ID NO: 14 is the nucleotide sequence of PHP16973 comprising the 1.7 kb ZmCAS1 promoter.

SEQ ID NO: 15 is the HindIII-Rca1 fragment (ZMCAS1HINDIIIPRO) comprising the 1.0 kb ZmCAS1 promoter of SEQ ID NO:9.

SEQ ID NO: 16 is the BamH1-Rca1 fragment (ZMCAS1BAMPRO) comprising the 1.7 kb ZmCAS1 promoter of SEQ ID NO:10.

SEQ ID NO: 17 is the amino acid sequence of a mannitol dehydrogenase (AAP52597) from rice (*Oryza sativa*).

SEQ ID NO: 18 is a nucleotide sequence from a mannitol dehydrogenase gene region (DP000086) from rice (*Oryza sativa*).

SEQ ID NO: 19 is a nucleotide sequence of a putative 5'UTR-Promoter region from a mannitol dehydrogenase gene (DP000086) from rice (*Oryza sativa*).

SEQ ID NO: 20 is the amino acid sequence of a mannitol dehydrogenase (XP-002436634) from Sorghum.

SEQ ID NO: 21 is a nucleotide sequence from a mannitol dehydrogenase gene region (NC-012879) from Sorghum.

SEQ ID NO: 22 is a nucleotide sequence of a putative 5'UTR-Promoter region from a mannitol dehydrogenase gene (NC-012879) from Sorghum.

DETAILED DESCRIPTION OF THE INVENTION

The disclosure of all patents, patent applications, and publications cited herein are incorporated by reference in their entirety.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes a plurality of such plants, reference to "a cell" includes one or more cells and equivalents thereof known to those skilled in the art, and so forth.

In the context of this disclosure, a number of terms shall be utilized.

As used herein, a "ZmCAS1 promoter" refers to one type of inducible promoter. The native ZmCAS1 promoter is the promoter of a maize gene isolated from a CBSU-Anther_Subtraction library with significant homology to mannitol dehydrogenase genes identified in various plant species including maize that are deposited in National Center for Biotechnology Information (NCBI) database. The "ZmCAS1 promoter", as used herein, also refers to fragments of the full-length native promoter that retain significant promoter activity. For example, a ZmCAS1 promoter can be 1.7 kb in length (SEQ ID NO:10) or a promoter-functioning fragment thereof, which includes, among others, the polynucleotide of SEQ ID NO: 9. A ZmCAS1 promoter also includes variants that are substantially similar and functionally equivalent to any portion of the nucleotide sequence, in increments of one base pair, between the 1.0 kb (SQE ID NO:9) and 1.7 kb (SEQ ID NO:10) fragments and sequences .

The term “Promoter” refers to a nucleotide sequence capable of regulating the expression of a coding sequence or functional RNA. Functional RNA includes, but is not limited to, transfer RNA (tRNA) and ribosomal RNA (rRNA). The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. The promoter usually comprises 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 can 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 region disclosed herein, it is within the state of the art to isolate and identify further regulatory elements in the region upstream of the TATA box from the particular promoter region identified herein. Accordingly, an “enhancer” is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or abiotic conditions.

The promoter elements which enable the inducible expression in the desired tissue can be identified, isolated, and used with other core promoters to confirm inducible expression. By core promoter is meant the minimal sequence required to initiate transcription, such as the sequence called the TATA box which is common to promoters in genes encoding proteins. Thus, the ZmCAS1 promoter can optionally be used in conjunction with its own or core promoters from other sources. The promoter may be native or non-native to the cell in which it is found.

Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (Biochemistry

of Plants 15:1-82 (1989)). It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity.

High level, constitutive expression of the candidate gene under control of the 35S or UBI promoter may have pleiotropic effects, although candidate gene efficacy may be estimated when driven by a constitutive promoter. Use of inducible or stress-specific promoters may eliminate undesirable effects but retain the ability to enhance drought tolerance. This effect has been observed in *Arabidopsis* (Kasuga et al. (1999) Nature Biotechnol. 17:287-91).

The term “inducible promoter” refers to promoters that selectively express a coding sequence or functional RNA in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters induced or regulated by light, heat, stress, flooding or drought, salt stress, osmotic stress, phytohormones, wounding, or chemicals such as ethanol, abscisic acid (ABA), jasmonate, salicylic acid, or safeners.

An example of a stress-inducible is RD29A promoter (Kasuga et al. (1999) Nature Biotechnol. 17:287-91). One of ordinary skill in the art is familiar with protocols for simulating drought conditions and for evaluating drought tolerance of plants that have been subjected to simulated or naturally-occurring drought conditions. For example, one can simulate drought conditions by giving plants less water than normally required or no water over a period of time, and one can evaluate drought tolerance by looking for differences in physiological and/or physical condition, including (but not limited to) vigor, growth, size, or root length, or in particular, leaf color or leaf area size. Other techniques for evaluating drought tolerance include measuring chlorophyll fluorescence, photosynthetic rates and gas exchange rates. Also, one of ordinary skill in the art is familiar with protocols for simulating stress conditions such as osmotic stress, salt stress and temperature stress and for evaluating stress tolerance of plants that have been subjected to simulated or naturally-occurring stress conditions.

The sequences of the invention may be isolated from any plant, including, but not limited to corn (*Zea mays*), canola (*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*), millet (*Panicum spp.*), 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 (*Avena sativa*), barley (*Hordeum vulgare*), vegetables, ornamentals, and conifers. Preferably, plants include corn, soybean, sunflower, safflower, canola, wheat, barley, rye, alfalfa, rice, cotton and sorghum.

This invention concerns an isolated nucleic acid fragment comprising an inducible ZmCAS1 promoter. This invention also concerns an isolated nucleic acid fragment comprising a promoter wherein said promoter consists essentially of the nucleotide sequence set forth in SEQ ID NO:9, or said promoter consists essentially of a fragment that is substantially similar and functionally equivalent to the nucleotide sequence set forth in SEQ ID NO:10. A nucleic acid fragment that is functionally equivalent to the instant ZmCAS1 promoter is any nucleic acid fragment that is capable of controlling the expression of a coding sequence or functional RNA in a similar manner to the ZmCAS1 promoter. The expression patterns of ZmCAS1 gene and its promoter are set forth in Examples 1-3.

The promoter activity of the maize genomic DNA fragment SEQ ID NO:9 or SEQ ID NO:10 upstream of the ZmCAS1 protein coding sequence was assessed by linking the fragment to a GUS gene or a MS45 gene, transforming the promoter:GUS (or MS45) expression cassette into maize, and analyzing GUS (or MS45) expression in various cell types of the transgenic plants (Examples 1-3). These results indicated that the nucleic acid fragment contained an inducible promoter.

In one embodiment, the invention is an isolated polynucleotide comprising, or consisting essentially of or consisting of:

a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:9 or a full-length complement thereof;

b) a nucleotide sequence comprising a fragment of SEQ ID NO:10, or a full-length complement thereof

c) a nucleotide sequence comprising a sequence having at least 90% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence of (a) or (b);

d) a nucleotide sequence comprising all or a fragment of a 1.7 kb 5 ‘ non-coding sequence of a mannitol dehydrogenase; or,

e) a derivative of one of the nucleotide sequences indicated in (a), (b), or (c) obtained by substitution, addition and/or deletion of one or more nucleotides; and,

wherein said nucleotide sequence is an inducible promoter.

In another embodiment of the invention the ZmCAS1 promoter is induced by a safener treatment of N-(aminocarbonyl)-2-chlorobenzenesulfonamide (2-CBSU). In another embodiment of the invention the ZmCAS1 promoter is induced by a heat treatment of a temperature greater than 26 °C and up to and including 37 °C.

The terms “N-(aminocarbonyl)-2-chlorobenzenesulfonamide ” , 2-CBSU” and “CBSU” are used interchangeably herein.

The promoter nucleotide sequences and methods disclosed herein are useful in regulating inducible expression of any heterologous nucleotide sequences in a host plant in order to alter the phenotype of a plant.

Various changes in phenotype are of interest including, but not limited to, modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant’s pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products 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 or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

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 characteristics and traits such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, but are not limited to, 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. Other gene of interest are genes allowing for site specific gene integration and gene stacking include, but not limited to, double-strand break inducing genes and recombinase genes. More specific categories of transgenes, for example, include, but are not limited to, genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain or seed characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting seed size, plant development, plant growth regulation, and yield improvement. Plant development and growth regulation also refer to the development and growth regulation of various parts of a plant, such as the flower, seed, root, leaf and shoot.

Other commercially desirable traits are genes and proteins conferring cold, heat, salt, and drought resistance.

One embodiment of the invention relates to a recombinant DNA comprising the isolated polynucleotide of the invention operably linked to at least one heterologous nucleic acid sequence, wherein the heterologous nucleic acid sequence codes for a gene selected from the group consisting of: a double-strand break inducing gene, a recombinase gene, a reporter gene, a selection marker, a disease resistance conferring gene, a herbicide resistance conferring gene, an insect resistance conferring gene; a gene involved in carbohydrate metabolism, a gene involved in fatty acid metabolism, a gene involved in amino acid metabolism, a gene involved in plant development, a gene involved in plant growth regulation, a gene involved in yield improvement, a gene involved in drought resistance, a gene involved in cold resistance, a gene involved in heat and salt resistance in plants.

Another embodiment of the invention relates to a recombinant DNA comprising the isolated polynucleotide of the invention operably linked to at least one heterologous nucleic acid sequence, wherein the heterologous nucleic acid sequence encodes a protein selected from the group consisting of: a double-strand break inducing protein, a recombinase protein, a reporter protein, a selection marker, a protein conferring disease resistance, protein conferring herbicide resistance, protein conferring insect resistance; protein involved in carbohydrate metabolism, protein involved in fatty acid metabolism, protein involved in amino acid metabolism, protein involved in plant development, protein involved in plant growth regulation, protein involved in yield improvement, protein involved in drought resistance, protein involved in cold resistance, protein involved in heat resistance and salt resistance in plants.

One embodiment of the invention, comprises a plant (for example, maize or a soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to a promoter fragment of the invention, wherein said promoter fragment comprises at least 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 9 or 10, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant not comprising said recombinant DNA construct.

Another embodiment of the invention, comprises a plant (for example, maize or a soybean plant) comprising in its genome a suppression DNA construct comprising a promoter fragment of the invention, wherein said promoter fragment comprises at least 40%, 45%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NOs: 9 or 10, and wherein said plant exhibits an alteration of at least one

agronomic characteristic when compared to a control plant not comprising said recombinant DNA construct.

In any of the foregoing embodiments or any other embodiments of the present invention, the at least one agronomic characteristic may be selected from the group consisting of greenness, yield, growth rate, biomass, fresh weight at maturation, dry weight at maturation, fruit yield, seed yield, total plant nitrogen content, fruit nitrogen content, seed nitrogen content, nitrogen content in a vegetative tissue, total plant free amino acid content, fruit free amino acid content, seed free amino acid content, free amino acid content in a vegetative tissue, total plant protein content, fruit protein content, seed protein content, protein content in a vegetative tissue, drought tolerance, nitrogen uptake, root lodging, harvest index, stalk lodging, plant height, ear height, ear length, early seedling vigor and seedling emergence under low temperature stress. For example, the alteration of at least one agronomic characteristic may be an increase in yield, greenness or biomass.

Disease and /or insect resistance genes may encode resistance to pests that have great yield drag such as for example, anthracnose, soybean mosaic virus, soybean cyst nematode, root-knot nematode , brown leaf spot, Downy mildew, purple seed stain, seed decay and seedling diseases caused commonly by the fungi - *Pythium* sp., *Phytophthora* sp., *Rhizoctonia* sp., *Diaporthe* sp.. Bacterial blight caused by the bacterium *Pseudomonas syringae* pv. *Glycinea.* Genes conferring insect resistance include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent 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 include 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). The ALS-gene mutants encode resistance to the herbicide chlorsulfuron. Glyphosate acetyl transferase (GAT) is an *N*-acetyltransferase from *Bacillus licheniformis* that was optimized by gene shuffling for acetylation of the broad spectrum herbicide, glyphosate, forming the basis of a novel mechanism of

glyphosate tolerance in transgenic plants (Castle et al. (2004) *Science* 304, 1151-1154).

Antibiotic resistance genes include, for example, neomycin phosphotransferase (npt) and hygromycin phosphotransferase (hpt). Two neomycin phosphotransferase genes are used in selection of transformed organisms: the neomycin phosphotransferase I (nptI) gene and the neomycin phosphotransferase II (nptII) gene. The second one is more widely used. It was initially isolated from the transposon Tn5 that was present in the bacterium strain *Escherichia coli* K12. The gene codes for the aminoglycoside 3'-phosphotransferase (denoted aph(3')-II or NPTII) enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin and paroromycin. NPTII is widely used as a selectable marker for plant transformation. It is also used in gene expression and regulation studies in different organisms in part because N-terminal fusions can be constructed that retain enzyme activity. NPTII protein activity can be detected by enzymatic assay. In other detection methods, the modified substrates, the phosphorylated antibiotics, are detected by thin-layer chromatography, dot-blot analysis or polyacrylamide gel electrophoresis. Plants such as maize, cotton, tobacco, Arabidopsis, flax, soybean and many others have been successfully transformed with the *npt*II gene.

The hygromycin phosphotransferase (denoted *hpt, hph* or *aph*IV) gene was originally derived from *Escherichia coli*. The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B. A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, including monocotyledonous. Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals. Likewise, the *hpt* gene is used widely in selection of transformed mammalian cells. The sequence of the *hpt* gene has been modified for its use in plant transformation. Deletions and substitutions of amino acid residues close to the carboxy (C)-terminus of the enzyme have increased the level of resistance in certain plants, such as tobacco. At the same time, the hydrophilic C-terminus of the enzyme has been maintained and may be essential for the strong activity of HPT. HPT activity can be checked using an enzymatic assay.

A non-destructive callus induction test can be used to verify hygromycin resistance.

Genes involved in plant growth and development have been identified in plants. One such gene, which is involved in cytokinin biosynthesis, is isopentenyl transferase (IPT). Cytokinin plays a critical role in plant growth and development by stimulating cell division and cell differentiation (Sun et al. (2003), Plant Physiol. 131: 167-176).

Calcium-dependent protein kinases (CDPK), a family of serine-threonine kinase found primarily in the plant kingdom, are likely to function as sensor molecules in calcium-mediated signaling pathways. Calcium ions are important second messengers during plant growth and development (Harper et al. Science 252, 951-954 (1993); Roberts et al. Curr Opin Cell Biol 5, 242-246 (1993); Roberts et al. Annu Rev Plant Mol Biol 43, 375-414 (1992)).

Nematode responsive protein (NRP) is produced by soybean upon the infection of soybean cyst nematode. NRP has homology to a taste-modifying glycoprotein miraculin and the NF34 protein involved in tumor formation and hyper response induction. NRP is believed to function as a defense-inducer in response to nematode infection (Tenhaken et al. BMC Bioinformatics 6:169 (2005)).

The quality of seeds and grains is reflected in traits such as levels and types of fatty acids or oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of carbohydrates. Therefore, commercial traits can also be encoded on a gene or genes that could increase for example methionine and cysteine, two sulfur containing amino acids that are present in low amounts in soybeans. Cystathionine gamma synthase (CGS) and serine acetyl transferase (SAT) are proteins involved in the synthesis of methionine and cysteine, respectively.

Other commercial traits can encode genes to increase for example monounsaturated fatty acids, such as oleic acid, in oil seeds. Soybean oil for example contains high levels of polyunsaturated fatty acids and is more prone to oxidation than oils with higher levels of monounsaturated and saturated fatty acids. High oleic soybean seeds can be prepared by recombinant manipulation of the activity of oleoyl 12-desaturase (Fad2). High oleic soybean oil can be used in applications that require a high degree of oxidative stability, such as cooking for a long period of time at an elevated temperature.

Raffinose saccharides accumulate in significant quantities in the edible portion of many economically significant crop species, such as soybean (*Glycine max* L. Merrill), sugar beet (*Beta vulgaris*), cotton (*Gossypium hirsutum* L.), canola (*Brassica sp.*) and all of the major edible leguminous crops including beans (*Phaseolus sp.*), chick pea (*Cicer arietinum*), cowpea (*Vigna unguiculata*), mung bean (*Vigna radiata*), peas (*Pisum sativum*), lentil (*Lens culinaris*) and lupine (*Lupinus sp.*). Although abundant in many species, raffinose saccharides are an obstacle to the efficient utilization of some economically important crop species.

Down regulation of the expression of the enzymes involved in raffinose saccharide synthesis, such as galactinol synthase for example, would be a desirable trait.

In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or a single vector incorporating two or more gene coding sequences. Any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, and nematode) or drought resistance, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

The term “Anther” or “Anther tissue” refers to male plant tissue encompassing cells, cell-layers and cell types that give rise to pollen grains capable of effecting fertilization. These cells include but are not limited to archesporial cells, pollen mother cells, meiocytes, microspores, tapetum, supporting cell layers, pollen and cells derived from these cell types.

An “isolated nucleic acid fragment” refers to a polymer of ribonucleotides (RNA) or deoxyribonucleotides (DNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The terms “polynucleotide”, “polynucleotide sequence”, “nucleic acid sequence”, and “nucleic acid fragment”/“isolated nucleic acid fragment” are used interchangeably herein. These terms encompass nucleotide sequences and the

like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by a single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

A "heterologous nucleic acid fragment" refers to a sequence that is not naturally occurring with the plant promoter sequence of the invention. While this nucleotide sequence is heterologous to the promoter sequence, it may be homologous, or native, or heterologous, or foreign, to the plant host. However, it is recognized that the instant promoters may be used with their native coding sequences to increase or decrease expression resulting in a change in phenotype in the transformed seed.

The terms "fragment (or variant) that is functionally equivalent" and "functionally equivalent fragment (or variant)" are used interchangeably herein. These terms refer to a portion or subsequence or variant of the promoter sequence of the present invention in which the ability to initiate transcription or drive gene expression (such as to produce a certain phenotype) is retained. Fragments and variants can be obtained via methods such as site-directed mutagenesis and synthetic construction. As with the provided promoter sequences described herein, the contemplated fragments and variants operate to promote inducible expression of an operably linked heterologous nucleic acid sequence, forming a recombinant DNA construct (also, a chimeric gene). For example, the fragment or variant can be used in the design of recombinant DNA constructs to produce the desired phenotype in a transformed plant. Recombinant DNA constructs can be designed for use in co-suppression or antisense by linking a promoter fragment or variant thereof in the appropriate orientation relative to a heterologous nucleotide sequence.

A functional fragment of the regulatory sequence can be formed by one or more deletions from a larger sequence. For example, the 5' portion of a promoter up to the TATA box near the transcription start site can be deleted without abolishing promoter activity, as described by Opsahl-Sorteberg, H-G. et al., "Identification of a 49-bp fragment of the HvLTP2 promoter directing aleruone cell specific expression" Gene 341:49-58 (2004). Such variants should retain promoter activity. Activity can be measured by Northern blot analysis, reporter activity measurements when using transcriptional fusions, and the like. See, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), herein incorporated by reference.

Sequences which hybridize to the regulatory sequences of the present invention are within the scope of the invention. Sequences that correspond to the promoter sequences of the present invention and hybridize to the promoter sequences disclosed herein will be at least 40% homologous, 50% homologous, 70% homologous, and even 85% 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% homologous or more with the disclosed sequence.

Smaller fragments may yet contain the regulatory properties of the promoter so identified and deletion analysis is one method of identifying essential regions. Deletion analysis can occur from both the 5' and 3' ends of the regulatory region. Fragments can be obtained by site-directed mutagenesis, mutagenesis using the polymerase chain reaction and the like. (See, Directed Mutagenesis: A Practical Approach IRL Press (1991)).

In some aspects of the present invention, the promoter fragments can comprise at least about 20 contiguous nucleotides, or at least about 50 contiguous nucleotides, or at least about 75 contiguous nucleotides, or at least about 100, 150, 200, 250, 300, 350, 400, 450, 500 contiguous nucleotides of SEQ ID NO:8 or up to the number of nucleotides present in a full-length nucleotide sequence disclosed herein (for example 1746, SEQ ID NO: 10).

In another aspect, a promoter fragment is the nucleotide sequence set forth in SEQ ID NO: 9 or SEQ ID NO: 10. 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 sequences disclosed herein, by synthesizing a nucleotide sequence from the naturally occurring promoter DNA sequence, or may be obtained through the use of PCR technology. See particularly, Mullis et al., Methods Enzymol. 155:335-350 (1987), and Higuchi, R. In PCR Technology: Principles and Applications for DNA Amplifications; Erlich, H.A., Ed.; Stockton Press Inc.: New York, 1989.

The isolated promoter sequences of the present invention can be modified to provide a range of inducible expression levels of the heterologous nucleotide sequence. Thus, less than the entire promoter regions may be utilized and the ability to drive expression of the coding sequence retained. As described in Examples 1-3, the 1.0 kb ZmCAS1 promoter fragment as well as the longer 1.7 kb ZmCAS1 promoter fragment were able to drive gene expression when induced by a chemical or stress treatment.

Modifications of the isolated promoter sequences of the present invention can provide for a range of inducible expression of the heterologous nucleotide sequence. Thus, they may be modified to be weak inducible promoters or strong inducible promoters. Generally, by “weak promoter” is intended a promoter that drives expression of a coding sequence at a low level. By “low level” is intended at levels 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 high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

The terms “substantially similar” and “corresponding substantially” as used herein refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention

encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under moderately stringent conditions (for example, 0.5 X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the nucleotide sequences reported herein and which are functionally equivalent to the promoter of the invention. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (Hames and Higgins, Eds.; In Nucleic Acid Hybridisation; IRL Press: Oxford, U.K., 1985). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes partially determine stringency conditions. One set of conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45 °C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50 °C for 30 min. Another set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60 °C. Another set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65 °C.

In general, sequences that correspond to the nucleotide sequences of the present invention and hybridize to the nucleotide sequence disclosed herein will be at least 40% homologous, 50% homologous, 70% homologous, and even 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% homologous or more with the disclosed sequence. That is, the sequence similarity between probe and target may range, sharing at least about 40%, about 50%, about 70%, and even about 85% or more sequence similarity.

Preferred substantially similar nucleic acid sequences encompassed by this invention are those sequences that are 80% identical to the nucleic acid fragments reported herein or which are 80% identical to any portion of the nucleotide sequences reported herein. More preferred are nucleic acid fragments which are

90% identical to the nucleic acid sequences reported herein, or which are 90% identical to any portion of the nucleotide sequences reported herein. Most preferred are nucleic acid fragments which are 95% identical to the nucleic acid sequences reported herein, or which are 95% identical to any portion of the nucleotide sequences reported herein. It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying related polynucleotide sequences. Useful examples of percent identities are those listed above, or also preferred is any integer percentage from 80% to 100%, such as 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98 and 99%.

A “substantially homologous sequence” refers to variants of the disclosed sequences such as those that result from site-directed mutagenesis, as well as synthetically derived sequences. A substantially homologous sequence of the present invention also refers to those fragments of a particular promoter nucleotide sequence disclosed herein that operate to promote the inducible expression of an operably linked heterologous nucleic acid fragment. These promoter fragments will comprise at least about 20 contiguous nucleotides, preferably at least about 50 contiguous nucleotides, more preferably at least about 75 contiguous nucleotides, even more preferably at least about 100 contiguous nucleotides of the particular promoter nucleotide sequence disclosed herein. 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 sequences disclosed herein; by synthesizing a nucleotide sequence from the naturally occurring promoter DNA sequence; or may be obtained through the use of PCR technology. See particularly, Mullis et al., Methods Enzymol. 155:335-350 (1987), and Higuchi, R. In PCR Technology: Principles and Applications for DNA Amplifications; Erlich, H.A., Ed.; Stockton Press Inc.: New York, 1989. Again, variants of these promoter fragments, such as those resulting from site-directed mutagenesis, are encompassed by the compositions of the present invention.

“Codon degeneracy” refers to divergence in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of

an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a nucleic acid fragment for improved expression in a host cell, it is desirable to design the nucleic acid fragment such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

Sequence alignments and percent similarity calculations may be determined using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) or using the AlignX program of the Vector NTI bioinformatics computing suite (Invitrogen). Multiple alignment of the sequences are performed using the Clustal method of alignment (Higgins and Sharp, CABIOS 5:151-153 (1989)) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are GAP PENALTY=10, GAP LENGTH PENALTY=10, KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1993)) and Gapped Blast (Altschul, S. F. et al., Nucleic Acids Res. 25:3389-3402 (1997)). BLASTN refers to a BLAST program that compares a nucleotide query sequence against a nucleotide sequence database.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" or "recombinant expression construct", which are used interchangeably, refers to any gene that is not

a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

“Coding sequence” refers to a DNA sequence which codes for a specific amino acid sequence. “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

The “5' non-coding sequences” refer to DNA sequences located upstream of a coding sequence which influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

An “intron” is an intervening sequence in a gene that is transcribed into RNA but is then excised in the process of generating the mature mRNA. The term is also used for the excised RNA sequences. An “exon” is a portion of the sequence of a gene that is transcribed and is found in the mature messenger RNA derived from the gene, but is not necessarily a part of the sequence that encodes the final gene product.

The term "constitutive promoter" refers to promoters active in all or most tissues of a plant at all or most developing stages. As with other promoters classified as “constitutive” (e.g. ubiquitin), some variation in absolute levels of expression can exist among different tissues or stages.

The term “constitutive promoter” or “tissue-independent” are used interchangeably herein.

The term “tissue specific promoter” refers to promoters that have been shown to direct RNA synthesis at higher levels only in particular types of cells or tissues and are often referred to as “tissue specific promoters”, or “tissue-preferred promoters” if the promoters direct RNA synthesis preferably in certain tissues but also in other tissues at reduced levels.

Among the most commonly used promoters are the nopaline synthase (NOS) promoter (Ebert et al., Proc. Natl. Acad. Sci. U.S.A. 84:5745-5749 (1987)), the octapine synthase (OCS) promoter, caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S promoter (Lawton et al., Plant Mol. Biol. 9:315-324 (1987)), the CaMV 35S promoter (Odell et al., Nature 313:810-812 (1985)), and the figwort mosaic virus 35S promoter (Sanger et al., Plant Mol. Biol. 14:433-43 (1990)), the light inducible promoter from the small subunit of rubisco, the Adh promoter (Walker et al., Proc. Natl. Acad. Sci. U.S.A. 84:6624-66280 (1987), the sucrose synthase promoter (Yang et al., Proc. Natl. Acad. Sci. U.S.A. 87:4144-4148 (1990)), the R gene complex promoter (Chandler et al., Plant Cell 1:1175-1183 (1989)), the chlorophyll a/b binding protein gene promoter, etc. Other commonly used promoters are, the promoters for the potato tuber ADPGPP genes, the sucrose synthase promoter, the granule bound starch synthase promoter, the glutelin gene promoter, the maize waxy promoter, Brittle gene promoter, and Shrunken 2 promoter, the acid chitinase gene promoter, and the zein gene promoters (15 kD, 16 kD, 19 kD, 22 kD, and 27 kD; Perdersen et al., Cell 29:1015-1026 (1982)). A plethora of promoters is described in PCT Publication No. WO 00/18963 published on April 6, 2000, the disclosure of which is hereby incorporated by reference.

The “translation leader sequence” refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D., Molecular Biotechnology 3:225 (1995)).

The “3' non-coding sequences” refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other

sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., Plant Cell 1:671-680 (1989).

"RNA transcript" refers to a product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When an RNA transcript is a perfect complementary copy of a DNA sequence, it is referred to as a primary transcript or it may be a RNA sequence derived from posttranscriptional processing of a primary transcript and is referred to as a mature RNA. "Messenger RNA" ("mRNA") refers to RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to and synthesized from an mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded by using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes mRNA and so can be translated into protein within a cell or in vitro. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks expression or transcripts accumulation of a target gene (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e. at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

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

The term "expression", as used herein, refers to the production of a functional end-product e.g., an mRNA or a protein (precursor or mature).

The term “expression cassette” as used herein, refers to a discrete nucleic acid fragment into which a nucleic acid sequence or fragment can be moved.

Expression or overexpression of a gene involves transcription of the gene and translation of the mRNA into a precursor or mature protein. “Antisense inhibition” refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. “Overexpression” refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. “Co-suppression” refers to the production of sense RNA transcripts capable of suppressing the expression or transcript accumulation of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020). The mechanism of co-suppression may be at the DNA level (such as DNA methylation), at the transcriptional level, or at post-transcriptional level.

Co-suppression constructs in plants previously have been designed by focusing on overexpression of a nucleic acid sequence having homology to an endogenous mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the overexpressed sequence (see Vaucheret et al., Plant J. 16:651-659 (1998); and Gura, Nature 404:804-808 (2000)). The overall efficiency of this phenomenon is low, and the extent of the RNA reduction is widely variable. Recent work has described the use of “hairpin” structures that incorporate all, or part, of an mRNA encoding sequence in a complementary orientation that results in a potential “stem-loop” structure for the expressed RNA (PCT Publication No. WO 99/53050 published on October 21, 1999; and PCT Publication No. WO 02/00904 published on January 3, 2002). This increases the frequency of co-suppression in the recovered transgenic plants. Another variation describes the use of plant viral sequences to direct the suppression, or “silencing”, of proximal mRNA encoding sequences (PCT Publication No. WO 98/36083 published on August 20, 1998). Genetic and molecular evidences have been obtained suggesting that dsRNA mediated mRNA cleavage may have been the conserved mechanism underlying these gene silencing phenomena (Elmayan et al., Plant Cell 10:1747-1757 (1998); Galun, In Vitro Cell. Dev. Biol. Plant 41(2):113-123 (2005); Pickford et al, Cell. Mol. Life Sci. 60(5):871-882 (2003)).

As stated herein, “suppression” refers to a reduction of the level of enzyme activity or protein functionality (e.g., a phenotype associated with a protein) detectable in a transgenic plant when compared to the level of enzyme activity or protein functionality detectable in a non-transgenic or wild type plant with the native enzyme or protein. The level of enzyme activity in a plant with the native enzyme is referred to herein as “wild type” activity. The level of protein functionality in a plant with the native protein is referred to herein as “wild type” functionality. The term “suppression” includes lower, reduce, decline, decrease, inhibit, eliminate and prevent. This reduction may be due to a decrease in translation of the native mRNA into an active enzyme or functional protein. It may also be due to the transcription of the native DNA into decreased amounts of mRNA and/or to rapid degradation of the native mRNA. The term “native enzyme” refers to an enzyme that is produced naturally in a non-transgenic or wild type cell. The terms "non-transgenic" and "wild type" are used interchangeably herein.

“Altering expression” refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ significantly from the amount of the gene product(s) produced by the corresponding wild-type organisms (i.e., expression is increased or decreased).

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. The preferred method of soybean cell transformation is the use of particle-accelerated or “gene gun” transformation technology (Klein, T., Nature (London) 327:70-73 (1987); U.S. Patent No. 4,945,050).

“Transient expression” refers to the temporary expression of often reporter genes such as β-glucuronidase (GUS), fluorescent protein genes GFP, ZS-YELLOW1 N1, AM-CYAN1, DS-RED in selected certain cell types of the host organism in which the transgenic gene is introduced temporally by a transformation method. The transformed materials of the host organism are subsequently discarded after the transient gene expression assay.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J. et al., In Molecular

Cloning: A Laboratory Manual; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989 (hereinafter "Sambrook et al., 1989") or Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., Eds.; In Current Protocols in Molecular Biology; John Wiley and Sons: New York, 1990 (hereinafter "Ausubel et al., 1990").

"PCR" or "Polymerase Chain Reaction" is a technique for the synthesis of large quantities of specific DNA segments, consisting of a series of repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps comprises a cycle.

The terms "recombinant polynucleotide", "recombinant nucleotide", "recombinant DNA" , "recombinant DNA construct" and "recombinant expression construct" are used interchangeably herein. A recombinant DNA construct comprises an artificial or heterologous combination of nucleic acid sequences, e.g., regulatory and coding sequences that are not found together in nature. For example, a recombinant DNA construct can comprise a plasmid vector or a fragment thereof comprising the instant inducible promoter and a heterologous polynucleotide of interest. In other embodiments, a recombinant construct may comprise regulatory sequences and coding sequences that are derived from maize, rice, sorghum or different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments provided herein. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., EMBO J. 4:2411-2418 (1985); De Almeida et al., Mol. Gen.

Genetics 218:78-86 (1989)), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

It is demonstrated herein that the maize mannitol dehydrogenase gene promoter ZmCAS1 can, in fact, be used as an inducible promoter to drive efficient expression of transgenes, and that such promoter can be isolated and used by one skilled in the art. Induced GUS and MS45 expression has been observed in sink tissues such as anthers, callus, root and shoots of seedlings as well as developing leaves (Examples 1-3)`

Mannitol metabolism plays an important role in plant responses to both biotic and abiotic stresses. (Stoop et al.2001, Trends in Plant Science, Volume 1, Issue 5, May 1996, Pages 139–144). Celery plants exposed to high salinity showed an increased mannitol accumulation primarily caused by a decrease in mannitol dehydrogenase activity in sink tissues (Stoop and Pharr. 1993 Plant Physiol. 103:1001-1008). As shown in Figure 1 B, the ZmCAS1cDNA (SEQ ID NO:5) showed a high % identity with a maize mannitol dehydrogenase (GI:226528549; SEQ ID NO:6), Figure 1(B). Taken together with our observations that the ZmCAS1 promoter can be induced by a chemical such as a safener, or a stress such as a heat treatment, one can further test the ability of the ZmCAS1 promoter to be responsive to stresses such as, but not limited to, drought, osmotic or salt stress, or a combination thereof.

It is clear from the disclosure set forth herein that one of ordinary skill in the art could perform the following procedure:

1) operably linking the nucleic acid fragment containing the ZMCAS1 promoter sequence to a suitable reporter gene; there are a variety of reporter genes that are well known to those skilled in the art, including the bacterial GUS gene, the firefly luciferase gene, and the cyan, green, red, and yellow fluorescent protein genes; any gene for which an easy and reliable assay is available can serve as the reporter gene.

2) transforming a chimeric ZmCAS1 promoter:reporter gene expression

cassette into an appropriate plant for expression of the promoter. There are a variety of appropriate plants which can be used as a host for transformation that are well known to those skilled in the art, including the dicots, Arabidopsis, tobacco, soybean, oilseed rape, peanut, sunflower, safflower, cotton, tomato, potato, cocoa and the monocots, corn, wheat , rice, barley and palm.

3) testing for expression of the ZmCAS1 promoter in various cell types of transgenic plant tissues, e.g., leaves, roots, flowers, seeds, transformed with the chimeric ZmCAS1 promoter:reporter gene expression cassette by assaying for expression of the reporter gene product.

In another aspect, this invention concerns a recombinant DNA construct comprising at least one heterologous nucleic acid fragment operably linked to any promoter, or combination of promoter elements, of the present invention. Recombinant DNA constructs can be constructed by operably linking the nucleic acid fragment of the invention promoter or a fragment that is substantially similar and functionally equivalent to any portion of the nucleotide sequence set forth in SEQ ID NOs: 9 or 10 to a heterologous nucleic acid fragment. Any heterologous nucleic acid fragment can be used to practice the invention. The selection will depend upon the desired application or phenotype to be achieved. The various nucleic acid sequences can be manipulated so as to provide for the nucleic acid sequences in the proper orientation. It is believed that various combinations of promoter elements as described herein may be useful in practicing the present invention.

In another aspect, this invention concerns a recombinant DNA construct comprising at least one acetolactate synthase (ALS) nucleic acid fragment operably linked to ZmCAS1 promoter, or combination of promoter elements, of the present invention. The acetolactate synthase gene is involved in the biosynthesis of branched chain amino acids in plants and is the site of action of several herbicides including sulfonyl urea. Expression of a mutated acetolactate synthase gene encoding a protein that can no longer bind the herbicide will enable the transgenic plants to be resistant to the herbicide (U.S. Patent No. 5,605,011, U.S. Patent No. 5,378,824). The mutated acetolactate synthase gene is also widely used in plant transformation to select transgenic plants.

In another embodiment, this invention concerns host cells comprising either

the recombinant DNA constructs of the invention as described herein or isolated polynucleotides of the invention as described herein. Examples of host cells which can be used to practice the invention include, but are not limited to, yeast, bacteria, and plants.

Plasmid vectors comprising the instant recombinant expression construct can be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host cells. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene.

The method of transformation/transfection is not critical to the instant invention; various methods of transformation or transfection are currently available. As newer methods are available to transform crops or other host cells they may be directly applied. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription or transcript and translation of the sequence to effect phenotypic changes in the organism. Thus, any method which provides for efficient transformation/transfection may be employed.

Methods for introducing expression vectors into plant tissue available to one skilled in the art are varied and will depend on the plant selected. Procedures for transforming a wide variety of plant species are well known and described throughout the literature. See, for example, Miki et al, "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biotechnology, supra; Klein et al, Bio/Technology 10:268 (1992); and Weising et al., Ann. Rev. Genet. 22: 421-477 (1988). For example, the DNA construct may be introduced into the genomic DNA of the plant cell using techniques such as microprojectile-mediated delivery, Klein et al., Nature 327: 70-73 (1987); electroporation, Fromm et al., Proc. Natl. Acad. Sci. 82: 5824 (1985); polyethylene glycol (PEG) precipitation, Paszkowski et al., EMBO J. 3: 2717-2722 (1984); direct gene transfer WO 85/01856 and EP No. 0 275 069; in vitro protoplast transformation, U.S. Patent No. 4,684,611; and microinjection of plant cell protoplasts or embryogenic callus, Crossway, Mol. Gen. Genetics 202:179-185 (1985). Co-cultivation of plant tissue with Agrobacterium tumefaciens is another option, where the DNA constructs are placed into a binary

vector system. See e.g., U.S. Patent No. 5,591,616; Ishida et al., "High Efficiency Transformation of Maize (Zea mays L.) mediated by *Agrobacterium tumefaciens*" Nature Biotechnology 14:745-750 (1996). The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct into the plant cell DNA when the cell is infected by the bacteria. See, for example Horsch et al., Science 233: 496-498 (1984), and Fraley et al., Proc. Natl. Acad. Sci. 80: 4803 (1983).

Standard methods for transformation of canola are described at Moloney et al. "High Efficiency Transformation of Brassica napus using Agrobacterium Vectors" Plant Cell Reports 8:238-242 (1989). Corn transformation is described by Fromm et al, Bio/Technology 8:833 (1990). Agrobacterium is primarily used in dicots, but certain monocots such as maize can be transformed by Agrobacterium (U.S. Patent No. 5,550,318). Rice transformation is described by Hiei et al., "Efficient Transformation of Rice (Oryza sativa L.) Mediated by Agrobacterium and Sequence Analysis of the Boundaries of the T-DNA" The Plant Journal 6(2): 271-282 (1994, Christou et al, Trends in Biotechnology 10:239 (1992) and Lee et al, Proc. Nat'l Acad. Sci. USA 88:6389 (1991). Wheat can be transformed by techniques similar to those used for transforming corn or rice. Sorghum transformation is described at Casas et al, supra and sorghum by Wan et al, PlantPhysiol. 104:37 (1994). Soybean transformation is described in a number of publications, including U.S. Patent No. 5,015,580.

When referring to "introduction" of the nucleotide sequence into a plant, it is meant that this can occur by direct transformation methods, such as Agrobacterium transformation of plant tissue, microprojectile bombardment, electroporation, or any one of many methods known to one skilled in the art; or, it can occur by crossing a plant having the heterologous nucleotide sequence with another plant so that progeny have the nucleotide sequence incorporated into their genomes. Such breeding techniques are well known to one skilled in the art.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have been published, among others, for cotton (U.S. Patent No. 5,004,863, U.S. Patent No. 5,159,135); soybean (U.S. Patent No. 5,569,834, U.S. Patent No. 5,416,011); Brassica (U.S. Patent

No. 5,463,174); peanut (Cheng et al., Plant Cell Rep. 15:653-657 (1996), McKently et al., Plant Cell Rep. 14:699-703 (1995)); papaya (Ling et al., Bio/technology 9:752-758 (1991)); and pea (Grant et al., Plant Cell Rep. 15:254-258 (1995)). For a review of other commonly used methods of plant transformation see Newell, C.A., Mol. Biotechnol. 16:53-65 (2000). One of these methods of transformation uses *Agrobacterium rhizogenes* (Tepfler, M. and Casse-Delbart, F., Microbiol. Sci. 4:24-28 (1987)). Transformation of soybeans using direct delivery of DNA has been published using PEG fusion (PCT Publication No. WO 92/17598), electroporation (Chowrira et al., Mol. Biotechnol. 3:17-23 (1995); Christou et al., Proc. Natl. Acad. Sci. U.S.A. 84:3962-3966 (1987)), microinjection, or particle bombardment (McCabe et al., BiolTechnology 6:923 (1988); Christou et al., Plant Physiol. 87:671-674 (1988)).

There are a variety of methods for the regeneration of plants from plant tissues. 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, Eds.; In Methods for Plant Molecular Biology; Academic Press, Inc.: San Diego, CA, 1988). 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 or 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. Preferably, the regenerated plants are 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 present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

In addition to the above discussed procedures, practitioners are familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA

molecules, plasmids, etc.), generation of recombinant DNA fragments and recombinant expression constructs and the screening and isolating of clones, (see for example, Sambrook, J. et al., In Molecular Cloning: A Laboratory Manual; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989; Maliga et al., In Methods in Plant Molecular Biology; Cold Spring Harbor Press, 1995; Birren et al., In Genome Analysis: Detecting Genes, 1; Cold Spring Harbor: New York, 1998; Birren et al., In Genome Analysis: Analyzing DNA, 2; Cold Spring Harbor: New York, 1998; Clark, Ed., In Plant Molecular Biology: A Laboratory Manual; Springer: New York, 1997).

The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression of the chimeric genes (Jones et al., EMBO J. 4:2411-2418 (1985); De Almeida et al., Mol. Gen. Genetics 218:78-86 (1989)). Thus, multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis. Also of interest are seeds obtained from transformed plants displaying the desired gene expression profile.

Inducible expression of chimeric genes in most plant cells makes the ZmCAS1 promoter of the instant invention especially useful when inducible expression of a target heterologous nucleic acid fragment is required.

Another general application of the ZmCAS1 promoter of the invention is to construct chimeric genes that can be used to reduce expression of at least one heterologous nucleic acid fragment in a plant cell. To accomplish this, a chimeric gene designed for gene silencing of a heterologous nucleic acid fragment can be constructed by linking the fragment to the ZmCAS1 promoter of the present invention. (See U.S. Patent No. 5,231,020, and PCT Publication No. WO 99/53050 published on October 21, 1999, PCT Publication No. WO 02/00904 published on January 3, 2002, and PCT Publication No. WO 98/36083 published on August 20, 1998, for methodology to block plant gene expression via cosuppression.)

Alternatively, a chimeric gene designed to express antisense RNA for a heterologous nucleic acid fragment can be constructed by linking the fragment in reverse orientation to the ZmCAS1 promoter of the present invention. (See U.S. Patent

No. 5,107,065 for methodology to block plant gene expression via antisense RNA.) Either the cosuppression or antisense chimeric gene can be introduced into plants via transformation. Transformants wherein expression of the heterologous nucleic acid fragment is decreased or eliminated are then selected.

This invention also concerns a method of altering (increasing or decreasing) the expression of at least one heterologous nucleic acid fragment in a plant cell which comprises:

(a) transforming a plant cell with the recombinant expression construct of described herein;

(b) induction of the inducible promoter by chemical or stress treatment on the cell of (a)

(c) growing fertile mature plants from the transformed plant cell of step (a); and,

(d) selecting plants containing the transformed plant cell wherein the expression of the heterologous nucleic acid fragment is increased or decreased.

Transformation and selection can be accomplished using methods well-known to those skilled in the art including, but not limited to, the methods described herein.

Non-limiting examples of compositions and methods disclosed herein are as follows:

1. An isolated polynucleotide comprising:
   a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:9 or SEQ ID NO:10, or a full-length complement thereof;
   b) a nucleotide sequence comprising a functional fragment of SEQ ID NO:10, or a full-length complement thereof;
   c) a nucleotide sequence comprising a sequence having at least 85% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence of (a) or (b);
   d) a nucleotide sequence which hybridizes to SEQ ID NO:9 under highly stringent conditions of a wash of 0.1 SSC, 0.1% (w/v) SDS at 65°C ;
   e) a nucleotide sequence comprising all or a fragment of a 1.7 kb 5 ‘ non-coding sequence of a mannitol dehydrogenase; or,

f) a derivative of one of the nucleotide sequences indicated in (a), (b), (c), (d) or (e) obtained by substitution, addition and/or deletion of one or more nucleotides; and,

wherein said nucleotide sequence is an inducible promoter.

2. The isolated polynucleotide of embodiment 1, wherein the nucleotide sequence of c) has at least 90% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

3. The isolated polynucleotide of embodiment 1, wherein the nucleotide sequence of c) has at least 95% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

4. The isolated polynucleotide of embodiment 1, wherein the nucleotide sequence of c) has at least 98% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

5. The isolated polynucleotide of embodiment 1 wherein said inducible promoter is induced by a chemical or stress treatment.

6. The isolated polynucleotide of embodiment 1 wherein said inducible promoter is induced by a safener or heat treatment.

7. The isolated polynucleotide of embodiment 6, wherein the safener is N-(aminocarbonyl)-2-chlorobenzenesulfonamide.

8. The isolated polynucleotide of embodiment 6, wherein said heat treatment comprises a temperature greater than 26°C.

9. A recombinant DNA construct comprising the isolated polynucleotide of embodiment 1 operably linked to at least one heterologous nucleic acid sequence.

10. The recombinant DNA construct of embodiment 9 , wherein the heterologous nucleic acid sequence codes for a gene selected from the group consisting of: a double-strand break inducing gene, a recombinase gene, a reporter gene, a selection marker, a disease resistance conferring gene, a herbicide resistance conferring gene, an insect resistance conferring gene; a gene involved in carbohydrate metabolism, a gene involved in fatty acid metabolism, a gene involved in amino acid metabolism, a gene involved in plant development, a gene involved in plant growth regulation, a gene

involved in yield improvement, a gene involved in drought resistance, a gene involved in cold resistance, a gene involved in heat and salt resistance in plants.

11. The recombinant DNA construct of embodiment 9, wherein the heterologous nucleic acid sequence encodes a protein selected from the group consisting of: a double-strand break inducing protein, a recombinase protein, a reporter protein, a selection marker, a protein conferring disease resistance, protein conferring herbicide resistance, protein conferring insect resistance; protein involved in carbohydrate metabolism, protein involved in fatty acid metabolism, protein involved in amino acid metabolism, protein involved in plant development, protein involved in plant growth regulation, protein involved in yield improvement, protein involved in drought resistance, protein involved in cold resistance, protein involved in heat resistance and salt resistance in plants.

12. A vector comprising the recombinant DNA construct of embodiment 9.

13. A cell comprising the recombinant DNA construct of embodiment 9.

14. The cell of embodiment 13, wherein the cell is a plant cell.

15. The plant cell of embodiment 14 having stably incorporated into its genome the recombinant DNA construct of embodiment 9.

16. A transgenic plant having stably incorporated into its genome the recombinant DNA construct of embodiment 9.

17. The transgenic plant of embodiment 16 wherein said plant is a monocot plant.

18. The transgenic plant of embodiment 17, wherein said monocot is selected from the group comprising: maize, wheat, rice, barley, sorghum, millet, sugarcane and rye.

19. The transgenic plant of embodiment 16, wherein said plant is a dicot plant.

20. The transgenic plant of embodiment 19, wherein said dicot is selected from the group comprising: soy, Brassica sp., cotton, safflower, tobacco, alfalfa and sunflower.

21. Transgenic seed produced by the transgenic plant of embodiment 16.

22. A plant stably transformed with a recombinant expression construct comprising a plant promoter and a heterologous nucleic acid fragment operably linked to said promoter, wherein said promoter is an inducible promoter and capable of controlling expression of said heterologous nucleic acid fragment in a plant cell, and further wherein said promoter comprises a fragment of SEQ ID NO:10.

23. A method of expressing a coding sequence or a functional RNA in a plant cell comprising:

a) introducing the recombinant DNA construct of embodiment 9 into a plant cell, wherein the at least one heterologous sequence comprises a coding sequence or a functional RNA;

b) growing the plant cell of step a);

c) induction of the inducible promoter by chemical or stress treatment on the plant cell of b); and,

d) selecting a plant cell displaying expression of the coding sequence or the functional RNA of the recombinant DNA construct.

24. The method of embodiment 23, wherein the chemical is a safener.

25. The method of embodiment 23 wherein the stress treatment is a heat treatment.

26. The method of embodiment 23 further comprising growing the plant cell of d) into a plant.

27. A method of expressing a coding sequence or a functional RNA in anther cells, said method comprising:

a) introducing the recombinant DNA construct of embodiment 9 into a plant cell, wherein the at least one heterologous sequence comprises a coding sequence or a functional RNA;

b) growing the plant cell of step a);

c) induction of the inducible promoter by chemical or stress treatment on the plant cell of b); and,

d) identification of anther cells displaying expression of the coding sequence or the functional RNA of the recombinant DNA construct.

28. The method of embodiment 23 or embodiment 27 wherein the at least one heterologous sequence is transiently expressed.

29. The method of embodiment 23 or embodiment 27 wherein the at least one heterologous sequence is stably incorporated in the plant cell.

30. A method for altering expression of at least one heterologous nucleic acid fragment in a plant comprising:

(a) transforming a plant cell with the recombinant expression construct of embodiment 9;

(b) induction of the inducible promoter by chemical or stress treatment on the cell of (a)

(c) growing fertile mature plants from the transformed plant cell of step (a); and,

(d) selecting plants containing the transformed plant cell wherein the expression of the heterologous nucleic acid fragment is increased or decreased.

31. A method of transgenically altering a marketable plant trait, comprising:

a) introducing a recombinant DNA construct of embodiment 9 into a plant;

b) induction of the inducible promoter by chemical or stress treatment on the plant of (a);

c) growing a fertile, mature plant resulting from step b); and

d) selecting a plant expressing the at least one heterologous nucleotide sequence in at least one plant tissue based on the altered marketable trait.

32. The method of embodiment 31 wherein the marketable trait is selected from the group consisting of: disease resistance, herbicide resistance, insect resistance carbohydrate metabolism, fatty acid metabolism, amino acid metabolism, plant development, plant growth regulation, yield improvement, drought resistance, cold resistance, heat resistance, and salt resistance.

33. An isolated polynucleotide comprising:

a) a nucleotide sequence comprising all or a functional fragment of SEQ ID NO:19 or SEQ ID NO:22;

b) a nucleotide sequence comprising a full-length complement of the nucleotide sequence (a); or,

c) a nucleotide sequence comprising a sequence having at least 90% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence of (a) or (b); and,

wherein said nucleotide sequence is a promoter.

34. The isolate polynucleotide of embodiment 33 wherein said promoter is an inducible promoter.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. Sequences of promoters, cDNA, adaptors, and primers listed in this invention all are in the 5' to 3' orientation unless described otherwise. Techniques in molecular biology were typically performed as described in Ausubel, F. M. et al., In Current Protocols in Molecular Biology; John Wiley and Sons: New York, 1990 or Sambrook, J. et al., In Molecular Cloning: A Laboratory Manual; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989 (hereinafter "Sambrook et al., 1989"). It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention 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

Identification of safener-inducible cDNAs expressed in microspores and/or tapetum.

Strategy design for the identification of safener-inducible cDNAs .

The isolation of conditionally regulated promoters with tissue specificity in plants which are different than the safener induced promoter ZmIN2-2 (Hershey et al. US patent 5,364,780 Nov. 15, 1994) would enable conditional regulation of genes in microspores and/or the tapetum. Previously, it has been demonstrated that while ZmIN2-2 transcript expression increases in callus, leaf and anther tissues in maize after safener treatment, genes regulated by this promoter do not express in maize tapetal cells (Cigan et al. 2001. Sex. Plant Reprod. 14, 135–142). Immunolocalization studies demonstrated that genes regulated by ZmIN2-2 are present in all anther cell types except the tapetum or microspores. To date, no promoters that respond to CBSU (Chlorobenzenesulfonamide) safener and are specifically expressed in tapetal cells or microspores at the tetrad stage of microsporogenesis have been identified. To enable the isolation of safener-inducible candidate promoters that are expressed in microspores or tapetum, a strategy was designed which takes advantage of two fundamental observations made of plants transformed with the E.coli DAMethylase gene expressed from the maize anther-specific promoter 5126 (5126:DAM; Unger et al., 2001, Transgenic Res. 10, 409–422). First, cytological examination of tetrad staged anthers from male-sterile plants expressing 5126:DAM revealed abnormal microspores and nearly ablated tapetal cells in otherwise structurally normal appearing anthers. Second, Northern analysis of mRNA isolated from 5126:DAM sterile anthers indicates a loss of two tapetal-specific transcripts, 5126 and MS45, while a transcript not expected to be the tapetal-specific (maize actin), is easily detected (Cigan et al. 2001. Sex. Plant Reprod. 14, 135–142). Therefore, anthers isolated from 5126:DAM sterile plants should be reduced or perhaps completely devoid of tapetal- and/or microspore-specific mRNAs.

In addition, comparison of the ZmIN2-2 transcript expression from RNAs isolated from wild-type male-fertile CBSU-treated plants to RNAs isolated from male-sterile CBSU-treated 5126:DAM plants showed, that in contrast the MS45 and

5126 tapetal-specific mRNAs, the ZmIN2-2 was not reduced in anther RNAs isolated from 5126:DAM CBSU-treated plants (Cigan et al. 2001. Sex. Plant Reprod. 14, 135–142).

A strategy was designed using sterile plants which were reduced or devoid of tapetal-and/or microspore-specific mRNAs. The strategy involved treating maize plants with CBSU and comparing anther mRNA transcript profiles from these treated control plants with treated 5126:DAM plants. Such a strategy did lead to the identification and isolation of mRNAs and, ultimately, promoters which are responsive to the safener and are microspore- or tapetum-expressed as described below.

Toward this end, differential RNA hybridization was used to enrich for maize anther or callus mRNAs that are increased by safener or heat treatment. Subsequently, these mRNAs were used as probes to isolate cDNAs from anther cDNA libraries prepared from CBSU-treated maize plants. These cDNAs were then used to screen mRNAs isolated from male-fertile and male-sterile 5126:DAM control and safener-treated plants as a means to identify transcripts which are induced by CBSU or heat treatment or and expressed in the tapetum or microspores as described below.

<u>Maize anther cDNA library construction from CBSU-treated wild-type plants and isolation of safener inducible cDNA's.</u>

Wild type maize plants were grown to the meiosis stage of microspore development. Plants were watered with 30 mg 2-CBSU and allowed to develop to the quartet and early vacuolate stage of microspore development. PolyA+ anther RNA was isolated from wild type control and CBSU treated plants and stored. A cDNA library was constructed from mRNAs isolated from CBSU-treated plants, arrayed onto nylon filters and stored. A cDNA subtraction library was generated using the Clontech PCR-Select cDNA Subtraction Kit (#K1804-1) following the manufacturer's instructions to enrich for CBSU-specific transcripts. Using this approach anther PolyA+ mRNA from wild-type plants was used to enrich for transcripts found in the anther PolyA+ mRNA from CBSU treated plants. This cDNA subtraction library was subcloned into pSPORT (BRL) vector, colonies plated,

picked and sequenced. Among the vector inserts sequenced, two DNA sequences were present at a high proportion, at more than 10% of the inserts sequenced, and referred to as ZmCAS1c-1 (477 bp; SEQ ID NO: 1) and ZmCAS1c-2 (438 bp; SEQ ID NO: 2) (CBSU-Anther-Subtract 1). Both ZmCAS1c-1 and ZmCAS1c-2 had sequence identity to mannitol dehydrogenases from plants. ZmCAS1c-1 and ZmCAS1c-2 DNA fragments were used as hybridization probes to screen the filter arrayed cDNA CBSU-anther library described above to isolate full-length cDNA containing hybridizing clones. Both ZmCAS1c-1 and ZmCAS1c-2 identified identical cDNA clones. One cDNA clone contained a 1354 bp SalI-NotI insert (SEQ ID NO: 3) that was sequenced and identified as a 1338 bp full length cDNA clone referred to ZmCAS1cDNA (SEQ ID NO: 4). ZmCAS1cDNA is capable of encoding a 354 amino acid sequence (SEQ ID NO: 5) with 99.7 % identity to a maize mannitol dehydogenase (GI number 226528549, NP_001147757.1, SEQ ID NO:6 ; Figure 1).

To determine whether the ZmCAS1 cDNA was 1) induced in maize anthers by CBSU-treatment and 2) reduced or absent in tapetum and microspore-ablated maize anthers from CBSU-treated 5126:DAM plants, a 477 bp ZmCAS1c-1 DNA fragment (SEQ ID NO:1), as well as DNA fragments from ZmMS45, Zm5126, ZmActin, and ZmUbiquitin (Cigan et al. 2001. Sex. Plant Reprod. 14, 135–142) were used as a hybridization probes against maize anther mRNAs isolated from male-fertile (F) and male-sterile (S), control (-) or CBSU-treated (+) plants. As shown in Figure 2, the constitutively expressed maize actin (ACTIN) and ubiquitin (UBI) transcripts were easily detected and did not change their steady-state level across all anther RNA samples and treatments. MS45 and 5126 transcripts were easily detected in anther RNAs from male-fertile plants but absent in anther RNAs from male-sterile plants (Figure 2) further supporting the observation that these RNAs are localized to the maize tapetum (Cigan et al. 2001. Sex. Plant Reprod. 14, 135–142).

Anther RNAs from control male-fertile (-, F) and control sterile plants (-, S) did not reveal detectable levels of the IN2-2 transcript, while strong hybridization signals were detected in mRNAs from anthers derived from CBSU-treated control male-fertile and male-sterile plants (Figure 2). In contrast strong hybridization signals for ZmCAS1 are only revealed in mRNAs from anthers derived from male-fertile CBSU-treated plants (+, F). The reduced ZmCAS1 signal observed in

mRNAs from anthers of male-sterile CBSU-treated plants (+, S) indicates that this ZmCAS1 transcript was present in cell layers of anthers. This observation indicates that ZmCAS1 tissue specific expression is different from the IN2-2 expression and thus makes the ZmCAS1 promoter a candidate which differs from the IN2-2 promoter in spatial expression in anthers.

When the ZmCAS1 probe was used to hybridize to maize callus or maize leaf treated with CBSU, strong hybridization was observed in mRNAs from callus, leaf and anther (Figure 3), similar to the IN2-2 probe, suggesting that in addition to expression in anthers, ZmCAS1 transcript is also expressed in callus and leaf in response to safener, CBSU, treatment.

Isolation of the 1.7 kb and truncated 1.0 kb ZmCAS1 promoter fragments

In order to isolate DNA sequences which correspond to the ZmCAS1 promoter, subgenomic SauIIIA genomic phage libraries from the maize line B73 were screened using the 477 bp ZmCAS1c-1 DNA fragment (SEQ ID NO:1) as a hybridization probe. A phage which contained a 4069 bp maize B73 DNA fragment (SEQ ID NO: 7). and hybridized to the ZmCAS1c-1 probe was isolated, plasmid excised and sequenced. DNA sequence analysis of this 4069 bp genomic DNA identified several regions of sequence identity to the ZmCAS1C cDNA. For promoter studies, oligonucleotide directed mutagenesis was used to introduce an RcaI DNA restriction site at nucleotide positions 3447-3452 in SEQ ID NO:7 using the MORPH Site-Specific Plasmid DNA Mutagenesis Kit 5 Prime-3 Prime (Boulder, CO) according to the vendors instructions using the oligonucleotide 5'-GCAGTTCATTCCTCATGACTGCTGCAGCAGAGC-3'(SEQ ID NO:8). A HindIII-RcaI fragment (ZmCAS1HindIII Pro, SEQ ID NO: 15) comprising the truncated 1.0 kb maize ZmCAS1 promoter of SEQ ID NO:9 and a BamHI-RcaI (ZmCAS1BamPro, SEQ ID NO: 16) fragment comprising the 1.7 kb maize ZmCAS1 promoter of SEQ ID NO:10 were isolated and used for promoter studies in plants.

As shown in Figure 4, Example 2, and other examples described herein, both the 1.7 kb ZmCAS1 promoter (SEQ ID NO:10) and the truncated 1.0 kb ZmCAS1 promoter (SEQ ID NO:9) were active in plant cells and induced by both a safener (CBSU , Figure 4A, 4B) and/or a heat treatment (Figure 4B).

EXAMPLE 2

Increased expression of GUS and ZmMS45 is observed in maize cells and plants when these genes are placed under the transcriptional control of the ZmCAS1 promoter in response to the safener, CBSU or a heat treatment.

Agrobacterium-mediated transformation of immature embryos was used to generate integrated copies of PHP16974 (SEQ ID NO:11; ZmCAS1HindIII Pro:GUS/35SPAT) comprising the 1.0 kb ZmCAS1 promoter (SEQ ID NO:9), PHP16975 (SEQ ID NO:12, ZmCAS1BamPro:GUS/35SPAT) comprising the 1.7 kb ZmCAS1 promoter (SEQ ID NO:10) and PHP16972 (SEQ ID NO:13, ZmCAS1HindIII Pro:MS45/35SPAT) comprising the 1.0 kb ZmCAS1 promoter or PHP16973 (SEQ ID NO:14, ZmCAS1BamPro:MS45/35SPAT:) comprising the 1.7 kb ZmCAS1 promoter.

As described in Example 1 and Figure 3, in addition to expression in anthers, ZmCAS1 transcript was also expressed in callus and leaf tissue in response to safener, CBSU, treatment. Bialophos-resistant callus events were selected for analysis and plant regeneration.

To determine whether the 1.7 kb or the 1.0 kb ZmCAS1 promoter from maize could direct induced expression of the GUS reporter, three bialophos-resistant callus events were placed onto maintenance media and maintenance media containing increasing amounts of the safener CBSU-2 for at room temperature for 18 hours, removed and stained with X-Gluc to detect GUS activity. As shown in Figure 4A, slight GUS expression is detected in callus grown on maintenance media (Figure 4A: C). In contrast, low levels of GUS expression are detected in callus grown on 10 mg/l CBSU (Figure 4A: 10) while strong GUS expression is observed in PHP16975 callus events grown on 100 mg/l CBSU (Figure 4A: 100). This data indicated that the 1.7 kb ZmCAS1 promoter is active in maize callus and can be induced by safener treatment.

Seven random bialophos-resistant callus events containing PHP16974 which contains a truncated 1.0 kb fragment of the ZmCAS1 promoter driving the GUS reporter were capable of inducible GUS expression when incubated in the presence of 100 mg/liter CBSU at room temperature (Figure 4B; 26C+CBSU). In a separate experiment, when these 7 callus events were grown on maintenance media without

CBSU but incubated for 2 days at 37°C returned to room temperature and then stained with X-gluc, increased GUS expression was also observed (Figure 4B; 37C). This data indicated that the truncated 1.0 kb ZmCAS1 promoter is active in maize callus and can be induced by safener and / or heat treatment.

Plants were also regenerated from callus events containing PHP16975 and grown in the greenhouse to approximately the 5 leaf stage. At this stage of development, leaf punches from plants regenerated from the 3 bialophos-resistant events shown in Figure 5 were collected pre- (C) and post-watering (S) with 30 mg of 2-CBSU to examine GUS expression in leaf in response to application of the safener. As shown in Figure 5 strong GUS expression was detected in leaf punches 2 days after watering (S) with CBSU across the 3 PHP16975 transformed plants analyzed.). This data further indicates that the 1.7 kb ZmCAS1 promoter is active in maize leaves and can be induced by safener treatment.

T-DNA vectors PHP16972 (SEQ ID NO:13, ZmCAS1HindIII Pro: MS45/35S:PAT) and PHP16973 (SEQ ID NO: 14, ZmCAS1BamPro:MS45/35S:PAT) were used to transform maize callus which was generated to contain a segregating population of MS45/ms45 heterozygous and ms45/ms45 homozygous mutant plants. In order to detect MS45 RNA or protein expression under the control of the 1.0 or 1.7 kb ZmCAS promoter in maize anthers, plants containing a naturally occurring mutation in the maize MS45 gene which results in loss of MS45 RNA and protein were used for these studies. Pollen from MS45/ms45 plants were used to fertilize male-sterile ms45/ms45 plants for the purpose of generating embryos which would be ms45/ms45 as described in Cigan et al 2001. By placing the maize MS45 gene under the control of the ZmCAS1 promoter in these transformation vectors, genes other than GUS could be tested for transcriptional-induction in response to safener in callus, leaf and maize anthers as has been previously demonstrated for ZmIn2-2:MS45 regulated expression (Cigan et al 2001. Sex. Plant Reprod. 14, 135–142). Five random bialophos-resistant callus events containing integrated copies of PHP16972 were placed onto maintenance media (Figure 5 , (-)) and maintenance media containing 100 mg/liter safener CBSU-2 (Figure 5 (+)) at room temperature for 18 hours, removed and PolyA+ RNA prepared and used for RNA analysis as described (Cigan et al. 2001.

Sex. Plant Reprod. 14, 135–142) using the ZmMS45 and ZmActin probes for hybridization analysis. As shown in Figure 6, strong induction of a hybridization signal corresponding to the MS45 mRNA is detected within RNA transcripts from ms45/ms45 callus grown on CBSU (+). A very low signal was observed when callus was grown in the absence of CBSU. Actin was used as a control probe to show nearly equivalent RNA levels were present in all samples. Multiple plants were regenerated from ms45/ms45 callus events transformed with PHP16973 and grown in the greenhouse. Plants were watered with 30 mg of CBSU at the meiosis stage of microspore development. Leaf and anthers (quartet, early uninucleate microspore stage) were collected 2 days later from control and CBSU-treated plants and whole-cell protein extracts were prepared from 4 leaf punches or 6 anthers as described (Cigan et al 2001). Leaf and anther proteins were electrophoresed on 10% SDS-denaturing polyacrylamide gels, transferred to supported nitrocellulose, and used for Western analysis using antibodies directed against the maize MS45 protein. Examination of leaf extracts from PHP16973 control (C) and treated (+) plants (Figure 7) demonstrates increased steady-state levels of the MS45 protein in leaf extracts derived from CBSU-treated plants (lanes 3, 5, 7, 9). Increased MS45 protein is also detected in anther extracts (Figure 8) derived from PHP16973 CBSU treated plants (Lane 2, 4, 7). This data further supports that the ZmCAS1 promoter is active in maize cells such as anthers, callus and leaves when induced by a safener.

Taken together the GUS and MS45 results described herein support that genes can be transcriptionally-induced when placed under the control of either the 1.7 kb or the 1.0 Kb ZmCAS1 promoter in maize cells and plants and transcription can be increased in callus, leaf and anthers in response to application of the safener CBSU and or heat treatment.

EXAMPLE 3

Heat treatment of rice plants transformed with PHP16974 comprising the truncated 1.0 kb ZmCAS1 promoter driving GUS expression results in GUS expression in germinating seedlings.

To determine whether the ZmCAS1 promoter could conditionally regulate expression in response to safener treatment or heat treatment in plant species other

than maize, Agrobacterium-mediated transformation was used to generate integrated copies of PHP16974 (ZmCAS1HindIII Pro:GUS) comprising the truncated 1.0 kb ZmCAS1 promoter for studies in rice. Scutellum from 10-14 day old germinating seeds (*Oryza sativa* cv.Kitaake) was used for rice transformation experiment (Toki. 1997, Pl Mol. Biol Reporter 15:16-21). Bialophos-resistant callus events containing PHP16974 were selected and screened for their ability to respond to safener application. Four independent bialophos-resistant events were grown on maintenance media or maintenance media containing 100 mg/liter CBSU for 24 hours, removed and stained with X-Gluc. As shown Figure 9, strong GUS expression is observed in PHP16974 callus events when grown on media containing the CBSU safener (Figure 9). PHP16974 bialophos-resistant events were regenerated into plants. Leaf tissue was collected from these plants and used for DNA hybridization analyses to identify single-copy PHP16974 insertions. These plants were allowed to set selfed seed and were used for subsequent studies to monitor GUS expression under the transcriptional control of the ZmCAS1 promoter. Sixteen seed were selected from 2 single-copy PHP16974 events were sterilized, grown on hormone-free media at 28°C or 37°C for 48 hours and allowed to germinate. Germinating seed was then incubated at 28C for 2 additional days and histochemically stained with X-Gluc to detect GUS activity (Reference). As shown in Figure 10A and 10C, seedlings germinated at 28° C exhibit very low levels of detectable Gus staining. In contrast, rice seedlings germinated at 37°C show pronounced blue staining and root and at the base of shoots (Figure 10B and 10D). These results are consistent with observations in maize. That is when the GUS gene is regulated by the 1.0 kb ZmCAS1 promoter, incubation at 37C resulted in increased Gus activity even in the absence of safener treatment.

CLAIMS

What is claimed is:

1. An isolated polynucleotide comprising:
   a) a nucleotide sequence comprising the sequence set forth in SEQ ID NO:9 or SEQ ID NO:10, or a full-length complement thereof;
   b) a nucleotide sequence comprising a functional fragment of SEQ ID NO:10, or a full-length complement thereof;
   c) a nucleotide sequence comprising a sequence having at least 85% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence of (a) or (b);
   d) a nucleotide sequence which hybridizes to SEQ ID NO:9 under highly stringent conditions of a wash of 0.1 SSC, 0.1% (w/v) SDS at 65°C ;
   e) a nucleotide sequence comprising all or a fragment of a 1.7 kb 5 ‘ non-coding sequence of a mannitol dehydrogenase ; or,
   f) a derivative of one of the nucleotide sequences indicated in (a), (b), (c), (d) or (e) obtained by substitution, addition and/or deletion of one or more nucleotides; and,
   wherein said nucleotide sequence is an inducible promoter.

2. The isolated polynucleotide of claim 1, wherein the nucleotide sequence of c) has at least 90% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

3. The isolated polynucleotide of claim 1, wherein the nucleotide sequence of c) has at least 95% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

4. The isolated polynucleotide of claim 1, wherein the nucleotide sequence of c) has at least 98% identity, based on the BLASTN method of alignment, when compared to the sequence set forth in SEQ ID NO:1.

5. The isolated polynucleotide of claim 1 wherein said inducible promoter is induced by a chemical or stress treatment.

6. The isolated polynucleotide of claim 1 wherein said inducible promoter is induced by a safener or heat treatment.

7. The isolated polynucleotide of claim 6, wherein the safener is N-(aminocarbonyl)-2-chlorobenzenesulfonamide.

8. The isolated polynucleotide of claim 6, wherein said heat treatment comprises a temperature greater than 26°C.

9. A recombinant DNA construct comprising the isolated polynucleotide of claim 1 operably linked to at least one heterologous nucleic acid sequence.

10. The recombinant DNA construct of claim 9 , wherein the heterologous nucleic acid sequence codes for a gene selected from the group consisting of: a double-strand break inducing gene, a recombinase gene, a reporter gene, a selection marker, a disease resistance conferring gene, a herbicide resistance conferring gene, an insect resistance conferring gene; a gene involved in carbohydrate metabolism, a gene involved in fatty acid metabolism, a gene involved in amino acid metabolism, a gene involved in plant development, a gene involved in plant growth regulation, a gene involved in yield improvement, a gene involved in drought resistance, a gene involved in cold resistance, a gene involved in heat and salt resistance in plants.

11. The recombinant DNA construct of claim 9, wherein the heterologous nucleic acid sequence encodes a protein selected from the group consisting of: a double-strand break inducing protein, a recombinase protein, a reporter protein, a selection marker, a protein conferring disease resistance, protein conferring herbicide resistance, protein conferring insect resistance; protein

involved in carbohydrate metabolism, protein involved in fatty acid metabolism, protein involved in amino acid metabolism, protein involved in plant development, protein involved in plant growth regulation, protein involved in yield improvement, protein involved in drought resistance, protein involved in cold resistance, protein involved in heat resistance and salt resistance in plants.

12. A vector comprising the recombinant DNA construct of claim 9.

13. A cell comprising the recombinant DNA construct of claim 9.

14. The cell of claim 13, wherein the cell is a plant cell.

15. The plant cell of claim 14 having stably incorporated into its genome the recombinant DNA construct of claim 9.

16. A transgenic plant having stably incorporated into its genome the recombinant DNA construct of claim 9.

17. The transgenic plant of claim 16 wherein said plant is a monocot plant.

18. The transgenic plant of claim 17, wherein said monocot is selected from the group comprising: maize, wheat, rice, barley, sorghum, millet, sugarcane and rye.

19. The transgenic plant of claim 16, wherein said plant is a dicot plant.

20. The transgenic plant of claim 19, wherein said dicot is selected from the group comprising: soy, Brassica sp., cotton, safflower, tobacco, alfalfa and sunflower.

21. Transgenic seed produced by the transgenic plant of claim 16.

22. A plant stably transformed with a recombinant expression construct comprising a plant promoter and a heterologous nucleic acid fragment operably linked to said promoter, wherein said promoter is an inducible promoter and capable of controlling expression of said heterologous nucleic acid fragment in a plant cell, and further wherein said promoter comprises a fragment of SEQ ID NO:10.

23. A method of expressing a coding sequence or a functional RNA in a plant cell comprising:
    a) introducing the recombinant DNA construct of claim 9 into a plant cell, wherein the at least one heterologous sequence comprises a coding sequence or a functional RNA;
    b) growing the plant cell of step a);
    c) induction of the inducible promoter by chemical or stress treatment on the plant cell of b); and,
    d) selecting a plant cell displaying expression of the coding sequence or the functional RNA of the recombinant DNA construct.

24. The method of claim 23, wherein the chemical is a safener.

25. The method of claim 23 wherein the stress treatment is a heat treatment.

26. The method of claim 23 further comprising growing the plant cell of d) into a plant.

27. A method of expressing a coding sequence or a functional RNA in anther cells, said method comprising:
    a) introducing the recombinant DNA construct of claim 9 into a plant cell, wherein the at least one heterologous sequence comprises a coding sequence or a functional RNA;
    b) growing the plant cell of step a);

c) induction of the inducible promoter by chemical or stress treatment on the plant cell of b); and,

d) identification of anther cells displaying expression of the coding sequence or the functional RNA of the recombinant DNA construct.

28. The method of claim 23 or claim 27 wherein the at least one heterologous sequence is transiently expressed.

29. The method of claim 23 or claim 27 wherein the at least one heterologous sequence is stably incorporated in the plant cell.

30. A method for altering expression of at least one heterologous nucleic acid fragment in a plant comprising:

(a) transforming a plant cell with the recombinant expression construct of claim 9;

(b) induction of the inducible promoter by chemical or stress treatment on the cell of (a)

(c) growing fertile mature plants from the transformed plant cell of step (a); and,

(d) selecting plants containing the transformed plant cell wherein the expression of the heterologous nucleic acid fragment is increased or decreased.

31. A method of transgenically altering a marketable plant trait, comprising:

a) introducing a recombinant DNA construct of claim 9 into a plant;

b) induction of the inducible promoter by chemical or stress treatment on the plant of (a);

c) growing a fertile, mature plant resulting from step b); and

d) selecting a plant expressing the at least one heterologous nucleotide sequence in at least one plant tissue based on the altered marketable trait.

32. The method of claim 31 wherein the marketable trait is selected from the group consisting of: disease resistance, herbicide resistance, insect resistance carbohydrate metabolism, fatty acid metabolism, amino acid metabolism, plant development, plant growth regulation, yield improvement, drought resistance, cold resistance, heat resistance, and salt resistance.

33. An isolated polynucleotide comprising:

a) a nucleotide sequence comprising all or a functional fragment of SEQ ID NO: 19 or SEQ ID NO: 22;

b) a nucleotide sequence comprising a full-length complement of the nucleotide sequence (a); or,

c) a nucleotide sequence comprising a sequence having at least 90% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence of (a) or (b); and,

wherein said nucleotide sequence is a promoter.

CA 02873518 2014-11-12

FIGURE 1

A)

```
                10        20        30        40        50        60
SEQIDNO-5.pro   MAAAAEHGNCDAWAARDPSGVLSPYKFNRRAVQSSDVSLKIIYCGVCYGDVMW QNKHND  60
SEQIDNO-6.pro   MAAAAEHGNCDAWAARDPSGVLSPYKFNRRAVQSSDVSLKIIYCGVCYGDVMW QNKHND  60

                70        80        90       100       110       120
SEQIDNO-5.pro   SVYPLVPGHEIAGVVTEVGADVKAFKVGDHAGVGTYVNSCRHCENCNSSLENYCPETVFT  120
SEQIDNO-6.pro   SVYPLVPGHEIAGVVTEVGADVKAFKVGDHAGVGTYVNSCRHCENCNSSLENYCPETVFT  120

                130       140       150       160       170       180
SEQIDNO-5.pro   YNTTDADGTITKGGYSTHIVVHERYCFKIPDGYPLAQAAPLLCAGITVYTPMARHNMKQP  180
SEQIDNO-6.pro   YNTTDADGTITKGGYSTHIVVHERYCFKIPDGYPLAKAAPLLCAGITVYTPMARHNMKQP  180

                190       200       210       220       230       240
SEQIDNO-5.pro   GKSLGVIGLGGLGHMAVKFGKAFGLKVTVFSTSESKREEAINLLGADNFVISSDTQQMEA  240
SEQIDNO-6.pro   GKSLGVIGLGGLGHMAVKFGKAFGLKVTVFSTSESKREEAINLLGADNFVISSDTQQMEA  240

                250       260       270       280       290       300
SEQIDNO-5.pro   LKNSLHFIVDTASGDHPFDPYLSLLKVGGVMTIVCFPSEIKMHPASLNRGGRTLSGSIVG  300
SEQIDNO-6.pro   LKNSLHFIVDTASGDHPFDPYLSLLKVGGVMTIVCFPSEIKMHPASLNRGGRTLSGSIVG  300

                310       320       330       340       350
SEQIDNO-5.pro   GTKDIQEMVNFCAENKIYPEIEIIKMDYINEALARLVNRDVKYRFVIDIKNSFE        354
SEQIDNO-6.pro   GTKDIQEMVNFCAENKIYPEIEIIKMDYINEALARLVNRDVKYRFVIDIKNSFE        354
```

B)

divergence

Percent Identity

	1	2	
1		99.7	1
2	0.3		2
	1	2	

SEQIDNO-5.pro
SEQIDNO-6.pro

FIGURE 2

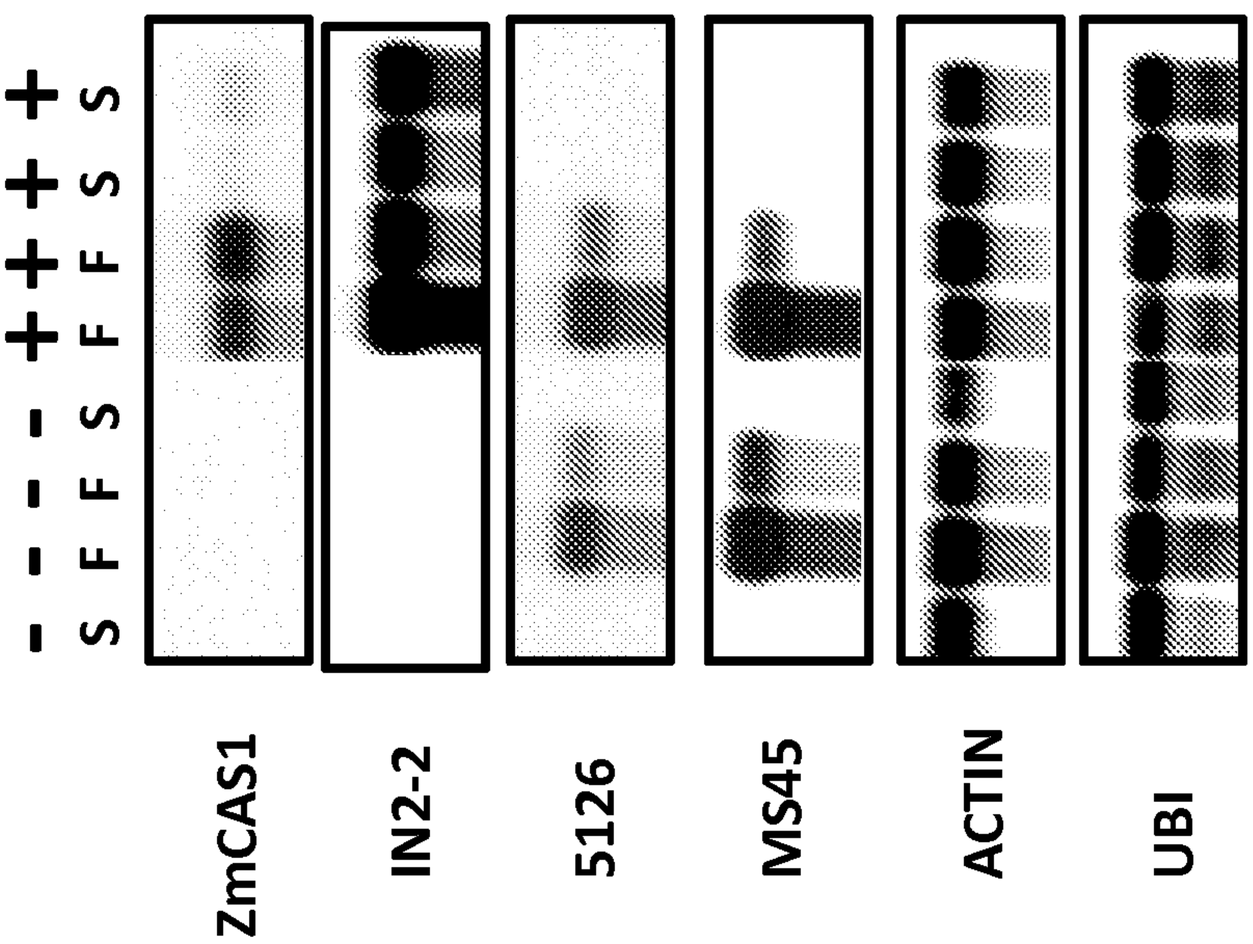


FIGURE 3

C= Callus, L= leaf, A= anther RNA, -/+ CBSU

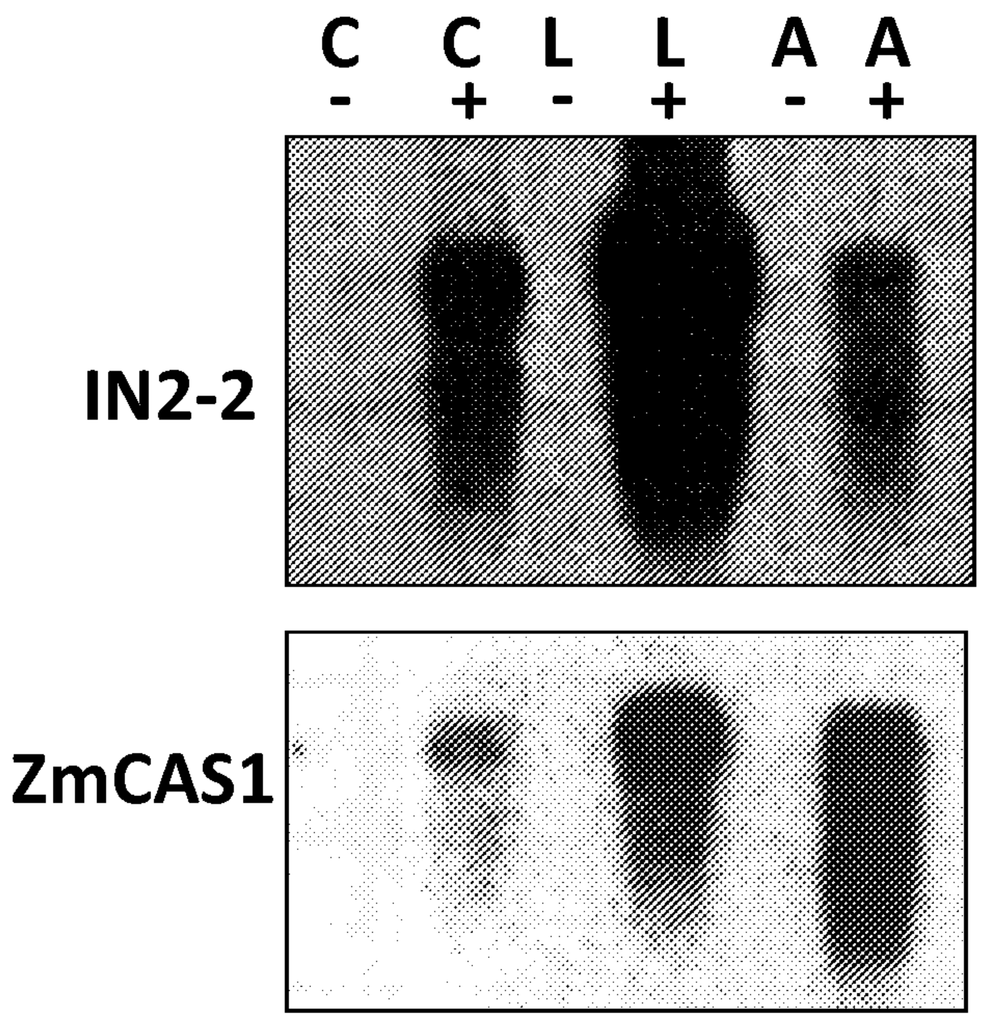


FIGURE 4

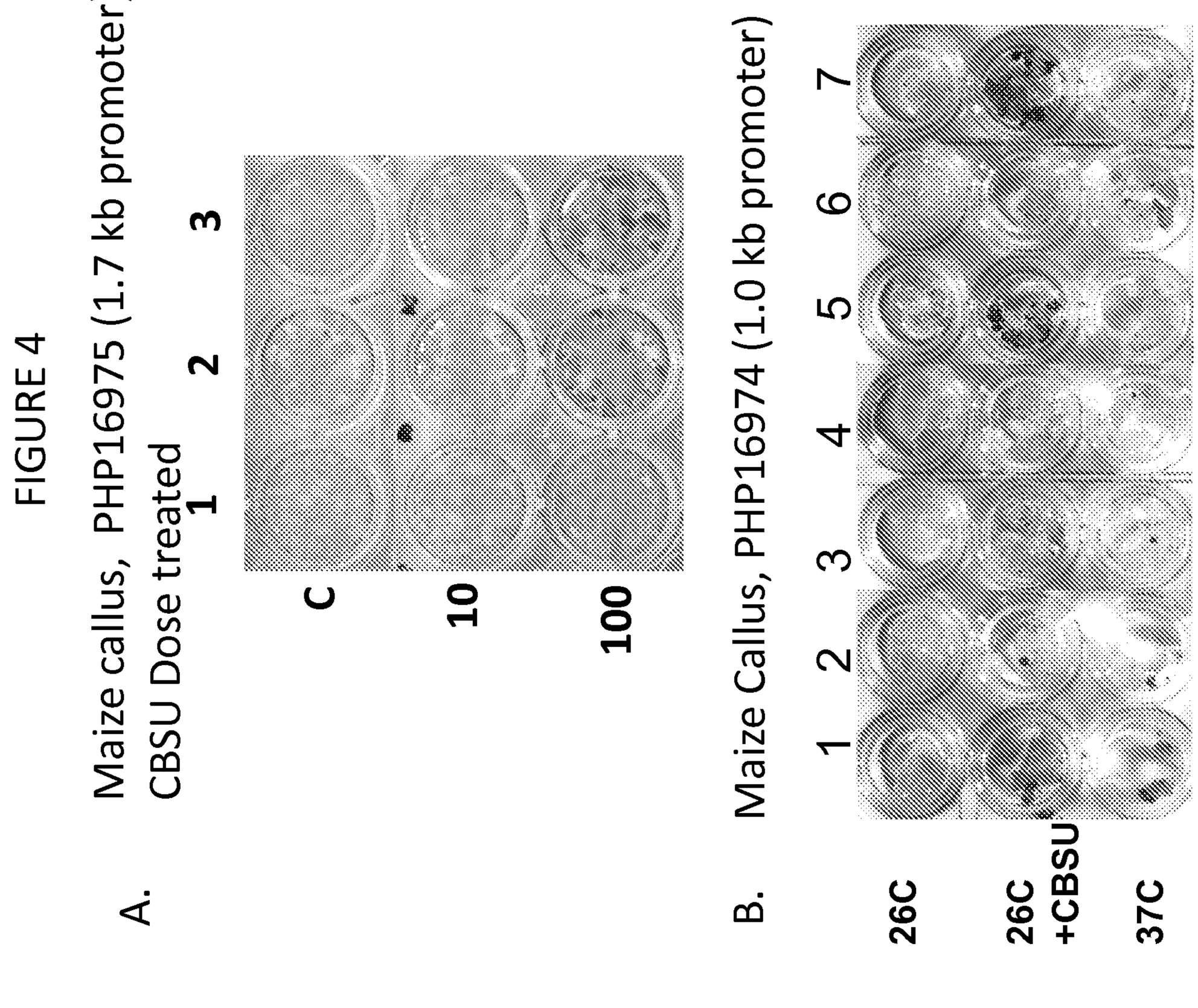
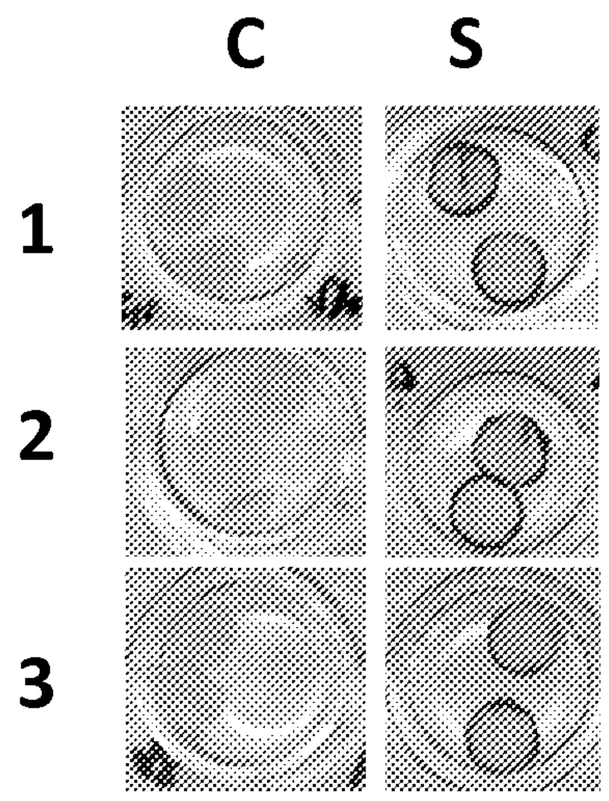

A.
Maize callus, PHP16975 (1.7 kb promoter)
CBSU Dose treated
1
2
3
C
10
100
B.
Maize Callus, PHP16974 (1.0 kb promoter)
1
2
3
4
5
6
7
26C
26C +CBSU
37C

FIGURE 5

Maize leaf, PHP16975, CBSU treated



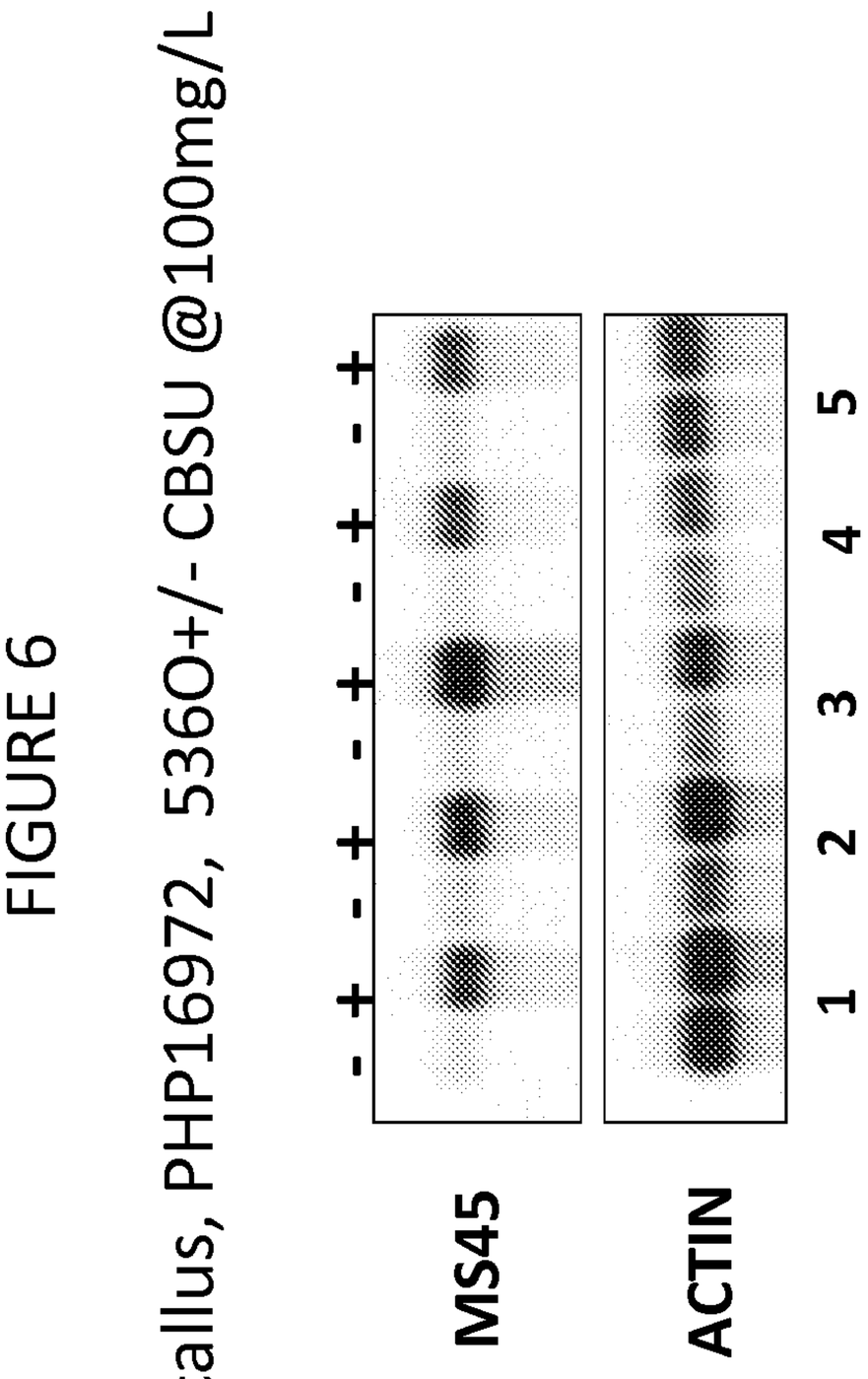
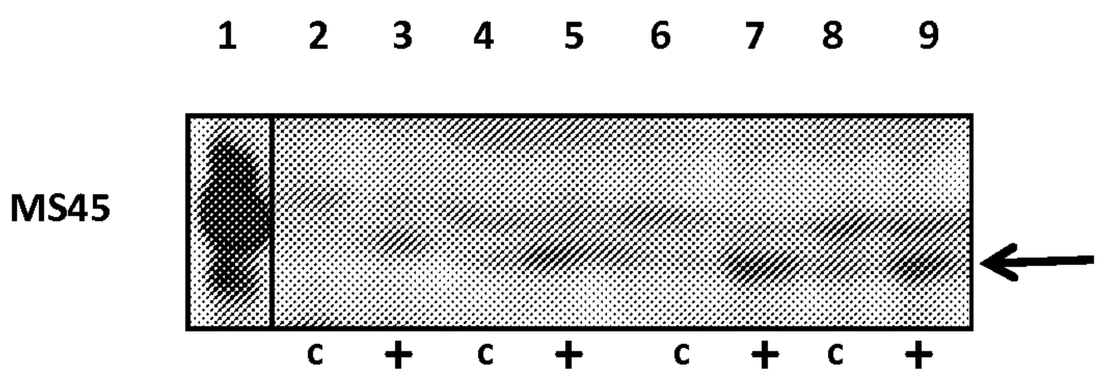


FIGURE 6

FIGURE 7

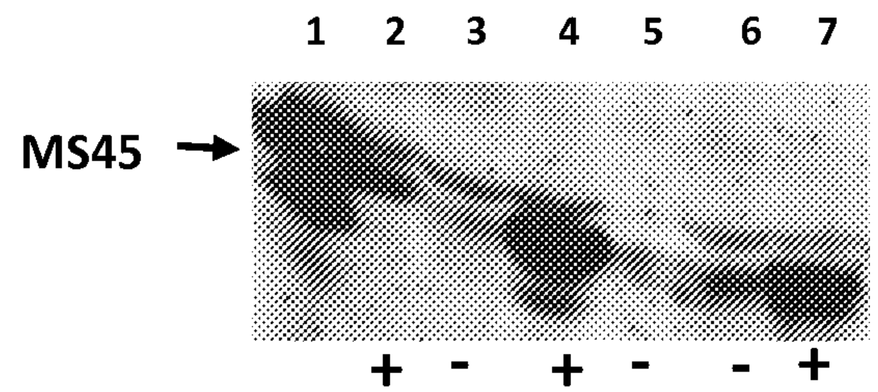
Maize leaf, PHP16973, CBSU induction



Lane 1, wildtype MS45 plant
Lane 2, Event 1, untreated
Lane 3, Event 1, CBSU
Lane 4, Event 2, untreated
Lane 5, Event 2, CBSU
Lane 6, Event 3, untreated
Lane 7, Event 3, CBSU
Lane 8, Event 4, untreated
Lane 9, Event 4, CBSU

FIGURE 8

Maize anther, PHP16973, CBSU induction



Lane 1, wildtype MS45 plant
Lane 2, Event 1, CBSU
Lane 3, Event 1, untreated
Lane 4, Event 2, CBSU
Lane 5, Event 2, untreated
Lane 6, Event 3, untreated
Lane 7, Event 3, CBSU

FIGURE 9

Rice events, PHP16974, CBSU induction

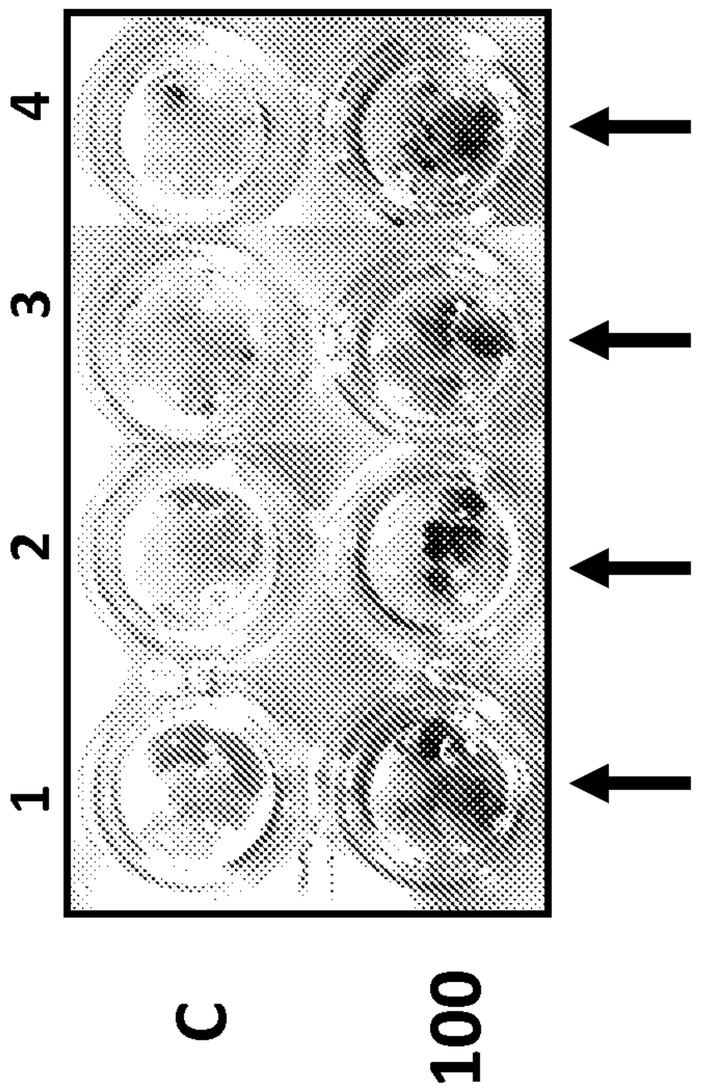


FIGURE 10

Rice Seedlings PHP16974

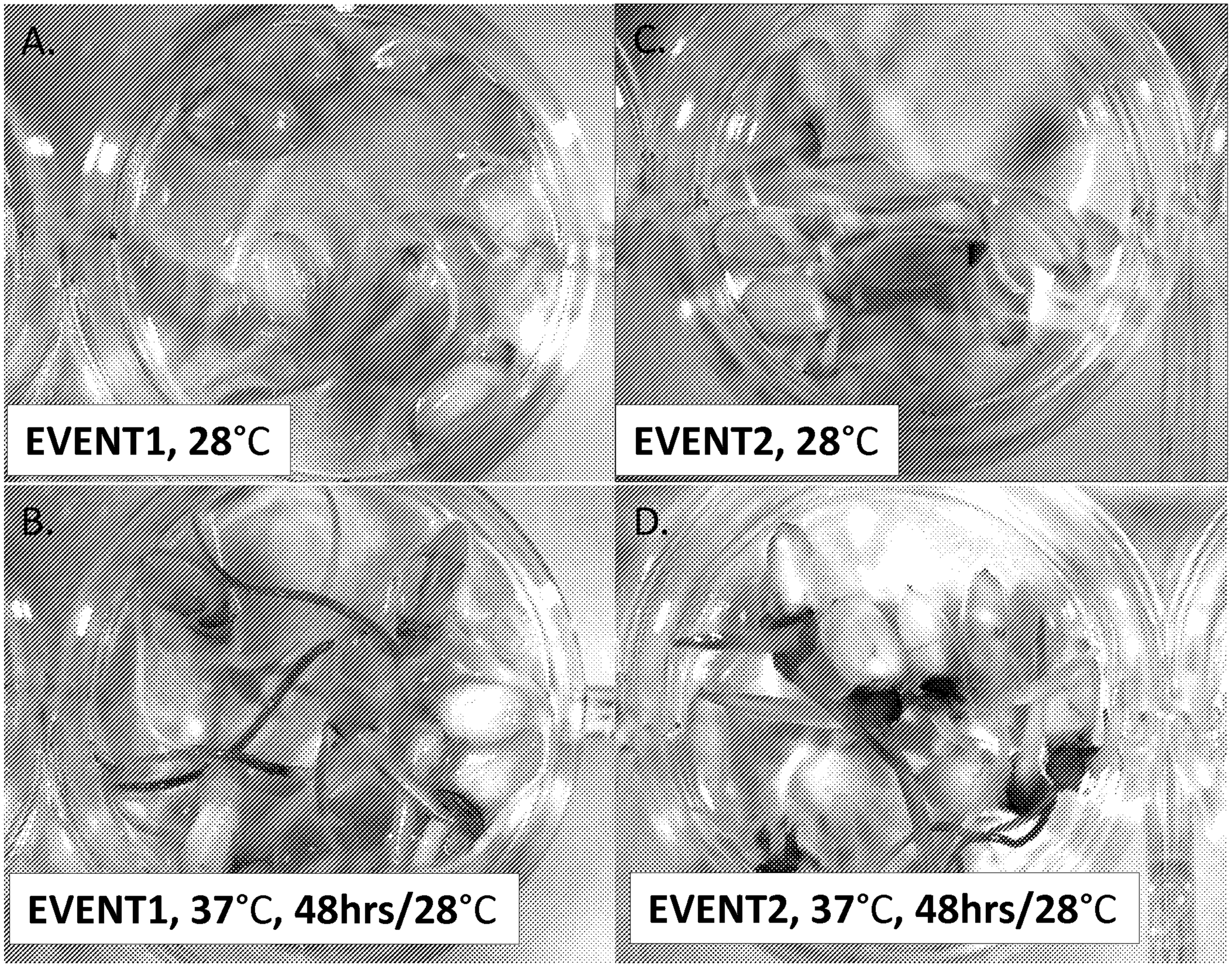


FIGURE 1

A)

```
                10        20        30        40        50        60
SEQIDNO-5.pro   MAAAAEHGNCDAWAARDPSGVLSPYKFNRRAVQSSDVSLKIIYCGVCYGDVMW QNKHND  60
SEQIDNO-6.pro   MAAAAEHGNCDAWAARDPSGVLSPYKFNRRAVQSSDVSLKIIYCGVCYGDVMW QNKHND  60

                70        80        90       100       110       120
SEQIDNO-5.pro   SVYPLVPGHEIAGVVTEVGADVKAFKVGDHAGVGTYVNSCRHCENCNSSLENYCPETVFT  120
SEQIDNO-6.pro   SVYPLVPGHEIAGVVTEVGADVKAFKVGDHAGVGTYVNSCRHCENCNSSLENYCPETVFT  120

               130       140       150       160       170       180
SEQIDNO-5.pro   YNTTDADGTITKGGYSTHIVVHERYCFKIPDGYPLAQAAPLLCAGITVYTPMARHNMKQP  180
SEQIDNO-6.pro   YNTTDADGTITKGGYSTHIVVHERYCFKIPDGYPLAKAAPLLCAGITVYTPMARHNMKQP  180

               190       200       210       220       230       240
SEQIDNO-5.pro   GKSLGVIGLGGLGHMAVKFGKAFGLKVTVFSTSESKREEAINLLGADNFVISSDTQQMEA  240
SEQIDNO-6.pro   GKSLGVIGLGGLGHMAVKFGKAFGLKVTVFSTSESKREEAINLLGADNFVISSDTQQMEA  240

               250       260       270       280       290       300
SEQIDNO-5.pro   LKNSLHFIVDTASGDHPFDPYLSLLKVGGVMTIVCFPSEIKMHPASLNRGGRTLSGSIVG  300
SEQIDNO-6.pro   LKNSLHFIVDTASGDHPFDPYLSLLKVGGVMTIVCFPSEIKMHPASLNRGGRTLSGSIVG  300

               310       320       330       340       350
SEQIDNO-5.pro   GTKDIQEMVNFCAENKIYPEIEIIKMDYINEALARLVNRDVKYRFVIDIKNSFE        354
SEQIDNO-6.pro   GTKDIQEMVNFCAENKIYPEIEIIKMDYINEALARLVNRDVKYRFVIDIKNSFE        354
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B)

divergence

Percent Identity

	1	2	
1		99.7	1
2	0.3		2
	1	2	

SEQIDNO-5.pro
SEQIDNO-6.pro