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(54) **Title:** MAIZE STRESS-RESPONSIVE NAC TRANSCRIPTION FACTORS AND PROMOTER AND METHODS OF USE

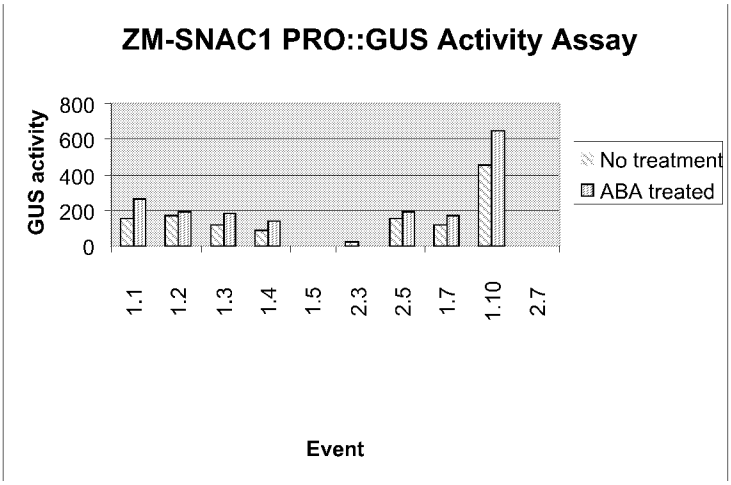


Figure 2

(57) **Abstract:** Methods and compositions for modulating plant development are provided. Polynucleotide sequences encoding ZmSNAC polypeptides are provided, as are the amino acid sequences of the encoded polypeptides. The sequences can be used in a variety of methods including modulating root development, modulating floral development, modulating leaf and/or shoot development, modulating senescence, modulating seed size and/or weight, and modulating tolerance of plants to abiotic stress. Transformed plants, plant cells, tissues, and seed are also provided. A stress-inducible ZmSNAC1 promoter is also provided.

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MAIZE STRESS-RESPONSIVE NAC TRANSCRIPTION FACTORS AND PROMOTER AND METHODS OF USE

FIELD OF THE INVENTION

The invention relates to the field of genetic manipulation of plants, particularly the modulation of gene activity to affect plant development and growth.

BACKGROUND OF THE INVENTION

Drought tolerance is a complex trait, controlled by multiple genes. Gene switches such as transcription factors, which can have significant and specific effects on plant physiology, are desirable modulation targets. Stress-responsive NAC transcription factors (SNACs) may control expression of numerous downstream genes important for adaptation to drought stresses and may ultimately enhance drought tolerance through one or more mechanisms such as stomatal aperture reduction, delayed senescence, and increased sink and source strength. It has been shown that ABA- and drought-responsive NACs can enhance drought tolerance in Arabidopsis (Tran, *et al.*, (2004) *Plant Cell* 16:2481-2498) and rice (Hu, *et al.*, (2006) *PNAS* 103(35):12987-12992). Therefore, SNAC genes from maize may also be used individually or in combination with other genes to enhance drought tolerance.

Expression of heterologous DNA sequences in a plant host is dependent upon the presence of operably linked regulatory elements that are functional within the plant host. Choice of the regulatory element will determine when and where within the organism the heterologous DNA sequence is expressed. Where continuous expression is desired throughout the cells of a plant, and/or throughout development, constitutive promoters are utilized. In contrast, where gene expression in response to a stimulus is desired, inducible promoters are the regulatory element of choice. Where expression in specific tissues or organs is desired, tissue-specific or tissue-preferred promoters may be used. That is, they may drive expression exclusively or preferentially in specific tissues or organs. Such tissue-specific promoters may be temporally constitutive or inducible. In any case, additional regulatory sequences upstream and/or downstream from a core promoter sequence may be included in expression constructs of transformation

vectors to bring about varying levels of expression of heterologous nucleotide sequences in a transgenic plant.

As this field develops and more genes become accessible, a greater need exists for plants transformed with multiple genes. These multiple exogenous genes typically need to be controlled by separate regulatory sequences. Further, some genes should be regulated constitutively whereas other genes should be expressed at certain developmental stages or locations in the transgenic organism. Accordingly, a variety of regulatory sequences having diverse effects is needed.

Multiple regulatory sequences are also needed in order to avoid undesirable molecular interactions which can result from using the same regulatory sequence to control more than one gene.

The inventor herein discloses the isolation and characterization of a promoter associated with a transcription factor which can serve as a regulatory element for expression of isolated nucleotide sequences of interest, thereby impacting various traits in plants. Alternatively or additionally, the promoter may be used to drive scorable markers.

BRIEF SUMMARY OF THE INVENTION

Compositions and methods of the invention comprise and employ maize SNAC transcription factors that are involved in modulating plant development, morphology, and physiology.

Compositions further include expression cassettes and vectors comprising the ZmSNAC sequences of the invention, and plants, plant cells, and plant parts in which ZmSNAC expression is modified. The plants, plant cells, and plant parts of the invention may exhibit enhanced abiotic stress tolerance through such phenotypic changes as delayed leaf rolling, reduced transpiration rate, increased stomatal closure, and improved cell membrane stability, all relative to a plant, plant cell, or plant part not modified per the invention.

Methods are provided for modifying the level of a ZmSNAC polypeptide in a plant comprising introducing into the plant a selected polynucleotide. The polynucleotide may be introduced within a construct designed to modulate the level or activity of the ZmSNAC polypeptide throughout the plant or in targeted tissues or at targeted developmental stages.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides an amino acid alignment of NAC transcription factors from various species. Included are ANAC (At1g52890, SEQ ID NO: 12); ATAF2 (At5g08790, SEQ ID NO: 13); ATAF1 (At1g01720, SEQ ID NO: 14); NAP (At1g69490, SEQ ID NO: 15); AtNAM (At1g52880, SEQ ID NO: 16); TIP (At5g24590, SEQ ID NO: 17); CUC2 (At5g53950, SEQ ID NO: 18); CUC1 (At3g15170, SEQ ID NO: 19); CUC3 (At1g76420, SEQ ID NO: 20); Arabidopsis NAC1 (At1g56010, SEQ ID NO: 21); ZmSNAC1 (SEQ ID NO: 2); ZmSNAC2 (SEQ ID NO: 4); ZmSNAC3 (SEQ ID NO: 6); ZmSNAC5 (SEQ ID NO: 10); ZmSNAC4 (SEQ ID NO: 8) and a consensus (SEQ ID NO: 22).

Figure 2 shows GUS expression driven by the ZmSNAC1 promoter.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 and 2 provide nucleotide and amino acid sequences for ZmSNAC1.

SEQ ID NO: 3 and 4 provide nucleotide and amino acid sequences for ZmSNAC2.

SEQ ID NO: 5 and 6 provide nucleotide and amino acid sequences for ZmSNAC3.

SEQ ID NO: 7 and 8 provide nucleotide and amino acid sequences for ZmSNAC4.

SEQ ID NO: 9 and 10 provide nucleotide and amino acid sequences for ZmSNAC5.

SEQ ID NO: 11 provides ZmSNAC1 promoter sequence.

SEQ ID NOS: 12 through 21 provide Arabidopsis NAC proteins as noted in the brief description of the drawings.

SEQ ID NO: 22 provides a consensus of Arabidopsis and Zea mays NAC proteins.

DETAILED DESCRIPTION OF THE INVENTION

The present invention now will be described more fully with reference to the accompanying drawings, in which some, but not all, embodiments of the invention are shown. Indeed, the invention may be embodied in many different forms and

should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the invention set forth herein will come to mind to one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

COMPOSITIONS

Compositions of the invention include maize SNAC transcription factor polypeptides and polynucleotides that are involved in modulating plant development, morphology, and physiology. In particular, the present invention provides for isolated polynucleotides comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NO: 2, 4, 6, 8 and 10. Further provided are isolated polypeptides having an amino acid sequence encoded by a polynucleotide described herein, for example those set forth in SEQ ID NO: 1, 3, 5, 7 and 9.

NAC transcription factors are encoded by genes present in a wide range of plant species, the name being derived from the NAM (no apical meristem; Souer, *et al.*, (1996) *Cell* 85:159-170), ATAF1,2 and CUC2 (cup-shaped cotyledon 2; Aida, *et al.*, (1997) *Plant Cell* 9:841-857) transcription factors. Expression patterns of NAC transcription factors, and the mutant phenotypes conferred by modulation of their expression, are similar. A highly conserved N-terminal DNA-binding domain has been identified and its structure has been characterized (Ernst, *et al.*, (2004) *EMBO Reports* 5(3):297-303). The more diverse C-terminal regions comprise transcriptional activation domains (Xie, *et al.*, (2000) *Genes Dev.* 14:3024-3036; Duval, *et al.*, (2002) *Plant Mol. Bio.* 50:237-248). NAC transcription factors have been shown to interact with numerous genes involved in meristem formation, organ differentiation, auxin signalling, root growth, and in biotic and abiotic stress response (see, review by Olsen, *et al.*, (2005) *Trends in Plant*

Science 10(2):1360-1385). A NAC recognition sequence (NACRS), and a core binding sequence, have been identified in Arabidopsis (Tran, *et al.*, (2004) *Plant Cell* 16:2481-2498).

The invention encompasses isolated or substantially purified polynucleotide or protein compositions. An "isolated" or "purified" polynucleotide or protein, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide or protein as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide or protein is substantially free of other cellular material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Optimally, an "isolated" polynucleotide is free of sequences (optimally protein encoding sequences) that naturally flank the polynucleotide (i.e., sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. For example, in various embodiments, the isolated polynucleotide can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequence that naturally flank the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5% or 1% (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, optimally culture medium represents less than about 30%, 20%, 10%, 5% or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed polynucleotides and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a polynucleotide that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides and up to the full-length polynucleotide encoding the proteins of the invention.

A fragment of a ZmSNAC polynucleotide that encodes a biologically active portion of a NAC transcription factor of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 225, 250, 275, 300 or 339 contiguous amino acids, or up to the total number of amino acids present in a full-length polypeptide of the invention. Fragments of a SNAC polynucleotide that are useful as hybridization probes or PCR primers generally need not encode a biologically active polyeptide.

Thus, a fragment of a ZmSNAC polynucleotide may encode a biologically active portion of a SNAC transcription factor, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a SNAC transcription factor can be prepared by isolating a portion of one of the ZmSNAC polynucleotides of the invention, expressing the encoded portion of the ZmSNAC protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion. Activity could be assessed by DNA binding activity or protein interaction studies, and/or by expressing the ZMSNAC or portions of the protein in transgenic plants and evaluating the plants for a phenotype.

Polynucleotides that are fragments of a ZmSNAC nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900 or 1000 contiguous nucleotides, or up to the number of nucleotides present in a full-length ZmSNAC polynucleotide disclosed herein.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those which, because of the degeneracy of the genetic code, do not change the encoded amino acid sequence. Naturally occurring variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis but which still encode a ZmSNAC protein of the invention. Generally, variants of a particular

polynucleotide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

Variants of a particular polynucleotide of the invention (i.e., the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Thus, for example, isolated polynucleotides that encode a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 2, 4, 6, 8 or 10 are disclosed. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

"Variant" protein is intended to mean a protein derived from the native protein by deletion or addition of one or more amino acids at one or more sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Certain variant proteins encompassed by the present invention are biologically active, that is, they continue to possess the desired biological activity of the native protein, i.e., transcription factor activity, as described herein. Such variants may result from, for example, genetic polymorphism or human manipulation. Biologically active variants of a native SNAC protein of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2 or even 1 amino acid residue.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of the SNAC proteins can be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel, *et al.*, (1987) *Methods in Enzymol.* 154:367-382; US Patent Number 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff, *et al.*, (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal.

Thus, the genes and polynucleotides of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired transcription factor activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and optimally will not create complementary regions that could produce secondary mRNA structure.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by assaying for DNA binding activity or protein-protein interactions, the activation of gene expression in transient studies, or an effect on gene expression in transgenic plants.

Variant polynucleotides and proteins also encompass sequences and proteins derived from a mutagenic or recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different NAC coding sequences

can be manipulated to create a new NAC polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between a ZmSNAC gene of the invention and other known NAC genes to obtain a new gene coding for a protein with an improved property of interest. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri, *et al.*, (1997) *Nature Biotech.* 15:436-438; Moore, *et al.*, (1997) *J. Mol. Biol.* 272:336-347; Zhang, *et al.*, *(1997) Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri, *et al.*, (1998) *Nature* 391:288-291; PCT Publication Number WO97/20078; and US Patent Numbers 5,605,793 and 5,837,458.

By "promoter" is intended a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular polynucleotide sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. The promoter sequences of the present invention regulate (i.e., repress or activate) transcription.

The polynucleotides of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire NAC sequences set forth herein or to variants and fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. "Orthologs" is intended to mean genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share at least 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity. Functions of

orthologs are often highly conserved among species. Thus, isolated polynucleotides that encode a NAC protein and which hybridize under stringent conditions to the SNAC sequences disclosed herein, or to variants or fragments or complements thereof, are encompassed by the present invention.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also, Innis, *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known polynucleotide is used as a probe that selectively hybridizes to other corresponding polynucleotides present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the ZmSNAC polynucleotides of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire ZmSNAC polynucleotide disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding NAC polynucleotides. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among NAC polynucleotide sequences and are optimally at least about 10 nucleotides in

length, and most optimally at least about 20 nucleotides in length. Such probes may be used to amplify corresponding NAC polynucleotides from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

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

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12

hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: T_m = 81.5°C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3 or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9 or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15 or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is optimal to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel, *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See, Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

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

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

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

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith, *et al.*, (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 872264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys

Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins, *et al.*, (1988) *Gene* 73:237-244 (1988); Higgins, *et al.*, (1989) *CABIOS* 5:151-153; Corpet, *et al.*, (1988) *Nucleic Acids Res.* 16:10881-90; Huang, *et al.*, (1992) *CABIOS* 8:155-65; and Pearson, *et al.*, (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul, *et al.*, (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul, *et al.*, (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See, Altschul, *et al.*, (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See, www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap

creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see, Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such

conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

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

The invention further provides plants having altered levels and/or activities of the ZmNAC polypeptides of the invention. In some embodiments, a construct directing increased expression of the sequences of the invention is stably incorporated into the genome of a plant of the invention. Other embodiments provide plants that are genetically modified at a native genomic locus encoding a SNAC polypeptide of the invention. By "native genomic locus" is intended a naturally occurring genomic sequence. The genomic locus may be modified to increase, reduce or eliminate the activity of the SNAC polypeptide. The term "genetically modified" as used herein refers to a plant or plant part that is modified in its genetic information by the introduction of one or more foreign polynucleotides, and the introduction of the foreign polynucleotide leads to a phenotypic change in the plant. By "phenotypic change" is intended a measurable change in one or more cell, tissue, or organ functions. For example, plants having a genetic modification at the genomic locus encoding a ZmSNAC polypeptide can

show reduced or eliminated expression or activity of the NAC polypeptide. Various methods to generate such a genetically modified genomic locus are described elsewhere herein, as are the variety of phenotypes that can result from the modulation of the level and/or activity of one or more of the ZmSNAC sequences of the invention.

As used herein, the term plant includes reference to whole plants, plant parts or organs (e.g., leaves, stems, roots), plant cells, and seeds and progeny of same. Plant cell, as used herein, includes, without limitation, cells obtained from or found in seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores, as well as plant protoplasts and plant cell tissue cultures, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, grain and the like. As used herein, "grain" refers to the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

METHODS

I. Providing Sequences

The sequences of the present invention can be introduced into and expressed in a host cell such as bacteria, yeast, insect, mammalian, or optimally plant cells. It is expected that those of skill in the art are knowledgeable in the numerous systems available for the introduction of a polypeptide or a nucleotide sequence of the present invention into a host cell. No attempt to describe in detail the various methods known for providing proteins in prokaryotes or eukaryotes will be made.

By "host cell" is meant a cell which comprises a heterologous nucleic acid sequence of the invention. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Host cells can also be monocotyledonous or dicotyledonous plant cells. In certain embodiments, the monocotyledonous host cell is a maize host cell.

The use of the term "polynucleotide" is not intended to limit the present invention to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides of the invention also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

The ZmSNAC polynucleotides of the invention can be provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a ZmSNAC polynucleotide of the invention. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of interest and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, “operably linked” means that the coding regions are in the same reading frame. The cassette may contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. An expression cassette may be provided with a plurality of restriction sites and/or recombination sites for insertion of the ZmSNAC polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

In certain embodiments, the expression cassette will include, in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a ZmSNAC polynucleotide of the invention, and a transcriptional and translational termination region functional in plants. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the ZmSNAC polynucleotide of the invention may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the ZmNAC polynucleotide of the invention may be heterologous to the host cell or to each other. As used herein with reference to a sequence, “heterologous” means a sequence that originates from a foreign species, or, if from the same species, is

substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably-linked polynucleotide. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While heterologous promoters can be used to express the ZmSNAC sequences, the native promoter sequences or other NAC promoters may also be used. Such constructs can change expression levels of ZmSNAC sequences in the plant or plant cell. Thus, the phenotype of the plant or plant cell can be altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably-linked ZmSNAC polynucleotide of interest, may be native with the plant host, or may be derived from another source, i.e., foreign or heterologous with reference to the promoter, the ZmSNAC polynucleotide of interest, the plant host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau, *et al.*, (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon, *et al.*, (1991) *Genes Dev.* 5:141-149; Mogen, *et al.*, (1990) *Plant Cell* 2:1261-1272; Munroe, *et al.*, (1990) *Gene* 91:151-158; Ballas, *et al.*, (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi, *et al.*, (1987) *Nucleic Acids Res.* 15:9627-9639.

Where appropriate, the polynucleotides may be optimized for increased expression in the transformed plant. That is, the polynucleotides can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, US Patent Numbers 5,380,831 and 5,436,391, and Murray, *et al.*, (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

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

The expression cassettes may additionally contain 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, *et al.*, (1989) *Proc. Natl. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie, *et al.*, (1995) *Gene* 165(2):233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak, *et al.*, (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling, *et al.*, (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie, *et al.*, (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel, *et al.*, (1991) *Virology* 81:382-385). See also, Della-Cioppa, *et al.*, (1987) *Plant Physiol.* 84:965-968. Other methods known to enhance translation can also be utilized.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and

hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su, *et al.*, (2004) *Biotechnol Bioeng* 85:610-9 and Fetter, *et al.*, (2004) *Plant Cell 16:*215-28), cyan fluorescent protein (CYP) (Bolte, *et al.*, (2004) *J. Cell Science* 117:943-54 and Kato, *et al.*, (2002) *Plant Physiol* 129:913-42), and yellow fluorescent protein (PhiYFP™ from Evrogen, see, Bolte, *et al.*, (2004) *J. Cell Science* 117:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson, *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao, *et al.*, (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley, *et al.*, (1980) in *The Operon*, pp. 177-220; Hu, *et al.*, (1987) *Cell* 48:555-566; Brown, *et al.*, (1987) *Cell* 49:603-612; Figge, *et al.*, (1988) *Cell* 52:713-722; Deuschle, *et al.*, (1989) *Proc. Natl. Acad. Aci. USA* 86:5400-5404; Fuerst, *et al.*, (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle, *et al.*, (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow, *et al.*, (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti, *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim, *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski, *et al.*, (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb, *et al.*, (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt, *et al.*, (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen, *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva, *et al.*, (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka, *et al.*, (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill, *et al.*, (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting; any selectable marker gene can be used in the present invention.

Certain other marker genes for plant transformation require screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. These marker genes are particularly useful to quantify or visualize the spatial pattern of expression of a gene in specific tissues and are frequently referred to as reporter

genes because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression. Commonly used genes for screening presumptively transformed cells include β-glucuronidase *(GUS),* β-galactosidase, luciferase and chloramphenicol acetyltransferase. Jefferson, (1987) *Plant Mol. Biol. Rep.* 5:387 and US Patent Number 5,599,670; Teeri, *et al.*,(1989) *EMBO J.* 8:343, Koncz, *et al.*, (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:131, De Block, *et al.*, (1984) *EMBO J.* 3:1681. Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig, *et al.*, (1990) *Science* 247: 449.

A number of promoters can be used in the practice of the invention, including the native promoter of a polynucleotide sequence of interest. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, inducible, tissue-preferred or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and US Patent Number 6,072,050; the core CaMV 35S promoter (Odell, *et al.*, (1985) *Nature* 313:810-812); rice actin (McElroy, *et al.*, (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen, *et al.*, (1989) *Plant Mol. Biol.* 12:619-632 and Christensen, *et al.*, (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last, *et al.*, (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten, *et al.*, (1984) *EMBO J.* 3:2723-2730); ALS promoter (US Patent Number 5,659,026), dMMV (double-enhanced version of the mirabilis mosaic virus promoter; see, Dey and Maiti (1999) *Plant Molecular Biology* 40(5):771-782), LESVBV (enhanced strawberry vein banding virus promoter; see, US Patent Application Publication Number 2002/0182593) and the like. Other constitutive promoters include, for example, US Patent Numbers 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142 and 6,177,611.

Tissue-preferred promoters can be utilized to target enhanced ZmNAC expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto, *et al.*, (1997) *Plant J.* 12(2):255-265; Kawamata, *et al.*, (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen, *et al.*, (1997) *Mol. Gen Genet.* 254(3):337-343; Russell, *et al.*, (1997) *Transgenic Res.* 6(2):157-168; Rinehart, *et al.*, (1996)

Plant Physiol. 112(3):1331-1341; Van Camp, *et al.*, (1996) *Plant Physiol.* 112(2):525-535; Canevascini, *et al.*, (1996) *Plant Physiol.* 112(2):513-524; Yamamoto, *et al.*, (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco, *et al.*, (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka, *et al.*, (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590 and Guevara-Garcia, *et al.*, (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression. See, also, US Patent Application Number 2003/0074698, herein incorporated by reference.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto, *et al.*, (1997) *Plant J.* 12(2):255-265; Kwon, *et al.*, (1994) *Plant Physiol.* 105:357-67; Yamamoto, *et al.*, (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor, *et al.*, (1993) *Plant J.* 3:509-18; Orozco, *et al.*, (1993) *Plant Mol. Biol.* 23(6):1129-1138; Baszczynski, *et al.*, (1988) *Nucl. Acid Res.* 16:4732; Mitra, *et al.*, (1994) *Plant Molecular Biology* 26:35-93; Kayaya, *et al.*, (1995) *Molecular and General Genetics* 248:668-674; and Matsuoka, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590. Senecence regulated promoters are also of use, such as, SAM22 (Crowell, *et al.*, (1992) *Plant Mol. Biol.* 18:459-466). See, also, US Patent Number 5,689,042 herein incorporated by reference.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated *de novo* from various compatible species. See, for example, Hire, *et al.*, (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger, *et al.*, (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao, *et al.*, (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also, Bogusz, *et al.*, (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis

of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see. *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri, *et al.*, (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see, *EMBO J.* 8(2):343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster, *et al.*, (1995) *Plant Mol. Biol.* 29(4):759-772); rolB promoter (Capana, *et al.*, (1994) *Plant Mol. Biol.* 25(4):681-691; and the CRWAQ81 root-preferred promoter with the ADH first intron (US Patent Application Publication NUmber 2005/0097633). See also, US Patent Numbers 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732 and 5,023,179.

"Seed-preferred" promoters refers to those promoters active during seed development and may include expression in seed initials or related maternal tissue. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase) (see, WO 00/11177 and US Patent Number 6,225,529; herein incorporated by reference). Gamma-zein is an endosperm-specific promoter. Globulin-1 (Glob-1) is a representative embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, gamma-zein, waxy, shrunken 1 and shrunken 2. See also, WO 00/12733, where seed-preferred promoters from *end1* and *end2* genes are disclosed; herein incorporated by reference. Additional embryo specific promoters are disclosed in Sato, *et al.*, (1996) *Proc. Natl. Acad. Sci.* 93:8117-8122; Nakase, *et al.*, (1997) *Plant J* 12:235-46; and Postma-Haarsma, *et al.*, (1999) *Plant Mol. Biol.* 39:257-71. Additional endosperm specific promoters are disclosed in Albani, *et al.*, (1984) *EMBO* 3:1405-15; Albani, *et al.*, (1999) *Theor. Appl. Gen.* 98:1253-62; Albani, *et al.*, (1993) *Plant J.* 4:343-55; Mena, *et al.*, (1998) *The Plant Journal* 116:53-62, and Wu, *et al.*, (1998) *Plant Cell Physiology* 39:885-889.

Also of interest are promoters active in meristem regions, such as developing inflorescence tissues, and promoters which drive expression at or about the time of anthesis or early kernel development. This may include, for example, the maize Zag promoters, including Zag1 and Zag2 (see, Schmidt, *et al.*, (1993) *The Plant Cell* 5:729-37; GenBank X80206; Theissen, *et al.*, (1995) *Gene* 156:155-166; and US Patent Application Number 10/817,483); maize Zap promoter (also known as ZmMADS; US Patent Application Number 10/387,937; WO 03/078590); maize ckx1-2 promoter (US Patent Application Publication Number 2002/0152500 A1; WO 02/0078438); maize eep1 promoter (US Patent Application Number 10/817,483); maize end2 promoter (US Patent Number 6,528,704 and US Patent Application Number 10/310,191); maize lec1 promoter (US Patent Application Number 09/718,754); maize F3.7 promoter (Baszczynski, *et al.*, (1997) *Maydica* 42:189-201); maize tb1 promoter (Hubbarda, *et al.*, (2002) *Genetics* 162:1927-1935 and Wang, *et al.*, (1999) *Nature* 398:236-239); maize eep2 promoter (US Patent Application Number 10/817,483); maize thioredoxinH promoter (US Provisional Patent Application Number 60/514,123); maize Zm40 promoter (US Patent Number 6,403,862 and WO 01/2178); maize mLIP15 promoter (US Patent Number 6,479,734); maize ESR promoter (US Patent Application Number 10/786,679); maize PCNA2 promoter (US Patent Application Number 10/388,359); maize cytokinin oxidase promoters (US Patent Application Number 11/094,917); promoters disclosed in Weigal, *et al.*, (1992) *Cell* 69:843-859; Accession Number AJ131822; Accession Number Z71981; Accession Number AF049870; and shoot-preferred promoters disclosed in McAvoy, *et al.*, (2003) *Acta Hort. (ISHS)* 625:379-385. Other dividing cell or meristematic tissue-preferred promoters that may be of interest have been disclosed in Ito, *et al.*, (1994) *Plant Mol. Biol.* 24:863-878; Regad, *et al.*, (1995) *Mo. Gen. Genet.* 248:703-711; Shaul, *et al.*, (1996) *Proc. Natl. Acad. Sci.* 93:4868-4872; Ito, *et al.*, (1997) *Plant J.* 11:983-992; and Trehin, *et al.*, (1997) *Plant Mol. Biol.* 35:667-672, all of which are hereby incorporated by reference herein.

Inflorescence-preferred promoters include the promoter of chalcone synthase (Van der Meer, *et al.*, (1990) *Plant Mol. Biol.* 15:95-109), LAT52 (Twell, *et al.*, (1989) *Mol. Gen. Genet.* 217:240-245), pollen specific genes (Albani, *et al.*, (1990) *Plant Mol Biol.* 15:605, Zm13 (Buerrero, *et al.*, (1993) *Mol. Gen. Genet.* 224:161-168), maize pollen-specific gene (Hamilton, *et al.*, (1992) *Plant Mol. Biol.*

18:211-218), sunflower pollen expressed gene (Baltz, *et al.*, (1992) *The Plant Journal* 2:713-721), and B. napus pollen specific genes (Arnoldo, *et al.*, (1992) *J. Cell. Biochem*, Abstract Number Y101204).

Stress-inducible promoters include salt-inducible or water-stress-inducible promoters such as P5CS (Zang, *et al.*, (1997) *Plant Sciences* 129:81-89); cold-inducible promoters, such as, cor15a (Hajela, *et al.*, (1990) *Plant Physiol.* 93:1246-1252), cor15b (Wlihelm, *et al.*, (1993) *Plant Mol Biol* 23:1073-1077), wsc120 (Ouellet, *et al.*, (1998) *FEBS Lett.* 423-324-328), ci7 (Kirch, *et al.*, (1997) *Plant Mol Biol.* 33:897-909), ci21A (Schneider, *et al.*, (1997) *Plant Physiol.* 113:335-45); drought-inducible promoters, such as, Trg-31 (Chaudhary, *et al.*, (1996) *Plant Mol. Biol.* 30:1247-57); osmotic inducible promoters, such as, Rab17 (Vilardell, *et al.*, (1991) *Plant Mol. Biol.* 17:985-93) and osmotin (Raghothama, *et al.*, (1993) *Plant Mol Biol* 23:1117-28); and, heat inducible promoters, such as, heat shock proteins (Barros, *et al.*, (1992) *Plant Mol.* 19:665-75; Marrs, *et al.*, (1993) *Dev. Genet.* 14:27-41), and smHSP (Waters, *et al.*, (1996) *J. Experimental Botany* 47:325-338). Other stress-inducible promoters include rip2 (US Patent Number 5,332,808 and US Patent Application Publication Number 2003/0217393), rab17 (Busk (1997) *Plant J* 11(6):1285-1295) and rd29a (Yamaguchi-Shinozaki, *et al.*, (1993) *Mol. Gen. Genetics* 236:331-340). Manipulation of ZmNAC expression to improve abiotic stress tolerance may involve the use of tissue-preferred and/or stress-responsive promoters.

Stress-insensitive promoters can also be used in the methods of the invention. This class of promoters, as well as representative examples, are further described elsewhere herein.

Nitrogen-responsive promoters can also be used in the methods of the invention. Such promoters include, but are not limited to, the 22 kDa Zein promoter (Spena, *et al.*, (1982) *EMBO J* 1:1589-1594 and Muller, *et al.*, (1995) *J. Plant Physiol* 145:606-613); the 19 kDa zein promoter (Pedersen, *et al.*, (1982) *Cell* 29:1019-1025); the 14 kDa zein promoter (Pedersen, *et al.*, (1986) *J. Biol. Chem.* 261:6279-6284), the b-32 promoter (Lohmer, *et al.*, (1991) *EMBO J* 10:617-624); and the nitrite reductase (NiR) promoter (Rastogi, *et al.*, (1997) *Plant Mol Biol.* 34(3):465-76 and Sander, *et al.*, (1995) *Plant Mol Biol.* 27(1):165-77). For a review of consensus sequences found in nitrogen-induced promoters, see for example, Muller, *et al.*, (1997) *The Plant Journal* 12:281-291.

Chemically-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemically-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemically-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena, *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis, *et al.*, (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz, *et al.*, (1991) *Mol. Gen. Genet.* 227:229-237, and US Patent Numbers 5,814,618 and 5,789,156), herein incorporated by reference.

Additional inducible promoters include heat shock promoters, such as Gmhsp17.5-E (soybean) (Czarnecka, *et al.*, (1989) *Mol Cell Biol.* 9(8):3457–3463); APX1 gene promoter (Arabidopsis) (Storozhenko, *et al.*, (1998) *Plant Physiol.* 118(3):1005–1014): *Ha hsp17.7 G4* (*Helianthus annuus*) (Almoguera, *et al.*, (2002) *Plant Physiol.* 129(1):333–341; and Maize Hsp70 (Rochester, *et al.*, (1986) *EMBO J.* 5: 451-8.

The methods of the invention involve introducing a polypeptide or polynucleotide into a plant. "Introducing" is intended to mean presenting to the plant the polynucleotide or polypeptide in such a manner that the sequence gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a sequence into a plant, only that the polynucleotide or polypeptides gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotides or polypeptides into plants are known in the art and include, but are not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

"Stable transformation" is intended to mean that the introduced nucleotide construct integrates into the genome of the plant and is capable of being inherited

by the progeny thereof. "Transient transformation" is intended to mean that a sequence is introduced into the plant but is only temporarily expressed or present in the plant.

Transformation protocols, as well as protocols for introducing polypeptides or polynucleotide sequences into plants, may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing polypeptides and polynucleotides into plant cells include microinjection (Crossway, *et al.*, (1986) *Biotechniques* 4:320-334), electroporation (Riggs, *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606), *Agrobacterium*-mediated transformation (US Patent Number 5,563,055 and US Patent Number 5,981,840), direct gene transfer (Paszkowski, *et al.*, (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, US Patent Number 4,945,050; US Patent Number 5,879,918; US Patent Numbers 5,886,244 and 5,932,782; Tomes, *et al.*, (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe, *et al.*, (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058). Also see, Weissinger, *et al.*, (1988) *Ann. Rev. Genet.* 22:421-477; Sanford, *et al.*, (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou, *et al.*, (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe, *et al.*, (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh, *et al.*, (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta, *et al.*, (1990) *Biotechnology* 8:736-740 (rice); Hoque, *et al.*, (2005) *Plant Cell Tissue & Organ Culture* 82(1):45-55 (rice); Sreekala, *et al.*, (2005) *Plant Cell Reports* 24(2):86-94 (rice); Klein, *et al.*, (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein, *et al.*, (1988) *Biotechnology* 6:559-563 (maize); US Patent Numbers 5,240,855; 5,322,783 and 5,324,646; Klein, *et al.*, (1988) *Plant Physiol.* 91:440-444 (maize); Fromm, *et al.*, (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren, *et al.*, (1984) *Nature* (London) 311:763-764; US Patent Number 5,736,369 (cereals); Bytebier, *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet, *et al.*, (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman, *et al.*, (Longman, New York), pp. 197-209 (pollen); Kaeppler, *et al.*, (1990) *Plant Cell Reports* 9:415-418 and Kaeppler, *et al.*, (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin, *et al.*, (1992) *Plant Cell* 4:1495-1505

(electroporation); Li, *et al.*, (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda, *et al.*, (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

In specific embodiments, the ZmSNAC sequences of the invention can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the ZmSNAC protein or variants and fragments thereof directly into the plant, or the introduction of a ZmSNAC transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway, *et al.*, (1986) *Mol Gen. Genet.* 202:179-185; Nomura, *et al.*, (1986) *Plant Sci.* 44:53-58; Hepler, *et al.*, (1994) *Proc. Natl. Acad. Sci.* 91:2176-2180 and Hush, *et al.*, (1994) *The Journal of Cell Science* 107:775-784, all of which are herein incorporated by reference. Alternatively, the ZmSNAC polynucleotide can be transiently transformed into the plant using other techniques known in the art. Such techniques include viral vector system and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, transcription from the particle-bound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethyleneimine (PEI; Sigma #P3143).

In other embodiments, the polynucleotide of the invention may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. It is recognized that a ZmSNAC polynucleotide of the invention may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that promoters useful for the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing polynucleotides into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, US Patent Numbers 5,889,191, 5,889,190, 5,866,785, 5,589,367, 5,316,931 and Porta, *et al.*, (1996) *Molecular Biotechnology* 5:209-221; herein incorporated by reference.

Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for example, WO 99/25821, WO 99/25854, WO 99/25840, WO 99/25855, and WO 99/25853, and US Patent Numbers 6,187,994; 6,552,248; 6,624,297; 6,331,661; 6,262,341; 6,541,231; 6,664,108; 6,300,545; 6,528,700 and 6,911,575, all of which are herein incorporated by reference. Briefly, the polynucleotide of the invention can be contained in a transfer cassette flanked by two non-recombinogenic recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its genome a target site which is flanked by two non-recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

The cells that have been transformed may be grown into plants in accordance with conventional means. See, for example, McCormick, *et al.*, (1986) *Plant Cell Reports* 5:81-84. These plants may then be pollinated with either the same transformed strain or different strains, and the resulting progeny having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure that expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a polynucleotide of the invention, for example, an expression cassette of the invention, stably incorporated into its genome.

Pedigree breeding starts with the crossing of two genotypes, such as an elite line of interest and one other inbred line having one or more desirable characteristics (i.e., having stably incorporated a polynucleotide of the invention, having a modulated activity and/or level of the polypeptide of the invention, etc) which complements the elite line of interest. If the two original parents do not provide all the desired characteristics, other sources can be included in the breeding population. In the pedigree method, superior plants are selfed and selected in successive filial generations. In the succeeding filial generations the

heterozygous condition gives way to homogeneous lines as a result of self-pollination and selection. Typically in the pedigree method of breeding, five or more successive filial generations of selfing and selection are practiced: F1 → F2; F2→ F3; F3 → F4; F4 → F_5, etc. After a sufficient amount of inbreeding, successive filial generations will serve to increase seed of the developed inbred. In specific embodiments, the inbred line comprises homozygous alleles at about 95% or more of its loci.

In addition to being used to create a backcross conversion, backcrossing can also be used in combination with pedigree breeding to modify an elite line of interest and a hybrid that is made using the modified elite line. Backcrossing can be used to transfer one or more specifically desirable traits from one line, the donor parent, to an inbred called the recurrent parent, which has overall good agronomic characteristics yet lacks that desirable trait or traits. However, the same procedure can be used to move the progeny toward the genotype of the recurrent parent but at the same time retain many components of the non-recurrent parent by stopping the backcrossing at an early stage and proceeding with selfing and selection. For example, an F1, such as a commercial hybrid, is created. This commercial hybrid may be backcrossed to one of its parent lines to create a BC1 or BC2. Progeny are selfed and selected so that the newly developed inbred has many of the attributes of the recurrent parent and yet several of the desired attributes of the non-recurrent parent. This approach leverages the value and strengths of the recurrent parent for use in new hybrids and breeding.

Therefore, an embodiment of this invention is a method of making a backcross conversion of a maize inbred line of interest, comprising the steps of crossing a plant of a maize inbred line of interest with a donor plant comprising a mutant gene or transgene conferring a desired trait (i.e., a modulation in the expression of a ZmSNAC polynucleotide, or any plant phenotype resulting from the modulated ZmSNAC expression level such as those phenotypes discussed elsewhere herein, including improved abiotic stress tolerance); selecting an F1 progeny plant comprising the mutant gene or transgene conferring the desired trait, and backcrossing the selected F1 progeny plant to a plant of the maize inbred line of interest. This method may further comprise the step of obtaining a molecular marker profile of the maize inbred line of interest and using the

molecular marker profile to select for a progeny plant with the desired trait and the molecular marker profile of the inbred line of interest. In the same manner, this method may be used to produce F1 hybrid seed by adding a final step of crossing the desired trait conversion of the maize inbred line of interest with a different maize plant to make F1 hybrid maize seed comprising a mutant gene or transgene conferring the desired trait.

Recurrent selection is a method used in a plant breeding program to improve a population of plants. The method entails individual plants cross pollinating with each other to form progeny. The progeny are grown and the superior progeny selected by any number of selection methods, which include individual plant, half-sib progeny, full-sib progeny, selfed progeny and topcrossing. The selected progeny are cross-pollinated with each other to form progeny for another population. This population is planted and again superior plants are selected to cross pollinate with each other. Recurrent selection is a cyclical process and therefore can be repeated as many times as desired. The objective of recurrent selection is to improve the traits of a population. The improved population can then be used as a source of breeding material to obtain inbred lines to be used in hybrids or used as parents for a synthetic cultivar. A synthetic cultivar is the resultant progeny formed by the intercrossing of several selected inbreds.

Mass selection is a useful technique when used in conjunction with molecular marker enhanced selection. In mass selection seeds from individuals are selected based on phenotype and/or genotype. These selected seeds are then bulked and used to grow the next generation. Bulk selection requires growing a population of plants in a bulk plot, allowing the plants to self-pollinate, harvesting the seed in bulk and then using a sample of the seed harvested in bulk to plant the next generation. Instead of self pollination, directed pollination could be used as part of the breeding program.

Mutation breeding is one of many methods that could be used to introduce new traits into an elite line. Mutations that occur spontaneously or are artificially induced can be useful sources of variability for a plant breeder. The goal of artificial mutagenesis is to increase the rate of mutation for a desired characteristic. Mutation rates can be increased by many different means including temperature, long-term seed storage, tissue culture conditions, radiation such as

X-rays, Gamma rays (e.g., cobalt 60 or cesium 137), neutrons, (product of nuclear fission by uranium 235 in an atomic reactor), Beta radiation (emitted from radioisotopes such as phosphorus 32 or carbon 14) or ultraviolet radiation (preferably from 2500 to 2900nm) or chemical mutagens (such as base analogues (5-bromo-uracil), related compounds (8-ethoxy caffeine), antibiotics (streptonigrin), alkylating agents (sulfur mustards, nitrogen mustards, epoxides, ethylenamines, sulfates, sulfonates, sulfones, lactones), azide, hydroxylamine, nitrous acid or acridines. Once a desired trait is observed through mutagenesis the trait may then be incorporated into existing germplasm by traditional breeding techniques, such as backcrossing. Details of mutation breeding can be found in "Principals of Cultivar Development," Fehr, 1993 Macmillan Publishing Company, the disclosure of which is incorporated herein by reference. In addition, mutations created in other lines may be used to produce a backcross conversion of elite lines that comprises such mutations.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn or maize (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats (*Avena sativa)*, barley (*Hordeum vulgare*), vegetables, ornamentals and conifers.

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

Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). In specific embodiments, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean plants are optimal, and in yet other embodiments corn plants are optimal.

Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as maize, wheat, barley, rice, sorghum, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the nucleic acid of interest can be isolated in significant quantities for introduction into the desired plant cells. In one embodiment, plant promoters that do not cause expression of the polypeptide in bacteria are employed.

Prokaryotes most frequently are represented by various strains of *E. coli;* however, other microbial strains may also be used. Commonly used prokaryotic

control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang, *et al.*, (1977) *Nature* 198:1056), the tryptophan (trp) promoter system (Goeddel, *et al.*, (1980) *Nucleic Acids Res.* 8:4057) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake, *et al.*, (1981) *Nature* 292:128). The inclusion of selection markers in DNA vectors transfected in *E coli.* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, (1983) *Gene* 22:229-235); Mosbach, *et al.*, (1983) *Nature* 302:543-545).

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra,* are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous polynucleotides in yeast is well known (Sherman, *et al.*, (1982) *Methods in Yeast Genetics,* Cold Spring Harbor Laboratory). Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris.* Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired. A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lists. The monitoring of the purification process can be accomplished by using

Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

The sequences of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, (1983) *J. Virol.* 45:773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors (Saveria-Campo (1985) *DNA Cloning Vol. II a Practical Approach,* D.M. Glover, Ed., IRL Press, Arlington, Virginia, pp. 213-238).

II. Modulating the Level and/or Activity of a ZmSNAC Polypeptide

A method for modulating the level and/or activity of the polypeptide of the present invention in a plant is provided. In general, the level and/or activity of the ZmSNAC polypeptide is increased or reduced by at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% or more, relative to a native control plant, plant part, or cell which does not comprise the introduced sequence. Modulation of the level and/or activity may occur at one or more stages of development. In specific embodiments, the polypeptides of the present invention are modulated in monocots, such as maize.

The expression level of the ZmSNAC polypeptide may be measured directly, for example, by assaying for the level of the ZmSNAC polypeptide in the plant, or indirectly, for example by measuring DNA binding activity and/or transgenic activation of gene expression in vitro or in vivo.

In specific embodiments, the polypeptide or the polynucleotide of the invention is introduced into the plant cell. Subsequently, a plant cell having the introduced sequence of the invention is selected using methods known to those of skill in the art such as, but not limited to, Southern blot analysis, DNA sequencing, PCR analysis, or phenotypic analysis. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time

sufficient to modulate the concentration and/or activity of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art and discussed briefly elsewhere herein.

It is also recognized that the level and/or activity of the polypeptide may be modulated by employing a polynucleotide that is not capable of directing, in a transformed plant, the expression of a protein or RNA. For example, the polynucleotides of the invention may be used to design polynucleotide constructs that can be employed in methods for altering or mutating a genomic nucleotide sequence in an organism. Such polynucleotide constructs include, but are not limited to, RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use are known in the art. See, US Patent Numbers 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972 and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham, *et al.*, (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; herein incorporated by reference.

It is therefore recognized that methods of the present invention do not depend on the incorporation of the entire polynucleotide into the genome, only that the plant or cell is altered as a result of the introduction of the polynucleotide into a cell. In one embodiment of the invention, the genome may be altered following the introduction of a polynucleotide into a cell. For example, the polynucleotide, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome include, but are not limited to, additions, deletions, and substitutions of nucleotides into the genome. While the methods of the present invention do not depend on additions, deletions, and substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprise at least one nucleotide.

It is further recognized that modulating the level and/or activity of the ZmSNAC sequence can be manipulated so as to occur only during certain developmental stages. Control of ZmSNAC expression can be obtained via the use of inducible promoters, tissue-preferred promoters, or promoters active exclusively or preferentially at one or more developmental stages. Alternatively,

the gene could be inverted or deleted using site-specific recombinases, transposons or recombination systems.

A "subject plant or plant cell" is one in which genetic alteration, such as transformation, has been effected as to a gene of interest, or is a plant or plant cell which is descended from a plant or cell so altered and which comprises the alteration. A "control" or "control plant" or "control cell" or "control plant cell" provides a reference point for measuring changes in phenotype of the subject plant or plant cell.

A control plant or plant cell may comprise, for example: (a) a wild-type plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (i.e. with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to conditions or stimuli that would induce expression of the gene of interest; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

In the present case, for example, changes in ZmSNAC transcription factor levels or activity could be measured by comparing performance of a subject plant or plant cell to a control plant or plant cell under drought or other abiotic stress conditions. In addition, changes in gene expression induced by ZmSNAC modulation could be assayed using gene-expression profiling technologies.

In certain embodiments the nucleic acid constructs of the present invention can be used in combination ("stacked") with other polynucleotide sequences of interest in order to create plants with a desired phenotype. The polynucleotides of the present invention may be stacked with any gene or combination of genes, and the combinations generated can include multiple copies of any one or more of the polynucleotides of interest. The desired combination may affect one or more traits; for example, the constructs and methods of the invention could be used in combination with other genes that confer tolerance to drought or other abiotic stresses, such as salt or heat stress, as well as with genes designed to enhance

yield. Other combinations may be designed to produce plants with a variety of desired traits, such as those described elsewhere herein.

A. Increasing the Activity and/or Level of a ZmSNAC Polypeptide

Methods are provided to increase the activity and/or level of a ZmSNAC polypeptide of the invention. An increase in the level of concentration and/or activity of a ZmSNAC polypeptide of the invention can be achieved by providing to the plant a ZmSNAC polypeptide. As discussed elsewhere herein, many methods are known in the art for providing a polypeptide to a plant including, but not limited to, direct introduction of the polypeptide into the plant, and introducing into the plant (transiently or stably) a polynucleotide construct encoding a polypeptide having SNAC transcription factor activity. Thus, the level and/or activity of a ZmSNAC polypeptide may be increased by altering the gene encoding the ZmSNAC polypeptide or its promoter. It is also recognized that the methods of the invention may employ a polynucleotide that is not capable of directing, in the transformed plant, the expression of a protein or RNA. See, e.g., Kmiec, US Patent Number 5,565,350; Zarling, *et al.*, PCT/US93/03868. Plants are provided which carry mutations affecting one or more ZmSNAC genes, wherein the mutations increase expression of at least one ZmSNAC gene or increase the level or activity of at least one encoded ZmSNAC polypeptide. As described elsewhere herein, methods to assay for an increase in level or activity of a ZmSNAC polypeptide are known.

B. Reducing the Activity and/or Level of a ZmNAC Polypeptide

Methods are provided to reduce or eliminate the activity and/or level (concentration) of a ZmSNAC polypeptide by transforming a plant cell with an expression cassette that expresses a polynucleotide that inhibits the expression of the ZmSNAC polypeptide. The polynucleotide may inhibit the expression of a ZmSNAC polypeptide directly, by preventing translation of the ZmSNAC polypeptide messenger RNA, or indirectly, by encoding a molecule that inhibits the transcription or translation of a gene encoding a ZmSNAC polypeptide. Methods for inhibiting or eliminating the expression of a gene in a plant are well known in the art, and any such method may be used in the present invention to inhibit the expression of the ZmSNAC polypeptides.

In accordance with the present invention, the expression of a ZmSNAC polypeptide is inhibited if the level of the ZmSNAC polypeptide is statistically lower than the level of the same ZmSNAC polypeptide in a plant that has not been genetically modified to inhibit the expression of that ZmSNAC polypeptide. In particular embodiments of the invention, the level or activity of the ZmSNAC polypeptide in a modified plant according to the invention is less than 95%, less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10% or less than 5% of the level or activity of the same ZmSNAC polypeptide in a control plant. The expression level of the ZmSNAC polypeptide may be measured directly, for example, by assaying for the level of the ZmSNAC polypeptide expressed in the cell or plant, or indirectly, for example, by measuring DNA binding activity or protein interaction in the cell or plant.

In other embodiments of the invention, the activity of one or more ZmSNAC polypeptides is reduced or eliminated by transforming a plant cell with an expression cassette comprising a polynucleotide encoding a polypeptide that inhibits the activity of one or more ZmSNAC polypeptides. The activity of a ZmSNAC polypeptide is inhibited according to the present invention if the transcription factor activity of the ZmSNAC polypeptide is statistically lower than the corresponding activity of the same ZmSNAC polypeptide in a control plant or cell. In particular embodiments of the invention, the activity of the ZmSNAC polypeptide in a modified plant according to the invention is less than 95%, less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% of the activity of the same ZmSNAC polypeptide in a control plant or cell. The activity of a ZmSNAC polypeptide is "eliminated" according to the invention when it is not detectable by the assay methods described elsewhere herein.

In other embodiments, the activity of a ZmSNAC polypeptide may be reduced or eliminated by disrupting the gene encoding the ZmSNAC polypeptide. The invention encompasses mutagenized plants that carry mutations in ZmSNAC genes, where the mutations reduce expression of the ZmSNAC gene or inhibit the activity of the encoded ZmSNAC polypeptide.

Thus, many methods may be used to reduce or eliminate the activity of a ZmSNAC polypeptide. More than one method may be used to reduce the activity

of a single ZmSNAC polypeptide. In addition, combinations of methods may be employed to reduce or eliminate the activity of two or more different ZmSNAC polypeptides.

Non-limiting examples of methods of reducing or eliminating the expression of a ZmSNAC polypeptide are given below.

1. Polynucleotide-Based Methods

In some embodiments of the present invention, a plant cell is transformed with an expression cassette that is capable of expressing a polynucleotide that inhibits the expression of a ZmSNAC sequence. The term "expression" as used herein refers to the biosynthesis of a gene product, including the transcription and/or translation of said gene product. For example, for the purposes of the present invention, an expression cassette capable of expressing a polynucleotide that inhibits the expression of at least one ZmSNAC sequence is an expression cassette capable of producing an RNA molecule that inhibits the transcription and/or translation of at least one ZmSNAC polypeptide. The "expression" or "production" of a protein or polypeptide from a DNA molecule refers to the transcription and translation of the coding sequence to produce the protein or polypeptide, while the "expression" or "production" of a protein or polypeptide from an RNA molecule refers to the translation of the RNA coding sequence to produce the protein or polypeptide.

Examples of polynucleotides that inhibit the expression of a ZmSNAC sequence are given below.

i. Sense Suppression/Cosuppression

In some embodiments of the invention, inhibition of the expression of a ZmSNAC polypeptide may be obtained by sense suppression or cosuppression. For cosuppression, an expression cassette is designed to express an RNA molecule corresponding to all or part of a messenger RNA encoding a ZmSNAC polypeptide in the "sense" orientation. Over expression of the RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the cosuppression expression cassette are screened to identify those that show the greatest inhibition of ZmSNAC polypeptide expression.

The polynucleotide used for cosuppression may correspond to all or part of the sequence encoding the ZmSNAC polypeptide, all or part of the 5' and/or 3' untranslated region of a ZmSNAC polypeptide transcript, or all or part of both the coding sequence and the untranslated regions of a transcript encoding a ZmSNAC polypeptide. In some embodiments where the polynucleotide comprises all or part of the coding region for the ZmSNAC polypeptide, the expression cassette is designed to eliminate the start codon of the polynucleotide so that no protein product will be transcribed.

Cosuppression may be used to inhibit the expression of plant genes to produce plants having undetectable protein levels for the proteins encoded by these genes. See, for example, Broin, *et al.*, (2002) *Plant Cell* 14:1417-1432. Cosuppression may also be used to inhibit the expression of multiple proteins in the same plant. See, for example, US Patent Number 5,942,657. Methods for using cosuppression to inhibit the expression of endogenous genes in plants are described in Flavell, *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:3490-3496; Jorgensen, *et al.*, (1996) *Plant Mol. Biol.* 31:957-973; Johansen and Carrington (2001) *Plant Physiol.* 126:930-938; Broin, *et al.*, (2002) *Plant Cell* 14:1417-1432; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731; Yu, *et al.*, (2003) *Phytochemistry* 63:753-763; and US Patent Numbers 5,034,323, 5,283,184 and 5,942,657; each of which is herein incorporated by reference. The efficiency of cosuppression may be increased by including a poly-dT region in the expression cassette at a position 3' to the sense sequence and 5' of the polyadenylation signal. See, US Patent Application Publication Number 2002/0048814, herein incorporated by reference. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, optimally greater than about 65% sequence identity, more optimally greater than about 85% sequence identity, most optimally greater than about 95% sequence identity. See, US Patent Numbers 5,283,184 and 5,034,323, herein incorporated by reference.

ii. Antisense Suppression

In some embodiments of the invention, inhibition of the expression of the ZmSNAC polypeptide may be obtained by antisense suppression. For antisense suppression, the expression cassette is designed to express an RNA molecule

complementary to all or part of a messenger RNA encoding the ZmSNAC polypeptide. Overexpression of the antisense RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the antisense suppression expression cassette are screened to identify those that show the desired inhibition of ZmSNAC polypeptide expression.

The polynucleotide for use in antisense suppression may correspond to all or part of the complement of the sequence encoding the ZmSNAC polypeptide, all or part of the complement of the 5' and/or 3' untranslated region of the ZmSNAC polypeptide transcript, or all or part of the complement of both the coding sequence and the untranslated regions of a transcript encoding the ZmSNAC polypeptide. In addition, the antisense polynucleotide may be fully complementary (i.e., 100% identical to the complement of the target sequence) or partially complementary (i.e., less than 100% identical to the complement of the target sequence) to the target sequence. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, US Patent Number 5,942,657. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, 300, 400, 450, 500, 550 or greater may be used. Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu, *et al.*, (2002) *Plant Physiol.* 129:1732-1743 and US Patent Numbers 5,759,829 and 5,942,657, each of which is herein incorporated by reference. Efficiency of antisense suppression may be increased by including a poly-dT region in the expression cassette at a position 3' to the antisense sequence and 5' of the polyadenylation signal. See, US Patent Application Publication Number 2002/0048814, herein incorporated by reference.

iii. Double-Stranded RNA Interference

In some embodiments of the invention, inhibition of the expression of a ZmSNAC polypeptide may be obtained by double-stranded RNA (dsRNA) interference. For dsRNA interference, a sense RNA molecule like that described above for cosuppression and an antisense RNA molecule that is fully or partially complementary to the sense RNA molecule are expressed in the same cell,

resulting in inhibition of the expression of the corresponding endogenous messenger RNA.

Expression of the sense and antisense molecules can be accomplished by designing the expression cassette to comprise both a sense sequence and an antisense sequence. Alternatively, separate expression cassettes may be used for the sense and antisense sequences. Multiple plant lines transformed with the dsRNA interference expression cassette or expression cassettes are then screened to identify plant lines that show the desired level of inhibition of ZmSNAC polypeptide expression. Methods for using dsRNA interference to inhibit the expression of endogenous plant genes are described in Waterhouse, *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964, Liu, *et al.*, (2002) *Plant Physiol.* 129:1732-1743, and WO 99/49029, WO 99/53050, WO 99/61631 and WO 00/49035; each of which is herein incorporated by reference.

iv. Hairpin RNA Interference and Intron-Containing Hairpin RNA Interference

In some embodiments of the invention, inhibition of the expression of one or more ZmSNAC polypeptides may be obtained by hairpin RNA (hpRNA) interference or intron-containing hairpin RNA (ihpRNA) interference. These methods are highly efficient at inhibiting the expression of endogenous genes. See, Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38 and the references cited therein.

For hpRNA interference, the expression cassette is designed to express an RNA molecule that hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem. The base-paired stem region comprises a sense sequence corresponding to all or part of the endogenous messenger RNA encoded by the gene whose expression is to be inhibited, and an antisense sequence that is fully or partially complementary to the sense sequence. Thus, the base-paired stem region of the molecule generally determines the specificity of the RNA interference. hpRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants. See, for example, Chuang and Meyerowitz, (2000) *Proc. Natl. Acad. Sci. USA* 97:4985-4990; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731 and Waterhouse

and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38. Methods for using hpRNA interference to inhibit or silence the expression of genes are described, for example, in Chuang and Meyerowitz, (2000) *Proc. Natl. Acad. Sci. USA* 97:4985-4990; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731; Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38; Pandolfini, *et al.*, *BMC Biotechnology* 3:7, and US Patent Application Publication Number 2003/0175965; each of which is herein incorporated by reference. A transient assay for the efficiency of hpRNA constructs to silence gene expression *in vivo* has been described by Panstruga, *et al.*, (2003) *Mol. Biol. Rep.* 30:135-140, herein incorporated by reference.

Alternatively, the base-paired stem region may correspond to a portion of a promoter sequence controlling expression of the gene to be inhibited. Transcriptional gene silencing (TGS) may be accomplished through use of hpRNA constructs wherein the inverted repeat of the hairpin shares sequence identity with the promoter region driving expression of a gene to be silenced. See, for example, US Patent Application Publication 2005/0246796. Processing of the hpRNA into short RNAs which can interact with the homologous promoter region may trigger degradation or methylation to result in silencing (Aufsatz, *et al.*, (2002) *PNAS* 99(4):16499-16506; Mette, *et al.*, (2000) *EMBO J* 19(19):5194-5201). In other embodiments, the hairpin inverted repeat is based on, and homologous to, the 3' untranslated region of the gene to be inhibited.

For ihpRNA, the interfering molecules have the same general structure as for hpRNA, but the RNA molecule additionally comprises an intron that is capable of being spliced in the cell in which the ihpRNA is expressed. The use of an intron minimizes the size of the loop in the hairpin RNA molecule following splicing, and this increases the efficiency of interference. See, for example, Smith, *et al.*, (2000) *Nature* 407:319-320. In fact, Smith, *et al.*, show 100% suppression of endogenous gene expression using ihpRNA-mediated interference. Methods for using ihpRNA interference to inhibit the expression of endogenous plant genes are described, for example, in Smith, *et al.*, (2000) *Nature* 407:319-320; Wesley, *et al.*, (2001) *Plant J.* 27:581-590; Wang and Waterhouse (2001) *Curr. Opin. Plant Biol.* 5:146-150; Waterhouse and Helliwell (2003) *Nat. Rev. Genet.* 4:29-38; Helliwell and Waterhouse, (2003) *Methods* 30:289-295, and US Patent Application Publication Number 2003/0180945, each of which is herein incorporated by reference.

The expression cassette for hpRNA interference may also be designed such that the sense sequence and the antisense sequence do not correspond to an endogenous RNA. In this embodiment, the sense and antisense sequence flank a loop sequence that comprises a nucleotide sequence corresponding to all or part of the endogenous messenger RNA of the target gene. Thus, it is the loop region that determines the specificity of the RNA interference. See, for example, WO 02/00904, herein incorporated by reference.

v. Amplicon-Mediated Interference

Amplicon expression cassettes comprise a plant virus-derived sequence that contains all or part of the target gene but generally not all of the genes of the native virus. The viral sequences present in the transcription product of the expression cassette allow the transcription product to direct its own replication. The transcripts produced by the amplicon may be either sense or antisense relative to the target sequence (i.e., the messenger RNA for ZmSNAC polypeptide). Methods of using amplicons to inhibit the expression of endogenous plant genes are described, for example, in Angell and Baulcombe (1997) *EMBO J.* 16:3675-3684, Angell and Baulcombe (1999) *Plant J.* 20:357-362, and US Patent Number 6,635,805, each of which is herein incorporated by reference.

vi. Ribozymes

In some embodiments, the polynucleotide expressed by the expression cassette of the invention is catalytic RNA or has ribozyme activity specific for the messenger RNA of a ZmSNAC polypeptide. Thus, the polynucleotide causes the degradation of the endogenous messenger RNA, resulting in reduced expression of the ZmSNAC polypeptide. This method is described, for example, in US Patent Number 4,987,071, herein incorporated by reference.

vii. Small Interfering RNA or Micro RNA

In some embodiments of the invention, inhibition of the expression of one or more ZmSNAC polypeptides may be obtained by RNA interference by expression of a gene encoding a micro RNA (miRNA). miRNAs are regulatory agents consisting of about 22 ribonucleotides. miRNA are highly efficient at inhibiting the

expression of endogenous genes. See, for example, Javier, *et al.*, (2003) *Nature* 425:257-263, herein incorporated by reference.

For miRNA interference, the expression cassette is designed to express an RNA molecule that is modeled on an endogenous miRNA gene. The miRNA gene encodes an RNA that forms a hairpin structure containing a 22-nucleotide sequence that is complementary to another endogenous gene (target sequence). For suppression of ZmSNAC polypeptide expression, the 22-nucleotide sequence is selected from a ZmSNAC polypeptide transcript sequence and contains 22 nucleotides encoding said ZmSNAC polypeptide sequence in sense orientation and 21 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence. miRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants.

2. Polypeptide-Based Inhibition of Gene Expression

In one embodiment, the polynucleotide encodes a zinc finger protein that binds to a gene encoding a ZmSNAC polypeptide, resulting in reduced expression of the gene. In particular embodiments, the zinc finger protein binds to a regulatory region of a ZmSNAC polypeptide gene. In other embodiments, the zinc finger protein binds to a messenger RNA encoding a ZmSNAC polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described, for example, in US Patent Number 6,453,242, and methods for using zinc finger proteins to inhibit the expression of genes in plants are described, for example, in US Patent Application Publication Number 2003/0037355; each of which is herein incorporated by reference.

3. Polypeptide-Based Inhibition of Protein Activity

In some embodiments of the invention, the polynucleotide encodes an antibody that binds to at least one ZmSNAC polypeptide, and reduces the activity of the ZmSNAC polypeptide. In another embodiment, the binding of the antibody results in increased turnover of the antibody-ZmSNAC polypeptide complex by cellular quality control mechanisms. The expression of antibodies in plant cells and the inhibition of molecular pathways by expression and binding of antibodies

to proteins in plant cells are well known in the art. See, for example, Conrad and Sonnewald (2003) *Nature Biotech.* 21:35-36, incorporated herein by reference.

4. Gene Disruption

In some embodiments of the present invention, the activity of a ZmSNAC polypeptide is reduced or eliminated by disrupting the gene encoding the ZmSNAC polypeptide. The gene encoding the ZmSNAC polypeptide may be disrupted by any method known in the art. For example, in one embodiment, the gene is disrupted by transposon tagging. In another embodiment, the gene is disrupted by mutagenizing plants using random or targeted mutagenesis, and selecting for plants that have reduced ZmSNAC activity. ZmSNAC down-regulated plants could be assayed for a decrease in expression of either ZmSNAC itself or of genes regulated by it. In addition, downregulation of ZmSNAC should have a measureable phenotype related to drought and yield.

i. Transposon Tagging

In one embodiment of the invention, the expression of one or more ZmSNAC polypeptides is reduced or eliminated by inserting a transposon within a regulatory region or coding region of the gene encoding the ZmSNAC polypeptide. A transposon may be inserted within an exon, intron, 5' or 3' untranslated sequence, a promoter, or any other regulatory sequence of a ZmSNAC polynucleotide. Methods for the transposon tagging of specific genes in plants are well known in the art. See, for example, Maes, *et al.*, (1999) *Trends Plant Sci.* 4:90-96; Dharmapuri and Sonti (1999) *FEMS Microbiol. Lett*. 179:53-59; Meissner, *et al.*, (2000) *Plant J.* 22:265-274; Phogat, *et al.*, (2000) *J. Biosci.* 25:57-63; Walbot (2000) *Curr. Opin. Plant Biol.* 2:103-107; Gai, *et al.*, (2000) *Nucleic Acids Res.* 28:94-96; Fitzmaurice, *et al.*, (1999) *Genetics* 153:1919-1928). In addition, the TUSC process for selecting Mu insertions in selected genes has been described in Bensen, *et al.*, (1995) *Plant Cell* 7:75-84; Mena, *et al.*, (1996) *Science* 274:1537-1540; and US Patent Number 5,962,764; each of which is herein incorporated by reference.

ii. Mutant Plants with Reduced Activity

Additional methods for decreasing or eliminating the expression of endogenous genes in plants are also known in the art and can be similarly applied to the instant invention. These methods include other forms of mutagenesis, such as ethyl methanesulfonate-induced mutagenesis, deletion mutagenesis, and fast neutron deletion mutagenesis used in a reverse genetics sense (with PCR) to identify plant lines in which the endogenous gene has been deleted. For examples of these methods see Ohshima, *et al.*, (1998) *Virology* 243:472-481; Okubara, *et al.*, (1994) *Genetics* 137:867-874; and Quesada, *et al.*, (2000) *Genetics* 154:421-436; each of which is herein incorporated by reference. In addition, a fast and automatable method for screening for chemically induced mutations, TILLING (Targeting Induced Local Lesions In Genomes), using denaturing HPLC or selective endonuclease digestion of selected PCR products, is also applicable to the instant invention. See, McCallum, *et al.*, (2000) *Nat. Biotechnol.* 18:455-457, herein incorporated by reference.

Mutations that impact gene expression or that interfere with the function of the encoded protein are well known in the art. Insertional mutations in gene exons usually result in null mutants. Mutations in conserved residues are particularly effective in inhibiting the activity of the encoded protein. Conserved residues of NAC polypeptides have been described (Ernst, *et al.*, (2004) *EMBO Reports* 5(3):297-303) and provide a basis for designing mutations with the goal of eliminating the ZmSNAC transcription factor activity. See, for example, Figure 1. Such mutants can be isolated according to well-known procedures, and mutations in different ZmSNAC loci can be stacked by genetic crossing. See, for example, Gruis, *et al.*, (2002) *Plant Cell* 14:2863-2882.

In another embodiment of this invention, dominant mutants can be used to trigger RNA silencing due to gene inversion and recombination of a duplicated gene locus. See, for example, Kusaba, *et al.*, (2003) *Plant Cell* 15:1455-1467.

The invention encompasses additional methods for reducing or eliminating the activity of one or more ZmSNAC polypeptides. Examples of other methods for altering or mutating a genomic nucleotide sequence in a plant are known in the art and include, but are not limited to, the use of RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides, and recombinogenic

oligonucleobases. Such vectors and methods of use are known in the art. See, for example, US Patent Numbers 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972 and 5,871,984; each of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham, *et al.*, (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; each of which is herein incorporated by reference.

III. Modulating ZmSNAC Transcription Factor Level and/or Activity

"Modulating ZmSNAC transcription factor level and/or activity" includes any statistically significant decrease or increase in level and/or activity of said factor in the plant when compared to a control plant. The modulated level and/or activity of the ZmSNAC transcription factor can comprise either an increase or a decrease of about 0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more when compared to a control. It is further recognized that the modulation of the ZmSNAC level/activity need not be an overall increase/decrease in ZmSNAC level and/or activity, but may comprise a change in tissue distribution of the transcription factor activity. Moreover, the modulation of the transcription factor level/activity need not be an overall increase/decrease, but also includes a change in the ratio of activity of various transcription factors.

Methods for assaying a modulation in transcription factor level and/or activity are known in the art. As discussed elsewhere herein, modulation in ZmSNAC level and/or activity can further be detected by monitoring for particular plant phenotypes. Because ZmSNAC genes regulate the expression of other genes, modulation of a ZmSNAC gene should be measurable by changes in target gene expression.

In specific methods, the level and/or activity of a ZmSNAC transcription factor in a plant is increased by increasing the level or activity of the ZmSNAC polypeptide in the plant. Methods for increasing the level and/or activity of ZmSNAC polypeptides in a plant are discussed elsewhere herein. In certain embodiments, a ZmSNAC nucleotide sequence encoding a ZmSNAC polypeptide can be provided by introducing into the plant a polynucleotide comprising a ZmSNAC nucleotide sequence of the invention, expressing the ZmSNAC sequence, and thereby increasing the level and/or activity of said transcription

factor in the plant or plant part when compared to a control. In some embodiments, the ZmSNAC nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

In other methods, the level and/or activity of ZmSNAC transcription factors in a plant is decreased by decreasing the level and/or activity of one or more of the ZmSNAC polypeptides in the plant. Such methods are disclosed in detail elsewhere herein. In one such method, a ZmSNAC nucleotide sequence is introduced into the plant and expression of the ZmSNAC nucleotide sequence decreases the level and/or activity of the ZmSNAC polypeptide in the plant or plant part when compared to a control plant or plant part. In certain embodiments, the nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate the level/activity of a ZmSNAC transcription factor in the plant. Exemplary promoters for this embodiment have been disclosed elsewhere herein.

Accordingly, the present invention further provides plants having a modulated level/activity of a ZmSNAC transcription factor when compared to said level/activity in a control plant. In one embodiment, the plant of the invention has an increased level/activity of the ZmSNAC polypeptide of the invention. In other embodiments, the plant of the invention has a reduced or eliminated level of the ZmSNAC polypeptide of the invention. In certain embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising a ZmSNAC nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

A. Modulating Root Development

Modulation of the level/activity of a ZmSNAC transcription factor may result in modulated root development when compared to a control plant. Such alterations in root development include, but are not limited to, alterations in the size or growth rate of the primary root, the fresh root weight, the extent of lateral and adventitious root formation, the vasculature system, meristem development and radial expansion. Methods of measuring such developmental alterations in the root system are known in the art. See, for example, US Patent Application Publication

Number 2003/0074698 and Werner, *et al.*, (2001) *PNAS* 18:10487-10492, both of which are herein incorporated by reference.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate root development in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters. Exemplary root-preferred promoters have been disclosed elsewhere herein.

Modulated root development may also impact the standability of a plant. The term "resistance to lodging" or "standability" refers to the ability of a plant to fix itself to the soil. For plants with an erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, depth and morphology of the root system. In addition, stimulating root growth and increasing root mass at appropriate developmental stages also finds use in promoting *in vitro* propagation of explants.

Increased root biomass and/or altered root architecture may also find use in improving nitrogen-use efficiency of the plant. Such improved efficiency may lead to, for example, an increase in plant biomass and/or seed yield at an existing level of available nitrogen, or maintenance of plant biomass and/or seed yield when available nitrogen is limited. Thus, agronomic and/or environmental benefits may ensue.

Furthermore, higher root biomass production has an indirect effect on production of compounds produced by root cells or transgenic root cells or cell cultures of said transgenic root cells. One example of an interesting compound produced in root cultures is shikonin, the yield of which can be advantageously enhanced by said methods.

B. Modulating Shoot and Leaf Development

Methods are also provided for modulating vegetative tissue growth in plants. In one embodiment, shoot and/or leaf development in a plant is modulated when compared to a control plant or plant part. Such alterations in shoot and/or leaf development include, but are not limited to, alterations in shoot meristem development, in leaf number, leaf size, leaf and stem vasculature, internode length, and leaf senescence. As used herein, "leaf development" and "shoot development" encompasses all aspects of growth of the different parts that make

up the leaf system and the shoot system, respectively, at different stages of their development, both in monocotyledonous and dicotyledonous plants. Methods for measuring such developmental alterations in the shoot and leaf system are known in the art. See, for example, Werner, *et al.*, (2001) *PNAS* 98:10487-10492 and US Patent Application Publication Number 2003/0074698, each of which is herein incorporated by reference.

The method for modulating shoot and/or leaf development in a plant comprises modulating the activity and/or level of a ZmSNAC polypeptide of the invention. In one embodiment, a ZmSNAC sequence of the invention is provided. In other embodiments, the ZmSNAC nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising a ZmSNAC nucleotide sequence of the invention, expressing the ZmSNAC sequence, and thereby modifying shoot and/or leaf development. In certain embodiments, the ZmSNAC nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate shoot and leaf development of the plant. Exemplary promoters for this embodiment include constitutive promoters, shoot-preferred promoters, shoot meristem-preferred promoters, senescence-activated promoters, stress-induced promoters, nitrogen-induced promoters and leaf-preferred promoters. Exemplary promoters have been disclosed elsewhere herein.

Decreasing ZmSNAC activity in a plant generally results in shorter internodes and stunted growth. Thus, the methods of the invention find use in producing dwarf plants. In addition, as discussed above, modulation of ZmSNAC activity in the plant may modulate both root and shoot growth. Thus, the present invention further provides methods for altering the root/shoot ratio.

It is further recognized that increasing seed size and/or weight can be accompanied by an increase in the rate of growth of seedlings or an increase in vigor. In addition, modulating the plant's tolerance to stress, as discussed elsewhere herein, along with modulation of root, shoot and leaf development, can increase plant yield and vigor. As used herein, the term "vigor" refers to the relative health, productivity, and rate of growth of the plant and/or of certain plant parts, and may be reflected in various developmental attributes, including, but not limited to, concentration of chlorophyll, photosynthetic rate, total biomass, root

biomass, grain quality, and/or grain yield. In *Zea mays* in particular, vigor may also be reflected in ear growth rate, ear size, and/or rate or degree of silk exsertion. Vigor may relate to the ability of a plant to grow rapidly during early development and to the successful establishment of a well-developed root system and a well-developed photosynthetic apparatus. Vigor may be determined with reference to different genotypes under similar environmental conditions, or with reference to the same or different genotypes under different environmental conditions.

Accordingly, the present invention further provides plants having modulated shoot and/or leaf development when compared to a control plant. In some embodiments, the plant of the invention has an increased level/activity of a ZmSNAC polypeptide of the invention. In other embodiments, the plant of the invention has a decreased level/activity of the ZmSNAC polypeptide of the invention.

C. Modulating Reproductive Tissue Development

Methods for modulating reproductive tissue development are provided. In one embodiment, methods are provided to modulate floral development in a plant. By "modulating floral development" is intended any alteration in a structure of a plant's reproductive tissue as compared to a control plant or plant part. "Modulating floral development" further includes any alteration in the timing of the development of a plant's reproductive tissue (i.e., delayed or accelerated floral development) when compared to a control plant or plant part. Macroscopic alterations may include changes in size, shape, number, or location of reproductive organs, the developmental time period during which these structures form, or the ability to maintain or proceed through the flowering process in times of environmental stress. Microscopic alterations may include changes to the types or shapes of cells that make up the reproductive organs.

The method for modulating floral development in a plant comprises modulating (either increasing or decreasing) the level and/or activity of the ZmSNAC polypeptide in a plant. In one method, a ZmSNAC sequence of the invention is provided. A ZmSNAC nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising a ZmSNAC nucleotide sequence of the invention, expressing the ZmSNAC sequence, and thereby

modifying floral development. In some embodiments, the ZmSNAC nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate floral development in the plant. Exemplary promoters for this embodiment include constitutive promoters, inducible promoters, shoot-preferred promoters, and inflorescence-preferred promoters (including developing-female-inflorescence-preferred promoters), including those listed elsewhere herein.

Accordingly, the present invention further provides plants having modulated floral development when compared to the floral development of a control plant. Compositions include plants with a modulated level/activity of one or more ZmSNAC polypeptides of the invention and having an altered floral development, which may include the capacity to sustain normal reproductive development under abiotic stress conditions.

D. Modulating the Stress Tolerance of a Plant

Methods are provided for the use of the ZmSNAC sequences of the invention to modify the tolerance of a plant to abiotic stress. Promoters that can be used in this method are described elsewhere herein, including low-level constitutive, stress-insensitive, or inducible, particularly stress-inducible, promoters. Accordingly, in one method of the invention, a plant's tolerance to stress is increased or maintained when compared to a control plant by introducing into the plant a polynucleotide comprising a ZmSNAC nucleotide sequence of the invention, expressing the ZmSNAC sequence, and thereby increasing the plant's tolerance to stress. In certain embodiments, the ZmSNAC nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

Increased growth of seedlings or early vigor is often associated with an increase in stress tolerance. For example, faster development of seedlings, including the root system of seedlings upon germination, is critical for survival, particularly under adverse conditions such as drought.

Methods are also provided to increase or maintain seed set during abiotic stress episodes. During periods of stress (i.e., drought, salt, heavy metals, temperature, etc.) embryo development is often aborted. In maize, halted embryo development results in aborted kernels on the ear (Cheikh and Jones, (1994)

Plant Physiol. 106:45-51; Dietrich, *et al.*, (1995) *Plant Physiol Biochem* 33:327-336). In soy, abortion of pods prior to seed maturation can reduce seed yield and is observed during both optimal and stress conditions. Preventing this seed loss will maintain yield. Accordingly, methods are provided to increase the stress resistance in a plant (e.g., during flowering and seed development). Increasing expression of the ZmSNAC sequence of the invention can modulate floral development during periods of stress, and thus methods are provided to maintain or improve the flowering process in plants under stress. The method comprises increasing the level and/or activity of one or more of the ZmSNAC sequences of the invention. In one method, a ZmSNAC nucleotide sequence is introduced into the plant and the level and/or activity of the ZmSNAC polypeptide is increased, thereby maintaining or improving the tolerance of the plant under stress conditions. In certain methods, the ZmSNAC nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

Significant yield instability can occur as a result of unfavorable environments during the lag phase of seed development. During this period, seeds undergo dramatic changes in ultra structure, biochemistry, and sensitivity to environmental perturbation, yet demonstrate little change in dry mass accumulation. Two important events that occur during the lag phase are initiation and division of endosperm cells and amyloplasts (which are the sites for starch deposition). In crop species such as maize, kernel sink capacity is principally a function of the number of endosperm cells and starch granules established during the first 6 to 12 days after pollination. The final number of endosperm cells and amyloplasts formed is highly correlated with final kernel weight. (Capitanio, *et al.*, (1983); Reddy and Daynard, (1983); Jones, *et al.*, (1985) (1996); Engelen-Eigles, *et al.*, (2000)).

In this embodiment, a variety of promoters could be used to direct the expression of a sequence capable of increasing the level and/or activity of the ZmSNAC polypeptide, including but not limited to, constitutive promoters, seed-preferred promoters, developing-seed promoters, meristem-preferred promoters, stress-induced promoters, and inflorescence-preferred (such as developing female inflorescence promoters). In one method, a promoter that is stress-inducible and is expressed in a tissue of the developing seed during the lag phase of development is used. By “lag phase” promoter is intended a promoter that is

active in the lag phase of seed development. A description of this developmental phase is found elsewhere herein. By "developing-seed-preferred" is intended a promoter that allows for enhanced ZmSNAC expression within a developing seed. Such promoters that are stress insensitive and are expressed in a tissue of the developing seed during the lag phase of development are known in the art and include *Zag2.1* (Theissen, *et al.*, (1995) *Gene* 156:155-166, Genbank Accession Number X80206), and *mzE40 (Zm40)* (US Patent Number 6,403,862 and WO01/2178).

Methods to assay for an increase in seed set during abiotic stress are known in the art. For example, plants having the increased ZmSNAC activity can be monitored under various stress conditions and compared to control plants. For instance, the plant having the increased ZmSNAC transcription factor activity can be subjected to various degrees of stress during flowering and seed set. Under identical conditions, the genetically modified plant having the increased ZmSNAC transcription factor activity will have a higher number of developing pods and/or seeds than a control plant.

Accordingly, the present invention further provides plants having increased yield or a maintained yield and/or an increased or maintained flowering process during periods of abiotic stress (for example, drought, salt, heavy metals, temperature extremes). In some embodiments, the plants having an increased or maintained yield during abiotic stress have an increased level/activity of the ZmSNAC polypeptide of the invention. In some embodiments, the plant comprises a ZmSNAC nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell. In some embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising a ZmSNAC nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

IV. Antibody Creation and Use

Antibodies can be raised to a protein of the present invention, including variants and fragments thereof, in both their naturally-occurring and recombinant forms. Many methods of making antibodies are known to persons of skill. A variety of analytic methods are available to generate a hydrophilicity profile of a protein of the present invention. Such methods can be used to guide the artisan in

the selection of peptides of the present invention for use in the generation or selection of antibodies which are specifically reactive, under immunogenic conditions, to a protein of the present invention. See, e.g., Janin, (1979) *Nature*, 277:491-492; Wolfenden, *et al.*, (1981) B*iochemistry* 208:49-855; Kyte and Doolite, (1982) *J. Mol Biol.* 157:105-132; Rose, *et al.*, (1985) *Science* 229:834-838. The antibodies can be used to screen expression libraries for particular expression products such as normal or abnormal protein, or altered levels of the same, which may be useful for detecting or diagnosing various conditions related to the presence of the respective antigens. Assays indicating high levels of a ZmSNAC protein of the invention, for example, could be useful in detecting plants, or specific plant parts, with elevated ZmSNAC transcription factor levels. Usually the antibodies in such a procedure are labeled with a moiety which allows easy detection of presence of antigen/antibody binding.

The ZmSNAC1 regulatory element will be operably linked to a sequence of interest, which will provide for modification of the phenotype of the plant. Such modification includes modulating the production of an endogenous product, as to amount, relative distribution, or the like, or for providing a novel function or expression product. For example, such a promoter is useful for modulation of expression of sequences encoding stress-responsive proteins, including other transcription factors. Additionally, linking a stress-induced promoter with a marker, and, in particular, a visual marker, may be useful in tracking the expression of a linked gene of interest. Commonly used genes for screening presumptively transformed cells include β-glucuronidase *(GUS),* β-galactosidase, luciferase and chloramphenicol acetyltransferase. Jefferson, (1987) *Plant Mol. Biol. Rep.* 5:387 and US Patent Number 5,599,670; Teeri, *et al.*,(1989) *EMBO J.* 8:343, Koncz, *et al.*, (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:131, De Block, *et al.*, (1984) *EMBO J.* 3:1681. Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig, *et al.*, (1990) *Science* 247:449.

A method for expressing an isolated nucleotide sequence in a plant using the regulatory sequences disclosed herein is provided. The method comprises transforming a plant cell with a transformation vector that comprises an isolated nucleotide sequence operably linked to a plant regulatory sequence of the present

invention and regenerating a stably transformed plant from the transformed plant cell. In this manner, the regulatory sequences are useful for controlling the expression of endogenous as well as exogenous products in a stress-induced manner.

Frequently it is desirable to have preferential expression of a DNA sequence in a tissue of an organism, or under certain environmental conditions. For example, increased resistance of a plant to insect attack might be accomplished by genetic manipulation of the plant's genome to comprise a tissue-specific promoter operably linked to a heterologous insecticide gene such that the insect-deterring substances are specifically expressed in the susceptible plant tissues. Increased tolerance to abiotic stress might be accomplished by genetic manipulation of the plant's genome to comprise a stress-induced promoter operably linked to a heterologous gene encoding a biosynthetic or regulatory gene for a plant hormone such that the hormone is specifically synthesized or its synthesis is regulated under the stress conditions. Preferential expression of the heterologous nucleotide sequence in the appropriate tissue or under the appropriate conditions reduces the drain on the plant's resources that occurs when a constitutive promoter initiates transcription of a heterologous nucleotide sequence throughout the cells of the plant and/or under all conditions.

Alternatively, it might be desirable to inhibit expression of a native DNA sequence within a plant's tissues to achieve a desired phenotype. For example, a hairpin configuration comprising all or a portion of a ZmSNAC1 promoter may be used to downregulate the native stress-responsive ZmSNAC1.

By "regulatory element" is intended sequences responsible for expression of the associated coding sequence including, but not limited to, promoters, terminators, enhancers, introns, and the like.

By "terminator" is intended a regulatory region of DNA that causes RNA polymerase to disassociate from DNA, causing termination of transcription.

By “promoter” is intended a regulatory region of DNA capable of regulating the transcription of a sequence linked thereto. It 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 may additionally comprise other recognition sequences generally positioned upstream or 5’ to the TATA box, referred to as upstream

promoter elements, which influence the transcription initiation rate and further include elements which impact spatial and temporal expression of the linked nucleotide sequence. 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 5' region upstream from the particular promoter region identified herein. Thus the promoter region disclosed herein may comprise upstream regulatory elements such as those responsible for tissue and temporal expression of the coding sequence, and may include enhancers, the DNA response element for a transcriptional regulatory protein, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, activator sequence and the like.

In the same manner, the promoter elements which enable expression under stress conditions can be identified, isolated, and used with other core promoters. 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 upstream promoter of ZmSNAC1 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.

The isolated promoter sequence of the present invention can be modified to provide for a range of expression levels of the isolated nucleotide sequence. Less than the entire promoter region can be utilized and the ability to drive stress-induced expression retained. It is recognized that expression levels of mRNA can be modulated with specific deletions of portions of the promoter sequence. Thus, the promoter can be modified to be a weak or strong promoter. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts. Generally, at least about 20 nucleotides of an isolated promoter sequence will be used to drive expression of a nucleotide sequence.

It is recognized that to increase transcription levels, enhancers can be utilized in combination with the promoter regions of the invention. Enhancers are nucleotide sequences that act to increase the expression of a promoter region.

Enhancers are known in the art and include the SV40 enhancer region, the 35S enhancer element, and the like.

The promoter of the present invention can be isolated from the 5' region of its native coding region or 5' untranslated region (5' UTR). Likewise the terminator can be isolated from the 3' region flanking its respective stop codon. The term "isolated" refers to material, such as a nucleic acid or protein, (1) which is substantially or essentially free from components which normally accompany or interact with the material as found in its naturally occurring environment; or (2) if the material is in its natural environment, the material has been altered by deliberate human intervention to a composition and/or placed at a locus in a cell other than the locus native to the material. Methods for isolation of promoter regions are well known in the art.

The Zea mays SNAC1 promoter is set forth in SEQ ID NO: 11 and is 1625 nucleotides in length.

The promoter regions from genes homologous to ZmSNAC1 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 and sorghum.

Promoter sequences from other plants may be isolated according to well-known techniques based on sequence homology. In these techniques, all or part of the known coding sequence is used as a probe which selectively hybridizes to other sequences present in a population of cloned genomic DNA fragments (i.e., genomic libraries) from a chosen organism. Methods are readily available in the art for the hybridization of nucleic acid sequences. An extensive guide to the

hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

"Functional variants" of the regulatory sequences are also encompassed by the compositions of the present invention. Functional variants include, for example, the native regulatory sequences of the invention having one or more nucleotide substitutions, deletions or insertions and which drive expression of an operably-linked sequence under conditions similar to those under which the native promoter is active. Functional variants of the invention may be created by site-directed mutagenesis, induced mutation, or may occur as allelic variants (polymorphisms).

As used herein, a "functional fragment" is a truncated regulatory sequence formed by one or more deletions from a larger regulatory element. 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, *et al.*, (2004) “Identification of a 49-bp fragment of the HvLTP2 promoter directing aleruone cell specific expression” *Gene* 341:49-58. Such fragments should retain promoter activity, particularly the ability to drive stress-induced expression. 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.

Functional fragments can be obtained by use of restriction enzymes to cleave the naturally occurring regulatory element nucleotide sequences disclosed herein; by synthesizing a nucleotide sequence from the naturally occurring DNA sequence; or can be obtained through the use of PCR technology. See particularly, Mullis, *et al.*, (1987) *Methods Enzymol.* 155:335-350, and Erlich, ed. (1989) PCR Technology (Stockton Press, New York).

For example, a routine way to remove part of a DNA sequence is to use an exonuclease in combination with DNA amplification to produce unidirectional

nested deletions of double stranded DNA clones. A commercial kit for this purpose is sold under the trade name Exo-Size™ (New England Biolabs, Beverly, Mass.). Briefly, this procedure entails incubating exonuclease III with DNA to progressively remove nucleotides in the 3' to 5' direction at 5' overhangs, blunt ends or nicks in the DNA template. However, exonuclease III is unable to remove nucleotides at 3', 4-base overhangs. Timed digests of a clone with this enzyme produces unidirectional nested deletions.

The entire promoter sequence or portions thereof can be used as a probe capable of specifically hybridizing to corresponding promoter sequences in genomic DNA. Alternatively, the probe represents a fragment of the coding sequence natively associated with the promoter sequence. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes can be used to amplify corresponding sequences from a chosen organism by the well-known process of polymerase chain reaction (PCR). This technique can be used to isolate additional promoter sequences from a desired organism or as a diagnostic assay to determine the presence of the promoter sequence in an organism. Examples include hybridization screening of plated DNA libraries (either plaques or colonies; see, e.g., Innis, *et al.*, (1990) PCR Protocols, A Guide to Methods and Applications, eds., Academic Press).

The regulatory elements disclosed in the present invention, as well as variants and fragments thereof, are useful in the genetic manipulation of any plant when operably linked with an isolated nucleotide sequence of interest whose expression is to be controlled to achieve a desired phenotypic response.

By “operably linked” is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. The expression cassette will include a regulatory sequence of the invention operably linked to at least one sequence of interest.

In one typical embodiment, in the context of an over expression cassette, operably linked means that the nucleotide sequences being linked are contiguous and, where necessary to join two or more protein coding regions, contiguous and in the same reading frame. In the case where an expression cassette contains

two or more protein coding regions joined in a contiguous manner in the same reading frame, the encoded polypeptide is herein defined as a "chimeric polypeptide" or a "fusion polypeptide". The cassette may additionally contain at least one additional coding sequence to be co-transformed into the organism. Alternatively, the additional coding sequence(s) can be provided on multiple expression cassettes.

The regulatory elements of the invention can be operably linked to the isolated nucleotide sequence of interest in any of several ways known to one of skill in the art. The isolated nucleotide sequence of interest can be inserted into a site within the genome which is 3' to the promoter of the invention using site specific integration as described in US Patent Number 6,187,994, herein incorporated in its entirety by reference.

The regulatory elements of the invention can be operably linked in expression cassettes along with isolated nucleotide sequences of interest for expression in the plant. Such an expression cassette is provided with a plurality of restriction sites for insertion of the nucleotide sequence of interest under the transcriptional control of the regulatory elements.

The isolated nucleotides of interest expressed by the regulatory elements of the invention can be used for directing expression of a sequence in plant tissues. This can be achieved by increasing expression of endogenous or exogenous products. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors. This down regulation can be achieved through many different approaches known to one skilled in the art, including antisense, cosuppression, use of hairpin formations, or others, and discussed infra. It is recognized that the regulatory elements may be used with their native or other coding sequences to increase or decrease expression of an operably linked sequence in the transformed plant or seed.

General categories of genes of interest for the purposes of the present invention include for example, those genes involved in information, such as zinc fingers; those involved in communication, such as kinases; and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, and grain characteristics.

Still other categories of transgenes include genes for inducing synthesis of exogenous products such as enzymes, cofactors, and hormones from plants and other eukaryotes as well as prokaryotic organisms.

Modifications that affect grain traits include increasing the content of oleic acid, or altering levels of saturated and unsaturated fatty acids. Likewise, the level of proteins, particularly modified proteins that improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

Increasing the levels of lysine and sulfur-containing amino acids may be desired as well as the modification of starch type and content in the seed. Hordothionin protein modifications are described in WO 9416078 filed April 10, 1997; WO 9638562 filed March 26, 1997; WO 9638563 filed March 26, 1997 and US Patent Number 5,703,049 issued December 30, 1997. Another example is lysine and/or sulfur-rich root protein encoded by the soybean 2S albumin described in WO 9735023 filed March 20, 1996, and the chymotrypsin inhibitor from barley, Williamson, *et al.*, (1987) *Eur. J. Biochem.* 165:99-106.

Agronomic traits can be improved by altering expression of genes that: affect the response of root, plant or seed growth and development during environmental stress, Cheikh-N, *et al.*, (1994) *Plant Physiol.* 106(1):45-51, and genes controlling carbohydrate metabolism to reduce kernel abortion in maize, Zinselmeier, *et al.*, (1995) *Plant Physiol.* 107(2):385-391.

It is recognized that any gene of interest, including the native coding sequence, can be operably linked to the regulatory elements of the invention and expressed in the plant.

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

EXPERIMENTAL

Example 1: Maize transformation

For example, immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a sequence, for example ZmSNAC1, operably linked to a constitutive promoter and further containing a selectable marker gene. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

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

A plasmid vector comprising the ZmSNAC sequence operably linked to a constitutive promoter is made. This plasmid DNA plus plasmid DNA containing a selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows: 100 µl prepared tungsten particles in water; 10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA); 100 µl 2.5 M $CaC1_2$; and, 10 µl 0.1 M spermidine.

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

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

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred

to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for the maintenance or increase of seed set during an abiotic stress episode.

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_2O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite® gelling agent (added after bringing to volume with D-I H_2O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_2O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite® gelling agent (added after bringing to volume with D-I H_2O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_2O) (Murashige and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_2O after adjusting to pH 5.6); 3.0 g/l Gelrite® gelling agent (added after bringing to volume with D-I H_2O); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_2O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_2O after adjusting pH to 5.6); and 6 g/l bacto™-agar (added after bringing to volume with polished D-I H_2O), sterilized and cooled to 60°C.

Example 2: Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing the ZmSNAC1 polynucleotide operably linked to a constitutive promoter as follows. To induce

somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein, *et al.*, (1987) *Nature* (London) 327:70-73, US Patent Number 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell, *et al.*, (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz, *et al.*, (1983) *Gene* 25:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the ZmSNAC sequence as described above can be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of

tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Example 3: Rice Transformation

One method for transforming DNA into cells of higher plants that is available to those skilled in the art is high-velocity ballistic bombardment using metal particles coated with the nucleic acid constructs of interest (see, Klein, *et al.*, *Nature* (1987) (London) 327:70-73, and see, US Patent Number 4,945,050). A Biolistic PDS-1000/He (BioRAD Laboratories, Hercules, CA) is used for these complementation experiments.

The bacterial hygromycin B phosphotransferase (Hpt II) gene from *Streptomyces hygroscopicus* that confers resistance to the antibiotic may be used as the selectable marker for rice transformation. In the vector, the Hpt II gene may be engineered with the 35S promoter from Cauliflower Mosaic Virus and the termination and polyadenylation signals from the octopine synthase gene of Agrobacterium tumefaciens. For example, see the description of vector pML18 in WO 97/47731, published on December 18, 1997, the disclosure of which is hereby incorporated by reference.

Embryogenic callus cultures derived from the scutellum of germinating rice seeds serve as source material for transformation experiments. This material is

generated by germinating sterile rice seeds on a callus initiation media (MS salts, Nitsch and Nitsch vitamins, 1.0 mg/l 2,4-D and 10 μM $AgNO_3$) in the dark at 27-28°C. Embryogenic callus proliferating from the scutellum of the embryos is transferred to CM media (N6 salts, Nitsch and Nitsch vitamins, 1 mg/l 2,4-D, Chu, *et al.*, (1985) *Sci. Sinica* 18:659-668). Callus cultures are maintained on CM by routine sub-culture at two-week intervals and used for transformation within 10 weeks of initiation.

Callus is prepared for transformation by subculturing 0.5-1.0 mm pieces approximately 1 mm apart, arranged in a circular area of about 4 cm in diameter, in the center of a circle of Whatman® #541 paper placed on CM media. The plates with callus are incubated in the dark at 27-28°C for 3-5 days. Prior to bombardment, the filters with callus are transferred to CM supplemented with 0.25 M mannitol and 0.25 M sorbitol for 3 hr in the dark. The petri dish lids are then left ajar for 20-45 minutes in a sterile hood to allow moisture on tissue to dissipate.

Each genomic DNA fragment is co-precipitated with pML18 (containing the selectable marker for rice transformation) onto the surface of gold particles. To accomplish this, a total of 10 μg of DNA at a 2:1 ratio of trait:selectable marker DNAs are added to 50 μl aliquot of gold particles that have been resuspended at a concentration of 60 mg ml^{-1}. Calcium chloride (50 μl of a 2.5 M solution) and spermidine (20 μl of a 0.1 M solution) are then added to the gold-DNA suspension as the tube is vortexing for 3 min. The gold particles are centrifuged in a microfuge for 1 sec and the supernatant removed. The gold particles are washed twice with 1 ml of absolute ethanol and then resuspended in 50 μl of absolute ethanol and sonicated (bath sonicator) for one second to disperse the gold particles. The gold suspension is incubated at -70°C for five minutes and sonicated (bath sonicator) if needed to disperse the particles. Six μl of the DNA-coated gold particles are then loaded onto mylar macrocarrier disks and the ethanol is allowed to evaporate.

At the end of the drying period, a petri dish containing the tissue is placed in the chamber of the PDS-1000/He. The air in the chamber is then evacuated to a vacuum of 28-29 inches Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1080-1100 psi. The tissue is placed approximately 8 cm from the stopping screen and the callus is bombarded two times. Two to four plates of

tissue are bombarded in this way with the DNA-coated gold particles. Following bombardment, the callus tissue is transferred to CM media without supplemental sorbitol or mannitol.

Within 3-5 days after bombardment the callus tissue is transferred to SM media (CM medium containing 50 mg/l hygromycin). To accomplish this, callus tissue is transferred from plates to sterile 50 ml conical tubes and weighed. Molten top-agar at 40°C is added using 2.5 ml of top agar/100 mg of callus. Callus clumps are broken into fragments of less than 2 mm diameter by repeated dispensing through a 10 ml pipet. Three ml aliquots of the callus suspension are plated onto fresh SM media and the plates are incubated in the dark for 4 weeks at 27-28°C. After 4 weeks, transgenic callus events are identified, transferred to fresh SM plates and grown for an additional 2 weeks in the dark at 27-28°C.

Growing callus is transferred to RM1 media (MS salts, Nitsch and Nitsch vitamins, 2% sucrose, 3% sorbitol, 0.4% gelrite® gelling agent +50 ppm hyg B) for 2 weeks in the dark at 25°C. After 2 weeks the callus is transferred to RM2 media (MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 0.4% gelrite® gelling agent + 50 ppm hyg B) and placed under cool white light (~40 $\mu Em^{-2}s^{-1}$) with a 12 hr photoperiod at 25°C and 30-40% humidity. After 2-4 weeks in the light, callus begin to organize, and form shoots. Shoots are removed from surrounding callus/media and gently transferred to RM3 media (1/2 x MS salts, Nitsch and Nitsch vitamins, 1% sucrose + 50 ppm hygromycin B) in phytatrays™ culture vessels (Sigma Chemical Co., St. Louis, MO) and incubation is continued using the same conditions as described in the previous step.

Plants are transferred from RM3 to 4" pots containing Scotts Metromix® 350 growing medium after 2-3 weeks, when sufficient root and shoot growth have occurred.

Example 4: Variants of ZmSNAC

A. Variant ZmSNAC Nucleotide Sequences That Do Not Alter the Encoded Amino Acid Sequence

The ZmSNAC nucleotide sequences set forth in SEQ ID NO: 1, 3, 5, 7 and 9 are used to generate variant nucleotide sequences having the nucleotide sequence of the open reading frame with about 70%, 75%, 80%, 85%, 90% or 95% nucleotide sequence identity when compared to the corresponding starting

unaltered ORF nucleotide sequence. These functional variants are generated using a standard codon table. While the nucleotide sequence of the variant is altered, the amino acid sequence encoded by the open reading frame does not change.

B. Variant Amino Acid Sequences of ZmSNAC1-5

Variant amino acid sequences of ZmSNAC1, ZmSNAC2, ZmSNAC3, ZmSNAC4 and ZmSNAC5 are generated. In this example, one or more amino acids are altered. Specifically, the open reading frame set forth in SEQ ID NO: 2, 4, 6, 8 or 10 is reviewed to determine the appropriate amino acid alteration. The selection of an amino acid to change is made by consulting a protein alignment with orthologs and other gene family members from various species. See Figure 1. An amino acid is selected that is deemed not to be under high selection pressure (not highly conserved) and which is rather easily substituted by an amino acid with similar chemical characteristics (i.e., similar functional side-chain). Assays as outlined elsewhere herein may be followed to confirm functionality. Variants having about 70%, 75%, 80%, 85%, 90% or 95% nucleic acid sequence identity to each of SEQ ID NO: 2, 4, 6, 8 and 10 are generated using this method.

C. Additional Variant Amino Acid Sequences of ZmSNAC Polypeptides

In this example, artificial protein sequences are created having 80%, 85%, 90% and 95% identity relative to the reference protein sequence. This latter effort requires identifying conserved and variable regions from the alignment set forth in Figure 1 and then the judicious application of an amino acid substitutions table. These parts will be discussed in more detail below.

Largely, the determination of which amino acid sequences are altered is made based on the conserved regions among the ZmSNAC proteins or among the other ZmSNAC polypeptides. See Figure 1. Based on the sequence alignment, the various regions of the ZmSNAC polypeptides that can likely be altered can be determined. It is recognized that conservative substitutions can be made in the conserved regions without altering function. In addition, one of skill will understand that functional variants of the ZmSNAC sequence of the invention can have minor non-conserved amino acid alterations in the conserved domain.

Artificial protein sequences are then created that are different from the original in the intervals of 80-85%, 85-90%, 90-95% and 95-100% identity. Midpoints of these intervals are targeted, with liberal latitude of plus or minus 1%, for example. The amino acids substitutions will be effected by a custom Perl script. The substitution table is provided below in Table 1.

First, any conserved amino acids in the protein that should not be changed are identified and "marked off" for insulation from the substitution. The start methionine will of course be added to this list automatically. Next, the changes are made.

H, C and P are not changed. The changes will occur with isoleucine first, sweeping N-terminal to C-terminal. Then leucine, and so on down the list until the desired target is reached. Interim number substitutions can be made so as not to cause reversal of changes. The list is ordered 1-17, so start with as many isoleucine changes as needed before leucine, and so on down to methionine. Clearly many amino acids will in this manner not need to be changed. L, I and V will involve a 50:50 substitution of the two alternate optimal substitutions.

The variant amino acid sequences are written as output. Perl script is used to calculate the percent identities. Using this procedure, variants of ZmSNAC1-5 are generated having about 82%, 87%, 92% and 97% amino acid identity to the starting unaltered ORF nucleotide sequence of SEQ ID NO: 2, 4, 6, 8 or 10.

Table 1. Substitution Table

Amino Acid	Strongly Similar and Optimal Substitution	Rank of Order to Change	Comment
I	L,V	1	50:50 substitution
L	I,V	2	50:50 substitution
V	I,L	3	50:50 substitution
A	G	4	
G	A	5	
D	E	6	
E	D	7	
W	Y	8	
Y	W	9	
S	T	10	
T	S	11	
K	R	12	
R	K	13	
N	Q	14	
Q	N	15	
F	Y	16	
M	L	17	First methionine cannot change
H		Na	No good substitutes
C		Na	No good substitutes
P		Na	No good substitutes

Example 5: Amplification of additional ZmNAC genes from soybean or other plant species

Additional ZmNAC or ZmSNAC genes from plant species could be identified by PCR or RT-PCR methods using degenerate primers such as the ones described below. Degenerate primers can be designed against conserved amino acid motifs found in available ZmNAC proteins from soybean, maize, rice or Arabidopsis. Such motifs can be identified from an alignment of the protein sequences. Sense/antisense primers could be used in different combinations. Similarly, several rounds of PCR could be used. The product of amplification of one pair of sense/antisense primers could be used as template for PCR with another set of internal (nested) degenerate primers therefore maximizing the chances for amplification of an appropriate sequence, i.e., containing a sequence corresponding to the corresponding amino acid motif.

Example 6: Transformation of Zea mays by Agrobacterium

For *Agrobacterium*-mediated transformation of maize, the method of Zhao is employed (US Patent Number 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the expression cassette to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). The immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 7: Activity of the ZmSNAC1 Promoter

To demonstrate that the DNA sequence isolated as the ZmSNAC1 promoter functions as a promoter, transgenic maize assays were performed. These assays provided a rapid assessment of whether the DNA sequence tested is able to direct gene expression.

The full length promoter (see, SEQ ID NO: 11) was PCR amplified from genomic DNA and cloned in an expression cassette as a translational fusion with B-glucuronidase (GUS; see, Jefferson, *et al.*, (1987) *EMBO J* 16:3901 and US Patent Number 5,599,670). Transgenic plants were created by Agrobacterium-

mediated transformation (see, Example 7) of the expression cassette into rapid-cycling maize as described in US Patent Application Publication 2003/0221212.

Leaf or stalk tissue was excised from T0 plants for analysis of ZmSNAC1 promoter activity. GUS expression was determined by immersing the excised plant tissues in either (1) a GUS staining buffer modified from Jefferson, *et al.*, (1987 *Plant Mol. Biol. Rep.* 5:387-405) containing 1.36g NaH_2PO_4, 1.74g Na_2HPO_4, 164 mg $K_4Fe(CN)_63H_2O$, 211 mg $K_3Fe(CN)_6$, 0.06 ml Triton X-100 and 50 mg X-Gluc(Sodium Salt) in a final volume of 100 ml, or (2) a GUS staining buffer of McCabe, *et al.*, (McCabe and Martinell (1993) *Bio/Technol.* 11:596-598) containing 1.36g NaH_2PO_4, 1.74g Na_2HPO_4, 16.4 mg $K_4Fe(CN)_63H_2O$, 0.29 g EDTA, 0.2 ml Triton X-100 and 50 mg X-Gluc (Sodium Salt) in a final volume of 100 ml. The plant tissue was incubated in the dark overnight at 37°C. Replacing the GUS staining solution with 70% ethanol stopped the assay. GUS activity was quantified using plant extract and expressed as nmoles/mg total protein/hour; see, Côté and Rutledge (2003) *Plant Cell Rep* 21(6):619-624. Ten non-transgenic control events were also tested. Seven of the ten transgene-positive events (Events 1.1, 1.2, 1.3, 1.4, 2.5, 1.7, 1.10) showed significant GUS staining in a range of activity levels; see, Figure 2. The ten control events did not show any significant GUS staining.

To further confirm that the ZmSNAC1 promoter can be induced by stress, excised tissue was incubated in 5μM abscisic acid (ABA) for 16 hours and GUS expression was again evaluated as described above. ABA treatment resulted in increased staining in the seven previously-identified events, as shown in Figure 2.

These data confirm the promoter function and stress-inducible nature of SEQ ID NO: 11 and its endogenous gene ZmSNAC1.

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

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

THAT WHICH IS CLAIMED:

1. An isolated polypeptide having transcription factor activity and comprising an amino acid sequence at least 95% identical to SEQ ID NO: 2, 4, 6, 8 or 10.
2. An isolated polynucleotide comprising a nucleotide sequence at least 95% identical to SEQ ID NO: 1, 3, 5, 7 or 9, or the complement thereof.
3. A transgenic plant comprising a polynucleotide of Claim 2 operably linked to a promoter that drives expression in the plant, wherein transcription factor activity in said plant is modulated relative to a control plant.
4. The plant of claim 3, wherein said transcription factor activity is increased.
5. The plant of claim 3, wherein said promoter is a tissue-preferred promoter, a constitutive promoter, or an inducible promoter.
6. The plant of claim 3 wherein said tissue-preferred promoter is a root-preferred promoter, a leaf-preferred promoter, a shoot-preferred promoter or an inflorescence-preferred promoter.
7. The plant of claim 3, wherein said modulation of transcription factor activity affects floral development.
8. The plant of claim 3, wherein said modulation of transcription factor activity affects root development.
9. The plant of claim 3, wherein the stress tolerance of said plant is increased relative to a control.
10. The plant of claim 9, wherein the drought tolerance of said plant is increased relative to a control.
11. The plant of claim 3, wherein said promoter is stress-induced.
12. A transformed seed of the plant of claim 3.
13. The plant of claim 3, wherein said plant is maize, wheat, rice, barley, sorghum, rye, soybean, brassica or sunflower.
14. A plant in which expression of a native genomic locus is modified, said genomic locus comprising a polynucleotide of claim 2.
15. A method of modulating transcription factor activity in a plant, comprising transforming said plant with a polynucleotide of claim 2 operably linked to a promoter.
16. The method of claim 15 wherein said modulation of transcription factor activity improves stress tolerance.

17. The method of claim 15 wherein said promoter is a tissue-preferred or inducible promoter, or is both tissue-preferred and inducible.

18. The method of claim 17, wherein said promoter is stress-inducible.

19. The method of claim 17, wherein senescence is delayed.

20. An isolated nucleic acid molecule comprising a first polynucleotide which initiates transcription of a second, operably-linked polynucleotide in a plant cell, wherein the first polynucleotide is selected from the group consisting of:

a) SEQ ID NO: 11;

b) at least 500 contiguous nucleotides of SEQ ID NO: 11.

21. An expression cassette comprising the first and second polynucleotides of Claim 20.

22. A plant cell having stably incorporated into its genome the expression cassette of Claim 21.

23. A plant having stably incorporated into its genome the expression cassette of Claim 21.

FIGURE 1


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          (1)1         10        20        30        40        50        60        70        80
    ANAC (1) --------MGIQETDPLTQLSLPPGFRFYPTDEELMVQYLCRKAAGYDFSLQ-LIAEIDLYKFDPWVLPNKALFG--EKE
   ATAF2 (1) ---------------MKSELNLPAGFRFHPTDEELVKFYLCRKCASEQISAP-VIAEIDLYKFNPWELPEMSLYG--EKE
   ATAF1 (1) ---------------MSELLQLPPGFRFHPTDEELVMHYLCRKCASQSIAVP-IIAEIDLYKYDPWELPGLALYG--EKE
     NAP (1) -------------MEVTSQSTLPPGFRFHPTDEELIVYYLRNQTMSKPCPVS-IIPEVDIYKFDPWQLPEKTEFG--ENE
   AtNAM (1) -----MESTDSSGGPPPPQPNLPPGFRFHPTDEELVIHYLKRKADSVPLPVA-IIADVDLYKFDPWELPAKASFG--EQE
     TIP (1) ---------MKEDMEVLSLASLPVGFRFSPTDEELVRYYLRLKINGHDNDVR-VIREIDICKWEPWDLPDFSVVKTTDSE
    CUC2 (1) -----MDIPYYHYDHGGDSQYLPPGFRFHPTDEELITHYLLRKVLDGCFSSR-AIAEVDLNKCEPWQLPGRAKMG--EKE
    CUC1 (1) --MDVDVFNGWGRPRFEDESLMPPGFRFHPTDEELITYYLLKKVLDSNFSCA-AISQVDLNKSEPWELPEKAKMG--EKE
    CUC3 (1) MMLAVEDVLSELAGEERNERGLPPGFRFHPTDEELITFYLASKIFHGGLSGI-HISEVDLNRCEPWELPEMAKMG--ERE
    NAC1 (1) ---METEEEMKESSISMVEAKLPPGFRFHPKDDELVCDYLMRRSLHNNHRPPLVLIQVDLNKCEPWDIPKMACVG--GKD
ZM-SNAC1 (1) ---MGLPMRR--ERDAEAELNLPPGFRFHPTDDELVEHYLCRKAAGQRLPVP-IIAEVDLYRFDPWDLPERALFG--ARE
ZM-SNAC2 (1) ---MGLPVTRRRERDAEAELDLPPGFRFHPTDDELVEHYLCRKAAGQRLPVP-IIAEVDLYRFDPWDLPERALFG--ARE
ZM-SNAC3 (1) ---MGQPVTRRRERDAEAELDLPPGFRFHPTDDELVEHYLCRKAAGQRLPVP-IIAEVDLYRFDPWDLPERALFG--ARE
ZM-SNAC5 (1) MSSGGGGRVPAAAAAQSQELQLPPGFRFHPTDEELVVHYLCRRCAGLPISVP-IIAEVDLYKHDPWQLPRMALYG--EKE
ZM-SNAC4 (1) ------------MSGAGPDLQLPPGFRFHPTDEELVMHYLCRRCAGLPIAVP-IIAEIDLYKFDPWQLPRMALYG--EKE
Consensus(1)                    EL LPPGFRFHPTDEELV HYLCRK A LSVP IIAEVDLYKFDPWDLPEKALFG EKE

          (81) 81        90        100       110       120       130       140       150     160
    ANAC (70) WYFFSPRDRKYPNGSRPNRVAGSGYWKATGTDKIISTEG-----QRVGIKKALVFYIGKAPKGTKTNWIMHEYRLIEPSR
   ATAF2 (63) WYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGKPK------TLGIKKALVFYAGKAPKGIKTNWIMHEYRLANVDR
   ATAF1 (63) WYFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGLPK------PVGIKKALVFYAGKAPKGEKTNWIMHEYRLADVDR
     NAP (65) WYFFSPRERKYPNGVRPNRAAVSGYWKATGTDKAIHSGS-----SNVGVKKALVFYKGRPPKGIKTDWIMHEYRLHDSRK
   AtNAM (73) WYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVISTGGGG-SKKVGVKKALVFYSGKPPKGVKSDWIMHEYRLTDNKP
     TIP (71) WLFFCPLDRKYPSGSRMNRATVAGYWKATGKDRKIKSGK----TKIIGVKRTLVFYTGRAPKGTRTCWIMHEYRATEKDL
    CUC2 (73) WYFFSLRDRKYPTGLRTNRATEAGYWKATGKDREIFSSKT---CALVGMKKTLVFYKGRAPKGEKSNWVMHEYRLEGKFS
    CUC1 (76) WYFFTLRDRKYPTGLRTNRATEAGYWKATGKDREIKSSK---TKSLLGMKKTLVFYKGRAPKGEKSCWVMHEYRLDGKFS
    CUC3 (78) WYFYSLRDRKYPTGLRTNRATTAGYWKATGKDKEVFSGGGG---QLVGMKKTLVFYKGRAPRGLKTKWVMHEYRLENDHS
```

FIGURE 1 (Con't.)

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    NAC1 (76) WYFYSQRDRKYATGLRTNRATATGYWKATGKDRTILRKG-----KLVGMRKTLVFYQGRAPRGRKTDWVMHEFRLQGSHH
ZM-SNAC1 (73) WYFFTPRDRKYPNGSRPNRAAGNGYWKATGADKPVAPRG-----RTLGIKKALVFYAGKAPRGVKTDWIMHEYRLADAGR
ZM-SNAC2 (75) WYFFTPRDRKYPNGSRPNRAAGDGYWKATGADKPVAPRG----ARTLGIKKALVFYAGKAPRGVKTDWIMHEYRLADAGR
ZM-SNAC3 (75) WYFFTPRDRKYPNGSRPNRAAGDGYWKATGADKPVAPRAAAADARTLGIKKALVFYAGKAPRGVKTDWIMHEYRLADAGR
ZM-SNAC5 (78) WYFFSPRDRKYPNGSRPNRAAGAGYWKATGADKPVGTPK------PVAIKKALVFYAGKAPKGDKTNWIMHEYRLADVDR
ZM-SNAC4 (66) WYFFSPRDRKYPNGSRPNRAAGAGYWKATGADKPVGTPK------PLAIKKALVFYAGKAPKGEKTNWIMHEYRLADVDR
Consensus(81) WYFFSPRDRKYPNGSRPNRAAGAGYWKATG DKPI S         VGIKKALVFYAGKAPKG KT WIMHEYRL D  R


      (161) 161      170       180       190       200       210       220      230      240
   ANAC(145) RNGS--------TKLDDWVLCRIYKKQS------------------SAQKQVYDNGIANAREFS-------------NNG
  ATAF2(137) SASVNKK---NNLRLDDWVLCRIYNKKG----------------------TMEKYFPADEKP-----------------
  ATAF1(137) SVRKKKN----SLRLDDWVLCRIYNKKG------ATE-RRGPPPPVVYGDEIME-----EKP---------------KVT
    NAP(140) ASTKRN----GSMRLDEWVLCRIYKKRG--------------------ASKLLN----EQEG----------------FM
  AtNAM(152) THICDFGNKKNSLRLDDWVLCRIYKKNN------------------STASRHHHHLHHIHLDNDHHRHDMMI---GDDR
    TIP(147) DGTK--------SGQNPFVVCKLFKKQDIVNGAAE-------------PEESKSCEVEPAVSSPTVVDEVEMSEVSPVFP
   CUC2(150) YHFISR------SSKDEWVISRVFQKTTLASTGAVSEGGGG----GGATVSVSSGTGPSKKTKVPSTISRNYQEQPSSPS
   CUC1(153) YHYIS------SSAKDEWVLCKVCLKSG--------------------VVSRETNLISSSSS-----------------SA
   CUC3(155) HRHT-------CK--EEWVICRVFNKTG-----------------DRKNVGLIHNQISYLHN-----------------H
   NAC1(151) PPNHSL-----SSPKEDWVLCRVFHKNT--------------------EGVICRDNMGSCFD----------------ET
ZM-SNAC1(148) AAAAKK----GSLRLDDWVLCRLYNKKN----------------------EWEKMQLGKTAV----------------AG
ZM-SNAC2(151) RAKK------GSLRLDDWVLCRLYNKKN----------------------EWEKMRLGK--------------------
ZM-SNAC3(155) RAKK----GSLRLDDWVLCRLYNKKN----------------------EWEKMRLGKGAA----------------AZM-
   SNAC5(152) TARKKNN----SLRLDDWVLCRIYNKKG-ALEKPTGAGGRAEASSQGALGSMSMGSPPEQKPSVLPSATATAT---AAAG
ZM-SNAC4(140) SARKKN-----SLRLDDWVLCRIYNKKGGGLEKAAAPAAGGDHKPVFATAAVSS--PPEQKP-------------FVAA
Consensus(161)            SLRLDDWVLCRIYNKK                        V    L                    -
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FIGURE 1 (Con't.)


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      241) 241      250       260       270       280       290       300       310      320
  ANAC(186) TSSTTSSSSHFEDVLDSFHQEIDNRNFQFSNPNRISSLRPDLTEQKTGFHGLADTSNFDWASFAGNVEHNNSVPELGMSH
 ATAF2(174) -RTT--TMAEQSSSPFDT----SDSTYPTLQEDDSSSSGGHGHVVSPD------VLEVQSEPKWGELEDALEAFDTSMFG
 ATAF1(186) EMVMPPPPQQTSEFAYFD----TSDSVPKLHTTDSSCSEQVVSPEFTS--------EVQSEPKWKDWSAVSNDNNNT---
   NAP(176) DEVLMEDETKVVVNEAERRTEEEIMMMTSMKLPRTCSLAHLLEMDYMG--------PVSHIDNFSQFDHLHQPDS-----
 AtNAM(210) FRHVPPGLHFPAIFSDNNDPTAIYDGGGGGYGGGSYSMNHCFASGSKQEQ---LFPPVMMMTSLNQDSGIGSSSS-----
   TIP(206) KTEETNPCDVAESSLVIPSECRSGYSVPEVTTTGLDDIDWLSFMEFDSPKLFSPLHSQVQSELGSSFNGLQSESSELFKN
  CUC2(220) SVSLPPLLDPTTTLGYTDSSCSYDSRSTNTTVTASAITEHVSCFSTVPTTTTALGLDVNSFSRLPPPLGFDFDPFPRFVS
  CUC1(191) VTGEFSSAGSAIAPIINTFATEHVSCFSNNSAAHTDASFHTFLPAPPPSLPPRQPRHVGDGVAFGQFLDLGSSGQIDFDA
  CUC3(192) SLSTTHHHHHEALPLLIEPSNKTLTNFPSLLYDDPHQNYNNNNFLHGSSGHNIDELKALINPVVSQLNGIIFPSGNNNND
  NAC1(190) ASASLPPLMDPYINFDQEPSSYLSDDHHYIINEHVPCFSNLSQNQTLNSNLTNSVSELKIPCKNPNPLFTGGSASATLTG
ZM-SNAC1(186) VGATKEEAMDMATSHTHSHSQSHSHSWGETRTPESEIVDNDPFPELDSFPAFQDPAMMMTVPKEEQVDGCSAKSGNLFVD
ZM-SNAC2(182) EGAAKEEAMDMSTSH----------SWGETRTPESEIVDNDPPFPDPPA------PAMMVPKKERVDAGGSARSSDLFVD
ZM-SNAC3(190) GAVKEEEAMDMSTSHSQ-----ITHSWGETRTPESEIVDNDLPFPDP---------AMMVPKKERVDDGGSARTSDLFVD
ZM-SNAC5(224) TGYAPLPFSELAAYYEVR----PSDSMPRAHGADSSCSGHALAATSSCGGGGGERPEVQSQPKIAEWERTFAAGA--GPG
ZM-SNAC4(199) AGGLPPAFPELAAYYDR-----PSDSMPRLHADYSSCSEQVLSPEQLACDR-----EVQSQPKISEWERTFASDP-----
Consensus(241)            A            S       S                    V    K     G  A       -
```

```
      (321) 321      330       340       350       360       370       380       390      400
  ANAC(266) VVPNLEYNCGYLKTEEEVESSHGFNNSGELAQKGYGVDSFGYSGQVGGFGFM----------------------------
 ATAF2(241) SSMELLQPDAFVPQFLYQS--DYFTSFQDPPEQ---KPFLNWSFAPQG-------------------------------
 ATAF1(251) LDFGFNYIDATVDNAFG-----GGGSSNQMFPL---QDMFMYMQKPY---------------------------------
   NAP(243) -E----------------------SSWFGDLQFNQDEILNHHRQAMFKF-------------------------------
 AtNAM(282) PSKRFNGGG---VG-------DCSTSMAATPLMQNQGGIYQLPGLNWYS-------------------------------
   TIP(286) HNEDYIQTQYGTNDADEYMSKFLDSFLDIPYEPEQIPYEPQNLSSCNKINDESKRGIKIRARRAQAPGCAEQFVMQGDAS
  CUC2(300) RNVSTQSNFRSFQENFNQFPYFGSSSASTMTSAVNLPSFQGGGGVSGMNYWLPATAEENESKVGVLHAGLDCIWNY----
  CUC1(271) AAAAFFPNLPSLPPTVLPPPPSFAMYGGGSPAVSVWPFTL-----------------------------------------
```

FIGURE 1 (Con't.)


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     CUC3(272) EDDFDFNLGVKTEQSSNGNEIDVRDYLENPLFQEASYGLLGFSSSPGPLHMLLDSPCPLGFQL----------------
     NAC1(270) LDSFCSSDQMVLRALLSQLTKIDGSLGPKESQSYGEGSSESLLTDIGIPSTVWNC-------------------------
  ZM-SNAC1(266) LSYDDIQGMYSGLDMLPPPGEDFYSSLFASPRVKGNQPAGAAGLGQF---------------------------------
  ZM-SNAC2(246) LSYDDIQGMYSGLDMLPPPGEDFYSSLFASPRVRGNQPTGAAGLGPF---------------------------------
  ZM-SNAC3(256) LSYDDIQGMYSGLDMLPPAGEDLYSSLFASPRVRGNQPTGPAGLGPF---------------------------------
  ZM-SNAC5(298) INPAGSMLGLGGHQLGSAA--VGVGLPAGDPLL---QDILTYWGKPY---------------------------------
  ZM-SNAC4(264) VNPAGSMLDP------------VVGHAGGDPLL---QDILMYWGKPF---------------------------------
Consensus(321) L                      SS  A P V            F                                  -
```

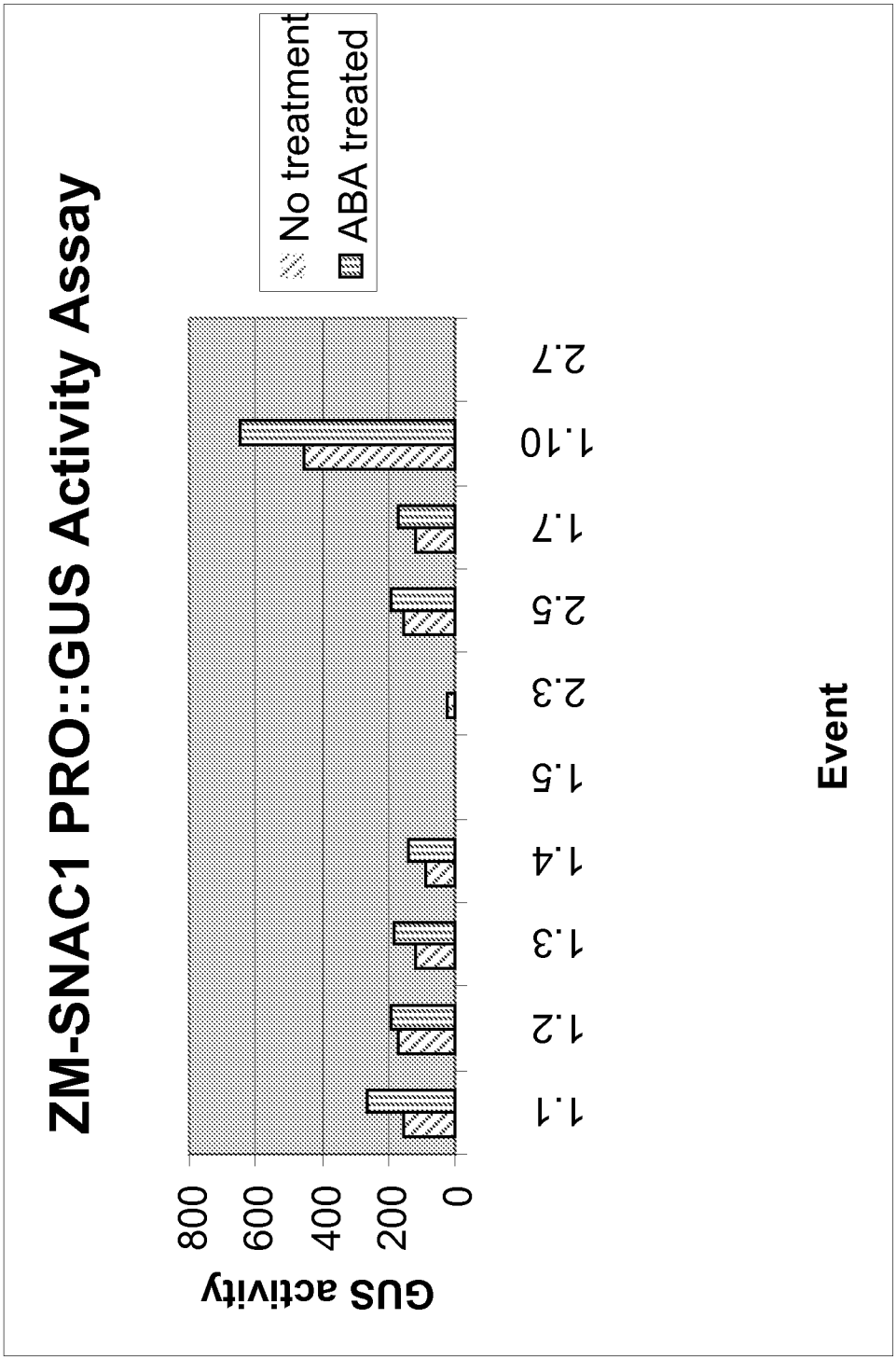

ZM-SNAC1 PRO::GUS Activity Assay
No treatment
ABA treated
GUS activity
0
200
400
600
800
1.1
1.2
1.3
1.4
1.5
2.3
2.5
1.7
1.10
2.7
Event

Figure 2