(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 December 2008 (11.12.2008)

PCT

(10) International Publication Number
WO 2008/148872 A1

(51) **International Patent Classification:**
C07K 14/415 (2006.01) ***A01H 5/00*** (2006.01)
C12N 15/82 (2006.01)

(21) **International Application Number:**
PCT/EP2008/057076

(22) International Filing Date: 6 June 2008 (06.06.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/942,304 6 June 2007 (06.06.2007) US

(71) **Applicant** *(for all designated States except US):* **CROPDESIGN N.V.** [BE/BE]; Technologiepark 3, B-9052 Zwijnaarde (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only):* **DANILEVSKAYA, Olga** [US/US]; 6004 Dogwood Circle, Johnston, Iowa 50131 (US). **BRUCE, Wesley** [US/US]; 7605 Sutcliffe Drive, Raleigh, North Carolina 27613 (US).

(74) Agent: MISTRY, Meeta; BASF SE, Global Intellectual Property, GVX - C006, 67056 Ludwigshafen (DE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, **BR,** BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, **HR,** HU, **ID,** IL, IN, IS, **JP,** KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, **DK,** EE, ES, FI, FR, GB, GR, **HR,** HU, IE, **IS, IT,** LT, LU, LV, MC, MT, NL, NO, PL, PT, **RO,** SE, **SI,** SK, TR), OAPI (BF, **BJ,** CF, CG, CI, CM, GA, GN, GQ, GW, ML, **MR,** NE, SN, TD, TG).

Declaration under Rule 4.17:
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2008/148872 A1

(54) Title: YIELD ENHANCEMENT IN PLANTS BY MODULATION OF MAIZE MADS BOX TRANSCRIPTION FACTOR ZMM28

(57) Abstract: Compositions and methods for modulating flower organ development, leaf formation, phototropism, apical dominance, fruit development, initiation of roots, and for increasing yield in a plant are provided. The compositions include a ZMM28 sequence. Compositions of the invention comprise amino acid sequences and nucleotide sequences selected from SEQ ID NOS: 1-2 as well as variants and fragments thereof. Nucleotide sequences encoding the ZMM28 sequences are provided in DNA constructs for expression in a plant of interest are provided for modulating the level of a ZMM28 sequence in a plant or a plant part are provided. The methods comprise introducing into a plant or plant part a heterologous polynucleotide comprising a ZMM28 sequence of the invention. The level of the ZMM28 polypeptide can be increased or decreased. Such method can be used to increase the yield in plants; in one embodiment, the method is used to increase grain yield in cereals.

YIELD ENHANCEMENT IN PLANTS BY MODULATION OF MAIZE MADS BOX TRANSCRIPTION FACTOR ZMM28

Field of the invention

The present invention is drawn to the field of genetics and molecular biology. More particularly, the compositions and methods are directed to modulation of transcription and improving yield in plants.

Background of the invention

Grain yield improvements by conventional breeding have nearly reached a plateau in maize. It is natural then to explore some alternative, non-conventional approaches that could be employed to obtain further yield increases. Since the harvest index in maize has remained essentially unchanged during selection for grain yield over the last hundred or so years, the yield improvements have been realized from the increased total biomass production per unit land area (Sinclair, *et al.,* (1998) *Crop Science* 38:638-643; Duvick, *et al.,* (1999) *Crop Science* 39:1622-1630; and, Tollenaar, *et al.,* (1999) *Crop Science* 39:1597-1604). This increased total biomass has been achieved by increasing planting density, which has led to adaptive phenotypic alterations, such as a reduction in leaf angle and tassel size, the former to reduce shading of lower leaves and the latter perhaps to increase harvest index (Duvick, *et al.,* (1999) *Crop Science* 39:1622-1630).

ZMM28 (Zea mays MADS) belongs to a family of MADS transcription factors that play critical roles in diverse developmental process in plants including flower and seed development (Münster, *et al.,* 2002; Parenicova, *et al.,* 2003). The highly conservative DNA binding MADS domain was named after MCM1 , AGAMOUS, DEFICIENS and SRF (serum response factor) proteins (Schwarz-Sommer, *et al.,* 1990). MADS box genes are major factors controlling the flower and seed development in plants. MADS box genes can modify significantly plant flower morphology and plant architecture in transgenic plants due to their universal role in plant development. ZMM28 is expressed predominantly in ears and may enhance yield through controlling a number of spikelets per ear, final kernel number and kernel size.

Methods and compositions are needed in the art which can employ such sequences to modulate organ development and yield in plants.

Brief summary of the invention

Compositions and methods for modulating flower organ development, leaf formation, phototropism, apical dominance, fruit development, initiation of roots, and for increasing yield in a plant are provided. The compositions include a ZMM28 sequence. Compositions of the invention comprise amino acid sequences and nucleotide sequences selected from SEQ ID NOS: 1-2 as well as variants and fragments thereof.

Nucleotide sequences encoding the ZMM28 sequences are provided in DNA constructs for expression in a plant of interest. Expression cassettes, plants, plant cells, plant parts, and seeds comprising the sequences of the invention are further provided. In specific embodiments, the polynucleotide is operably linked to a constitutive promoter.

Methods for modulating the level of a ZMM28 sequence in a plant or a plant part are provided. The methods comprise introducing into a plant or plant part a heterologous polynucleotide comprising a ZMM28 sequence or a ZMM28 domain of the invention. The level of the ZMM28 polypeptide can be increased or decreased. Such method can be used to increase the yield in plants; in one embodiment, the method is used to increase grain yield in cereals.

Brief description of the figures

Figure 1 provides an alignment of several ZMM28 sequences from *Zea mays* (SEQ ID NO: 2), *Arabidopsis thaliana* (SEQ ID NO: 6), *Oryza sativum* (SEQ ID NO: 3), *Hordeum vulgare* (SEQ ID NO: 5), *Triticum aestivum* (SEQ ID NO: 4) and *Antirrhinum majus SQUAMOSA* (SEQ ID NO: 7). The ZMM28 MADS consensus domain is single-underlined (SEQ ID NO: 8).

Detailed description of the invention

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions 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.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are 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.

I. Overview

5 Methods and compositions are provided to promote floral organ development, root initiation, and yield, and for modulating leaf formation, phototropism, apical dominance, fruit development and the like, in plants. The compositions and methods of the invention result in improved plant or crop yield by modulating in a plant the level of at least one ZMM28 polypeptide or a polypeptide having a biologically active variant or fragment of a ZMM28 10 polypeptide of the invention.

II. Compositions

Compositions of the invention include ZMM28 polynucleotides and polypeptides and variants and fragments thereof that are involved in regulating transcription. ZMM28 encodes a plant 15 protein with ZMM28 MADS domain. The ZMM28 MADS domain (SEQ ID NO: 8) in ZMM28 is from amino acid residues 1 to 57 corresponding to the nucleic acid positions of SEQ ID NO: 1 (nucleic acid positions 106 to 268 corresponding to SEQ ID NO: 2). By "corresponding to" is intended that the recited amino acid positions for each domain relate to the amino acid positions of the recited SEQ ID NO, and that polypeptides comprising these domains may be 20 found by aligning the polypeptides with the recited SEQ ID NO: using standard alignment methods.

The ZMM28 sequence of the invention act as a transcription factor that binds specifically to a target gene(s) to activate (or) repress their transcription.

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ZMM28 is predominantly expressed in the young ear during spikelet formation. As used herein, a "ZMM28" sequence comprises a polynucleotide encoding or a polypeptide having the ZMM28 MADS domain or a biologically active variant or fragment of the ZMM28 MADS domain. See, for example, Jurata and Gill (1997) *Mol. Cell. Biol.* 17:5688-98; and Franks, *et* 30 *a/.*, (2002) *Development* 129:253-63.

In one embodiment, the present invention provides isolated ZMM28 polypeptides comprising amino acid sequences as shown in SEQ ID NO: 2 and fragments and variants thereof. Further provided are polynucleotides comprising the nucleotide sequence set forth in SEQ ID NO: 1 35 and sequences comprising a polynucleotide encoding a ZMM28 MADS domain (SEQ ID NO: 8).

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 ZMM28 domain or ZMM28 polynucleotides and proteins encoded thereby are also encompassed by the methods and compositions of the present invention. By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the native protein and hence regulate transcription. For example, polypeptide fragments will comprise the ZMM28 MADS domain (SEQ ID NO: 8). Alternatively, fragments that are used for suppressing or silencing (i.e., decreasing the level of expression) of a ZMM28 sequence need not encode a protein fragment, but will retain the ability to suppress expression of the target sequence. In addition, fragments 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 18 nucleotides, 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 polynucleotide encoding a ZMM28 MADS domain or a ZMM28 polypeptide will encode at least 15, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 675, 700, 725, 750, 775, 800, 825 contiguous amino acids, or up to the total number of amino acids present in a full-length ZMM28 MADS domain, or ZMM28 protein (i.e., SEQ ID NO: 2).

Fragments of a ZMM28 MADS domain, or a ZMM28 polynucleotide that are useful as hybridization probes, PCR primers, or as suppression constructs generally need not encode a biologically active portion of a ZMM28 protein or a ZMM28 domain.

5 A biologically active portion of a polypeptide comprising a ZMM28 MADS domain, or a ZMM28 protein can be prepared by isolating a portion of a ZMM28 polynucleotide, expressing the encoded portion of the ZMM28 protein (e.g., by recombinant expression *in vitro),* and assessing the activity of the encoded portion of the ZMM28 protein. Polynucleotides that are fragments of a ZMM28 nucleotide sequence, or a polynucleotide sequence comprising a 10 ZMM28 MADS domain comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,050, 2,100, 2,150, 2,200, 2,250, 2,300, 2,350, 2,400, 2,450, 2,500 contiguous nucleotides, or up to the number of nucleotides present in a ZMM28 MADS domain or in a ZMM28 polynucleotide (i.e., SEQ ID NOS: 1, 1270 nucleotides).

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"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 internal 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 20 polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the ZMM28 polypeptides or of a ZMM28 MADS domain. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for 25 example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotide, such as those generated, for example, by using site-directed mutagenesis but which still encode a polypeptide comprising a ZMM28 MADS domain or a ZMM28 polypeptide that is capable of regulating transcription or that is capable of reducing the level of expression (i.e., suppressing 30 or silencing) of a ZMM28 polynucleotide. 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.

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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, an isolated polynucleotide that encodes a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 1 or SEQ ID NO: 2 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 internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, regulate transcription as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a ZMM28 protein of the invention or of a ZMM28 MADS domain 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 ZMM28 protein or the consensus ZMM28 MADS domain as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a ZMM28 protein of the invention or of a ZMM28 MADS domain 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 polynucleotides 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 ZMM28 proteins or ZMM28 MADS domains 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; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. 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 both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired activity (i.e., the ability to regulate transcription or decrease the level of expression of a target ZMM28 sequence). In specific embodiments, the mutations that will be made in the DNA encoding the variant do not place the sequence out of reading frame and do not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication Number 75,444.

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. For example, the activity of a ZMM28 polypeptide can be evaluated by assaying for the ability of the polypeptide to regulate transcription. Various methods can be used to assay for this activity, including, directly monitoring the level of expression of a target gene at the nucleotide or polypeptide level. Methods for such an analysis are known and include, for example, Northern blots, S1 protection assays, Western blots, enzymatic or colorimetric assays. Methods to assay for a modulation of transcriptional activity can include monitoring for an alteration in the phenotype of the plant. For example, as discussed in further detail elsewhere herein, modulating the level of a ZMM28 polypeptide can result in alterations in flower formation and yield. Methods to assay for these changes are discussed in further detail elsewhere herein.

Variant polynucleotides and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different ZMM28 coding sequences can be manipulated to create a new ZMM28 sequence or ZMM28 MADS domain 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 the ZMM28 gene of the invention and other known ZMM28 genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies

for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. ScL USA* 9 1:10747-10751 ; Stemmer (1994) *Nature* 370:389-391 ; Crameri, *et ai,* (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 ;
5 and U.S. Patent Numbers 5,605,793 and 5,837,458.

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
10 based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire ZMM28 sequences, or to ZMM28 MADS domains of the invention, 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
15 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 can silence or
20 suppress the expression of a ZMM28 sequence or a polynucleotide that encodes for a ZMM28 protein and which hybridize under stringent conditions to the ZMM28 sequences disclosed herein, or to variants or fragments thereof, are encompassed by the present invention.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to
25 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);
30 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.

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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
5 labeling synthetic oligonucleotides based on the ZMM28 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 ai,* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

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For example, the entire ZMM28 polynucleotide or a polynucleotide encoding a ZMM28 MADS domain disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding ZMM28 polynucleotide and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences
15 that are unique among ZMM28 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 ZMM28 polynucleotide 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
20 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
25 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
30 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.

35 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^{0}C for short probes (e.g., 10 to 50 nucleotides) and at

least about 60^{0}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 (2OX 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^{0}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^{0}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^{0}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^{0}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^{0}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* 4:1 1-17; 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; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

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
5 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
10 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
15 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
20 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
25 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
30 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
35 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).

5 (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
10 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.

15 **III. Plants**

In specific embodiments, the invention provides plants, plant cells, and plant parts having altered levels (i.e., an increase or decrease) of a ZMM28 sequence. In some embodiments, the plants and plant parts have stably incorporated into their genome at least one heterologous polynucleotide encoding a ZMM28 polypeptide comprising the ZMM28 MADS domain as set
20 forth in SEQ ID NO: 8, or a biologically active variant or fragment thereof. In one embodiment, the polynucleotide encoding the ZMM28 polypeptide is set forth in SEQ ID NO: 1 or a biologically active variant or fragment thereof.

In yet other embodiments, plants and plant parts are provided in which the heterologous
25 polynucleotide stably integrated into the genome of the plant or plant part comprises a polynucleotide which when expressed in a plant increases the level of a ZMM28 polypeptide comprising a ZMM28 MADS domain, or an active variant or fragment thereof. Sequences that can be used to increase expression of a ZMM28 polypeptide include, but are not limited to, the sequence set forth in SEQ ID NO: 1 or variants or fragments thereof.

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As discussed in further detail elsewhere herein, such plants, plant cells, plant parts, and seeds can have an altered phenotype including, for example, altered flower organ development, leaf formation, phototropism, apical dominance, fruit development, root initiation, and improved yield.

35

As used herein, the term plant includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, 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, and the like. Grain is intended to mean 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
5 included within the scope of the invention, provided that these parts comprise the introduced or heterologous polynucleotides disclosed herein.

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
10 to, corn *(Zea mays), Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea),* particularly those *Brassica* species useful as sources of seed oil, alfalfa *{Medicago sativa),* rice *{Oryza sativa),* rye *{Secale cereale),* sorghum *(Sorghum bicolor, Sorghum vulgare),* millet (e.g., pearl millet *(Pennisetum glaucum),* proso millet *(Panicum miliaceum),* foxtail millet *(Setaria italica),* finger millet *(Eleusine coracana)),* sunflower *(Helianthus annuus),* safflower *(Carthamus tinctorius),* wheat *(Triticum*
15 *aestivum),* soybean *(Glycine max),* tobacco *(Nicotiana tabacum),* potato *(Solarium 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
20 *(Psidium guajava),* mango *(Mangifera indica),* olive *(Olea europaea),* papaya *(Carica papaya),* cashew *(Anacardium occidentale),* macadamia *(Macadamia integrifolia),* almond *(Prunus amygdalus),* sugar beets *(Beta vulgaris),* sugarcane *(Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

25 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*
30 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),*
35 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 corn, 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.

A "subject plant or plant cell" is one in which an alteration, such as transformation or introduction of a polypeptide, has occurred, 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 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 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.

IV. Polynucleotide Constructs

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 various polynucleotides employed in the methods and compositions 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 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 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, by operably linked is intended that the coding regions are in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the ZMM28 polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette can include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a ZMM28 polynucleotide, and a transcriptional and translational termination region (i.e., termination region) functional in plants. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the ZMM28 polynucleotide may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the ZMM28 polynucleotides may be heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. 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 it may be optimal to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs can change expression levels of a ZMM28 transcript or protein 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 ZMM28 polynucleotide of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the ZMM28 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) *Ce//* 64:671-674; Sanfacon, *et al.,* (1991) *Genes Dev.* 5:141-149; Mogen, *et al.,* (1990) *Plant* Ce// 2:1261-1272; Munroe, *et al.,* (1990) *Gene* 9 1:151-158; 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.* 92:1-1 1 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. 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 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) (GalMe, *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) (GalMe, *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* 8 1:382-385). See also, Della-Cioppa, *et al.,* (1987) *Plant Physiol.* 84:965-968.

5 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*
10 mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention, including the native promoter of the polynucleotide sequence of interest. The promoters can be selected based on
15 the desired outcome. The nucleic acids can be combined with constitutive, 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 U.S. Patent Number
20 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.* 8 1:581-588); MAS (Velten, *et al.,* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent Number 5,659,026), GOS2 promoter (dePater, *et al.,* (1992)
25 *Plant J.* 2:837-44), and the like. Other constitutive promoters include, for example, U.S. 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 6,177,61 1.

The expression cassette can also comprise a selectable marker gene for the selection of
30 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
35 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 florescent protein (CYP) (Bolte, *et al.,* (2004) *J. Cell*

Science 117:943-54 and Kato, *et al.,* (2002) *Plant Physiol* 129:913-42), and yellow florescent 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.* 3:506-51 1; Christopherson, *et al.,* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao, *et al.,* (1992) *Cell* 7 1:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:241 9-2422; Barkley, *et al.,* (1980) in *The Operon,* pp. 177-220; Hu, *et al.,* (1987) Ce// 48:555-566; Brown, *et al.,* (1987) Ce// 49:603-612; Figge, *et al.,* (1988) Ce// 52:71 3-722; Deuschle, *etal.,* (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; Bairn, *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, *etal.,* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt, *et al.,* (1988) *Biochemistry* 27:1094-1 104; 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.

In certain embodiments the polynucleotides of the present invention can be stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired trait. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. The combinations generated can also include multiple copies of any one of the polynucleotides of interest. The polynucleotides of the present invention can also be stacked with traits desirable for disease or herbicide resistance (e.g., fumonisin detoxification genes (U.S. Patent Number 5,792,931); avirulence and disease resistance genes (Jones, *et al.,* (1994) *Science* 266:789; Martin, *et al.,* (1993) *Science* 262:1432; Mindrinos, *et al.,* (1994) Ce// 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)); and traits desirable for processing or process products such as high oil (e.g., U.S. Patent Number 6,232,529); modified oils (e.g., fatty acid desaturase genes (U.S. Patent Number 5,952,544; WO 94/1 1516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., U.S. Patent Number 5.602,321 ; beta-ketothiolase,

polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert, *et al.,* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoat.es (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides providing agronomic traits such as male sterility (e.g., see, U.S. Patent Number 5,583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (e.g., WO 99/61619, WO 00/17364, and WO 99/25821); the disclosures of which are herein incorporated by reference.

These stacked combinations can be created by any method including, but not limited to, cross-breeding plants by any conventional or TopCross methodology, or genetic transformation. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO99/25821 , WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

V. Method of Introducing

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 polynucleotide or polypeptides into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

"Stable transformation" is intended to mean that the nucleotide construct introduced into a plant 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 polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a 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, /Agrobacteπt/m-mediated transformation (U.S. Patent Number 5,563,055 and U.S. Patent Number 5,981 ,840), direct gene transfer (Paszkowski, *et al.,* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent Number 4,945,050; U.S. Patent Number 5,879,918; U.S. Patent Number 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 Led 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); Klein, *et al.,* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein, *et al.,* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Numbers 5,240,855; 5,322,783; and 5,324,646; Klein, *et al.,* (1988) *Plant Physiol.* 9 1:440-444 (maize); Fromm, *et al.,* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren, *et al.,* (1984) *Nature (London)* 3 1 1:763-764; U.S. 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 ZMM28 sequences or variants and fragments thereof 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 ZMM28 protein or variants and fragments thereof directly into the plant or the introduction of the ZMM28 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 ZMM28 polynucleotide can be transiently transformed into the plant using 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, the 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 particles coated with polyethylimine (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 the a ZMM28 sequence or a variant or fragment thereof 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 of 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, U.S. 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, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference. Briefly, the polynucleotide of the invention can be contained in 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 ways. See, for example, McCormick, *et al.,* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting progeny having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. 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 their genome.

VI. Methods of Use

A. Methods for Modulating Expression of at Least One ZMM28 Sequence or a Variant or Fragment Therefore in a Plant or Plant Part

A "modulated level" or "modulating level" of a polypeptide in the context of the methods of the present invention refers to any increase or decrease in the expression, concentration, or activity of a gene product, including any relative increment in expression, concentration or activity. Any method or composition that modulates expression of a target gene product, either at the level of transcription or translation, or modulates the activity of the target gene product can be used to achieve modulated expression, concentration, activity of the target gene product. In general, the level is increased or decreased by at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater relative to an appropriate control plant, plant part, or cell. Modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development. In specific embodiments, the polypeptides of the present invention are modulated in monocots, particularly grain plants such as rice, wheat, maize, and the like.

The expression level of a polypeptide having a ZMM28 MADS domain or a biologically active variant or fragment thereof may be measured directly, for example, by assaying for the level of the ZMM28 polypeptide in the plant, or indirectly, for example, by measuring the level of the polynucleotide encoding the protein or by measuring the activity of the ZMM28 polypeptide in the plant. Methods for determining the activity of the ZMM28 polypeptide are described elsewhere herein.

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 an 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, U.S. 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 thereof 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 the polynucleotide into a cell. For example, the polynucleotide, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome of the present invention 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 comprises at least one nucleotide.

In one embodiment, the activity and/or level of a ZMM28 polypeptide is increased. An increase in the level and/or activity of the ZMM28 polypeptide can be achieved by providing to the plant a ZMM28 polypeptide or a biologically active variant or fragment thereof. 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 ZMM28 polypeptide into the plant or introducing into the plant (transiently or stably) a polynucleotide construct encoding a polypeptide having ZMM28 activity. 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 an RNA. Thus, the level and/or activity of a ZMM28 polypeptide may be increased by altering the gene encoding the ZMM28 polypeptide or its promoter. See, e.g., Kmiec, U.S. Patent Number 5,565,350; Zarling, *et al.,* PCT/US93/03868. Therefore, mutagenized plants that carry mutations in ZMM28 genes, where the mutations increase expression of the ZMM28 gene or increase the activity of the encoded ZMM28 polypeptide, are provided.

In other embodiments, the activity and/or level of the ZMM28 polypeptide of the invention is reduced or eliminated by introducing into a plant a polynucleotide that inhibits the level or activity of a polypeptide. The polynucleotide may inhibit the expression of ZMM28 gene directly, by preventing translation of the ZMM28 messenger RNA, or indirectly, by encoding a polypeptide that inhibits the transcription or translation of a ZMM28 gene encoding a ZMM28 protein. 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 at least one ZMM28 sequence in a plant. In other embodiments of the invention, the activity of a ZMM28 polypeptide is reduced or eliminated by transforming a plant cell with a sequence encoding a polypeptide that inhibits the activity of the ZMM28 polypeptide. In other embodiments, the activity of a ZMM28 polypeptide may be reduced or eliminated by disrupting the gene encoding the ZMM28 polypeptide. The invention encompasses mutagenized plants that carry mutations in ZMM28 genes, where the mutations reduce expression of the ZMM28 gene or inhibit the ZMM28 activity of the encoded ZMM28 polypeptide.

Reduction of the activity of specific genes (also known as gene silencing or gene suppression) is desirable for several aspects of genetic engineering in plants. Many techniques for gene silencing are well known to one of skill in the art, including, but not limited to, antisense technology (see, e.g., Sheehy, *et al.,* (1988) *Proc. Natl. Acad. Sci. USA* 85:8805-8809; and U.S. Patent Numbers 5,107,065; 5,453,566; and 5,759,829); cosuppression (e.g., Taylor (1997) *Plant Cell* 9:1245; Jorgensen (1990) *Trends Biotech.* 8(12):340-344; Flavell (1994) *Proc. Natl. Acad. Sci. USA* 91:3490-3496; Finnegan, *et al.,* (1994) *Bio/Technology* 12:883-888; and Neuhuber, *et al.,* (1994) *Mol. Gen. Genet.* 244:230-241); RNA interference (Napoli, *et al.,* (1990) *Plant Cell* 2:279-289; U.S. Patent Number 5,034,323; Sharp (1999) *Genes Dev.* 13:139-141 ; Zamore, *et al.,* (2000) *Cell* 101 :25-33; and Montgomery, *et al.,* (1998) *Proc. Natl. Acad. Sci. USA* 95:15502-15507), virus-induced gene silencing (Burton, *et al.,* (2000) *Plant*

Cell 12:691-705; and Baulcombe (1999) *Curr. Op. Plant Bio.* 2:109-1 13); target-RNA-specific ribozymes (Haseloff, *et al.,* (1988) *Nature* 334:585-591); hairpin structures (Smith, *et al.,* (2000) *Nature* 407:319-320; WO 99/53050; WO 02/00904; WO 98/53083; Chuang and Meyerowitz (2000) *Proc. Natl. Acad. ScL USA* 97:4985-4990; Stoutjesdijk, *et al.,* (2002) *Plant*
5 *Physiol.* 129:1723-1731 ; Waterhouse and Helliwell (2003) *Nat. Rev. Genet.* 4:29-38; Pandolfini, *et al., BMC Biotechnology* 3:7, U.S. Patent Publication Number 20030175965; Panstruga, *et al.,* (2003) *Mol. Biol. Rep.* 30:135-140; Wesley, *et al.,* (2001) *Plant J.* 27:581-590; Wang and Waterhouse (2001) *Curr. Opin. Plant Biol.* 5:146-150; U.S. Patent Publication Number 20030180945; and WO 02/00904, all of which are herein incorporated by reference);
10 ribozymes (Steinecke, *et al.,* (1992) *EMBO J.* 11:1525; and Perriman, *et al.,* (1993) *Antisense Res. Dev.* 3:253); oligonucleotide-mediated targeted modification (e.g., WO 03/076574 and WO 99/25853); Zn-finger targeted molecules (e.g., WO 01/52620; WO 03/048345; and WO 00/42219); transposon tagging (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-
15 274; 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; Bensen, *et al.,* (1995) *Plant Cell* 7:75-84; Mena, *et al.,* (1996) *Science* 274:1537-1540; and U.S. Patent Number 5,962,764); each of which is herein incorporated by reference; and other methods or combinations of the above methods known to those of skill in the art.

20

It is recognized that with the polynucleotides of the invention, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the ZMM28 sequences can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences
25 hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, optimally 80%, more optimally 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, 300, 400, 450, 500,
30 550 or greater may be used.

The polynucleotides of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using polynucleotides in the sense orientation are known in the art. The
35 methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a polynucleotide that corresponds to the transcript of the endogenous gene. 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, U.S. Patent Numbers 5,283,184 and 5,034,323; herein incorporated by reference.

5

Thus, many methods may be used to reduce or eliminate the activity of a ZMM28 polypeptide or a biologically active variant or fragment thereof. In addition, combinations of methods may be employed to reduce or eliminate the activity of at least one ZMM28 polypeptide. It is further recognized that the level of a single ZMM28 sequence can be modulated to produce the
10 desired phenotype. Alternatively, is may be desirable to modulate (increase and/or decrease) the level of expression of multiple sequences having a ZMM28 MADS domain or a biologically active variant or fragment thereof.

As discussed above, a variety of promoters can be employed to modulate the level of the
15 ZMM28 sequence. In one embodiment, the expression of the heterologous polynucleotide which modulates the level of at least one ZMM28 polypeptide can be regulated by a tissue-preferred promoter, particularly, a leaf-preferred promoter (i.e., mesophyll-preferred promoter or a bundle sheath preferred promoter) and/or a seed-preferred promoter (i.e., an endosperm-preferred promoter or an embryo-preferred promoter).

20

B. Methods to Modulate Floral Organ Development and Yield in a Plant

The ZMM28 nucleic acid molecules of the invention encode a protein that may function as a transcription factor. Additionally, ZMM28 may play a role in floral development. ZMM28 has a phenotype that includes enhanced yield and yield components.

25

Accordingly, methods and compositions are provided to modulate ZMM28 and ZMM28 polypeptides and thus to modulate floral organ development and yield in plants. In one embodiment, the compositions of the invention can be used to increase grain yield in cereal plants. In this embodiment, the ZMM28 coding sequence is expressed in a cereal plant of
30 interest to increase expression of the ZMM28 transcription factor.

In this manner, the methods and compositions can be used to increase yield in a plant. As used herein, the term "improved yield" means any improvement in the yield of any measured plant product. The improvement in yield can comprise a 0.1%, 0.5%, 1%, 3%, 5%, 10%, 15%,
35 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater increase in measured plant product. Alternatively, the increased plant yield can comprise about a 0.5 fold, 1 fold, 2 fold, 4 fold, 8 fold, 16 fold or 32 fold increase in measured plant products. For example, an increase in the

bu/acre yield of soybeans or corn derived from a crop having the present treatment as compared with the bu/acre yield from untreated soybeans or corn cultivated under the same conditions would be considered an improved yield. By increased yield is also intended at least one of an increase in total seed numbers, an increase in total seed weight, an increase in root
5 biomass and an increase in harvest index. Harvest index is defined as the ratio of yield biomass to the total cumulative biomass at harvest.

Accordingly, various methods to increase yield of a plant are provided. In one embodiment, increasing yield of a plant or plant part comprises introducing into the plant or plant part a
10 heterologous polynucleotide; and, expressing the heterologous polynucleotide in the plant or plant part. In this method, the expression of the heterologous polynucleotide modulates the level of at least one ZMM28 polypeptide in the plant or plant part, where the ZMM28 polypeptide comprises a ZMM28 MADS domain having an amino acid sequence set forth in SEQ ID NO: 8 or a variant or fragment of the domain.

15

In specific embodiments, modulation of the level of the ZMM28 polypeptide comprises an increase in the level of at least one ZMM28 polypeptide. In such methods, the heterologous polynucleotide introduced into the plant encodes a polypeptide having a ZMM28 MADS domain or a biologically active variant or fragment thereof. In specific embodiments, the
20 heterologous polynucleotide comprises the sequence set forth in at least one SEQ ID NO: 1 and/or a biologically active variant or fragment thereof.

In other embodiments, modulating the level of at least one ZMM28 polypeptide comprises decreasing in the level of at least one ZMM28 polypeptide. In such methods, the heterologous
25 polynucleotide introduced into the plant need not encode a functional ZMM28 polypeptide, but rather the expression of the polynucleotide results in the decreased expression of a ZMM28 polypeptide comprising a ZMM28 MADS domain or a biologically active variant or fragment of the ZMM28 MADS domain. In specific embodiments, the ZMM28 polypeptide having the decreased level is set forth in SEQ ID NO: 2 or a biologically active variant or fragment thereof.

30

Items

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:
   (a) the nucleotide sequence set forth in SEQ ID NO: 1;
35 (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2;

(c) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1, wherein said nucleotide sequence encodes a polypeptide having ZMM28 protein activity;
(d) a nucleotide sequence comprising at least 50 consecutive nucleotides of SEQ ID NO: 1 or a complement thereof; and,
(e) a nucleotide sequence encoding an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2, wherein said nucleotide sequence encodes a polypeptide having ZMM28 protein activity.

2. An expression cassette comprising the polynucleotide of item 1.
3. The expression cassette of item 2, wherein said polynucleotide is operably linked to a promoter that drives expression in a plant.
4. The expression cassette of item 3, wherein said polynucleotide is operably linked to a constitutive promoter.
5. A plant comprising the expression cassette of item 3 or item 4.
6. The plant of item 5, wherein said plant is a monocot.
7. The plant of item 6, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.
8. The plant of item 7, wherein said monocot is rice.
9. The plant of item 7, wherein said monocot is maize.
10. The plant of item 5, wherein said plant has an increased level of a polypeptide selected from the group consisting of:
(a) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2;
(b) a polypeptide having at least 90% sequence identity to SEQ ID NO: 2, wherein said polypeptide has ZMM28 protein activity; and
(c) a polypeptide comprising a ZMM28 MADS domain set forth in SEQ ID NO: 8.
11. The plant of item 5, wherein said plant has a phenotype selected from the group consisting of:
(a) an increased total seed number;
(b) an increased total seed weight;
(c) an increased harvest index; and
(d) an increased root biomass.
12. A method of increasing the level of a polypeptide in a plant comprising introducing into said plant the expression cassette of item 3 or item 4.
13. The method of item 12, wherein the yield of the plant is increased.
14. The method of item 12, wherein increasing the level of said polypeptide produces a phenotype in the plant selected from the group consisting of:
(a) an increased total seed number;
(b) an increased total seed weight;

(c) an increased harvest index; and

(d) an increased root biomass.

15. The method of item 13, wherein said expression cassette is stably integrated into the genome of the plant.

5 16. The method of item 13, wherein said plant is a monocot.

17. The method of item 16, wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

18. The method of item 17, wherein said monocot is rice.

19. The method of item 17, wherein said monocot is maize.

10 20. A method of increasing yield in a plant comprising increasing expression of a ZMM28 polypeptide in said plant, wherein said ZMM28 polypeptide has ZMM28 protein activity and is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 80% sequence identity to the sequence set forth in SEQ ID NO: 2; and

15 (b) a polypeptide comprising a ZMM28 MADS domain set forth in SEQ ID NO: 8.

21. The method of item 20, wherein said polypeptide comprises an amino acid sequence having at least 95% sequence identity with the sequence set forth in SEQ ID NO: 2.

22. The method of item 20, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2.

20 23. The method of any one of items 20 through 22, comprising introducing into said plant an expression cassette comprising a polynucleotide encoding said ZMM28 polypeptide operably linked to a promoter that drives expression in a plant cell, wherein said polynucleotide comprises a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 1;

25 (b) a nucleotide sequence encoding the polypeptide of SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 95% sequence identity to the sequence set forth in SEQ ID NO: 1;

(d) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2; and,

30 (e) a nucleotide sequence encoding an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO: 2.

24. The method of item 23, comprising:

(a) transforming a plant cell with said expression cassette; and

(b) regenerating a transformed plant from the transformed plant cell of step (a).

35 25. The method of item 23 or item 24, wherein said expression cassette is stably incorporated into the sequence of the plant.

26. The method of item 23, wherein said promoter is a constitutive promoter.

27. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:
    (a) the amino acid sequence comprising SEQ ID NO: 2;
    (b) the amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2, wherein said polypeptide has the ability to modulate transcription; and,
    (c) the amino acid sequence comprising at least 30 consecutive amino acids of SEQ ID NO: 2, wherein said polypeptide retains the ability to modulate transcription.

Experimental

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

Example 1: Cloning of Maize ZMM28 Gene

The cDNA that encoded the ZMM28 polypeptide from maize was identified by sequence homology from a collection of ESTs generated from a maize cDNA library using BLAST 2.0 (Altschul, *et al.,* (1990) *J. Mol. Biol.* 215:403) against the NCBI DNA sequence database. From the EST plasmid, the maize ZMM28 cDNA fragment was amplified by PCR using Hifi Taq DNA polymerase in standard conditions with maize ZMM28-specific primers that included the AttB site for GATEWAY® recombination cloning. A PCR fragment of the expected length was amplified and purified using standard methods as described by Sambrook, *et al.,* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). The first step of the GATEWAY® procedure, the BP reaction, was then performed, during which the PCR fragment recombined *in vivo* with the pDONR201 plasmid to produce the "entry clone." Plasmid pDONR201 was purchased from Invitrogen, as part of the GATEWAY® technology (Invitrogen, Carlsbad, CA).

Example 2: Vector Construction (pGOS2::ZMM28)

The entry clone was subsequently used in an LR reaction with a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders, a plant selectable marker, a screenable marker, and a GATEWAY® cassette intended for LR in vivo recombination with the sequence of interest already cloned in the entry clone. Upstream of this GATEWAY® cassette is the rice GOS2 promoter (Hensgens, *et al.,* (1993) *Plant Mol. Biol.* 23:643-669) that confers moderate constitutive expression on the gene of interest. After the LR recombination step, the resulting expression vector pGOS2::ZMM28 was transformed into *Agrobacterium tumefaciens* strain LBA4044 and subsequently into *Oryza sativa* var. Nipponbare plants (see, Chan, M.T., *et al.,* (1993) *Plant Mol Biol,* 22(3):491-506, and Chan, MT., *et al.,* (1992) *Plant Cell Physiol,* 33(5):577-583). Transformed rice plants were grown and examined for various growth characteristics as described herein in Example 4.

Example 3: Rice Transformation Method

High-velocity ballistic bombardment using metal particles coated with the nucleic acid constructs was used to transform wild-type rice (Klein, *et al.,* (1987) *Nature* 327:70-73; U.S. Patent Number 4,945,050, incorporated by reference herein). A Biolistic PDS-1000/He (BioRAD Laboratories, Hercules, CA) was used for these complementation experiments. The particle bombardment technique was used to transform wild-type rice with the pGOS2::ZMM28. The bacterial hygromycin B phosphotransferase (Hpt II) gene from *Streptomyces hygroscopicus* (which confers resistance to the antibiotic) was used as the selectable marker for rice transformation. In the vector, pML18, the Hpt II gene was engineered with the 35S promoter from Cauliflower Mosaic Virus and the termination and polyadenylation signals from the octopine synthase gene of *Agrobacterium tumefaciens.* pML18 is described in WO 97/47731 , the disclosure of which is hereby incorporated by reference.

Embryogenic callus cultures derived from the scutellum of germinating rice seeds served 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 then 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 DNA fragment was 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 were added to a 50 µl aliquot of gold particles that had 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) were then added to the gold-DNA suspension as the tube was vortexing for 3 min. The gold particles were centrifuged in a microfuge for 1 second and the supernatant removed. The gold particles were then washed

twice with 1 ml of absolute ethanol and resuspended in 50 µl of absolute ethanol and sonicated (bath sonicator) for one second to disperse the gold particles. The gold suspension was incubated at -70^{0}C for five minutes and sonicated (bath sonicator) to disperse the particles. Six µl of the DNA-coated gold particles was then loaded onto mylar macrocarrier disks and the
5 ethanol was allowed to evaporate.

At the end of the drying period, a petri dish containing the tissue was placed in the chamber of the PDS-1000/He. The air in the chamber was then evacuated to a vacuum of 28-29 inches Hg. The macrocarrier was accelerated with a helium shock wave using a rupture membrane
10 that bursts when the He pressure in the shock tube reaches 1080-1 100 psi. The tissue was placed approximately 8 cm from the stopping screen and the callus was bombarded two times. Two to four plates of tissue were bombarded in this way with the DNA-coated gold particles. Following bombardment, the callus tissue was transferred to CM media without supplemental sorbitol or mannitol.
15

Three to five days after bombardment, the callus tissue was transferred to SM media (CM medium containing 50 mg/l hygromycin). To accomplish this, callus tissue was transferred from plates to sterile 50 ml conical tubes and weighed. Molten top-agar at 40^{0}C was added using 2.5 ml of top agar/100 mg of callus. Callus clumps were broken into fragments of less
20 than 2 mm diameter by repeated dispensing through a 10 ml pipette. Three ml aliquots of the callus suspension were plated onto fresh SM media and the plates were incubated in the dark for 4 weeks at 27-28°C. After 4 weeks, transgenic callus events were identified, transferred to fresh SM plates and grown for an additional 2 weeks in the dark at 27-28°C.
Growing callus was transferred to RM1 media (MS salts, Nitsch and Nitsch vitamins, 2%
25 sucrose, 3% sorbitol, 0.4% gelrite +50 ppm hyg B) for 2 weeks in the dark at 25°C. After 2 weeks the callus was transferred to RM2 media (MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 0.4% gelrite + 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 began to organize and form shoots. Shoots were removed from surrounding callus/media and gently
30 transferred to RM3 media (1/2 x MS salts, Nitsch and Nitsch vitamins, 1% sucrose + 50 ppm hygromycin B) in phytatrays (Sigma Chemical Co., St. Louis, MO) and incubation was continued using the same conditions as described in the previous step. The resultant TO transformants were transferred from RM3 to 4" pots containing Metro mix 350 after 2-3 weeks, when sufficient root and shoot growth had occurred.

35

Example 4: Overexpression of a ZMM28 Sequence to Increase Yield In Rice

Evaluation of TO, T1, and T2 Rice Plants Transformed with pGOS2::ZMM28

Approximately 15 to 20 independent TO transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. Six events of which the T1 progeny segregated 3/1 for presence/absence of the transgene were retained. "Null plants" or "Null segregants" or "Nullizygotes" are the plants treated in the same way as a transgenic plant, but from which the transgene has segregated. Null plants can also be described as the homozygous negative transformants. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homozygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by PCR.

Based on the results of the T1 evaluation (described herein), four events that showed improved growth and yield characteristics at the T1 level were chosen for further characterization in the T2 generation. To this extent, seed batches from the positive T1 plants (both hetero- and homozygotes), were screened by monitoring marker expression. For each chosen event, the heterozygote seed batches were then selected for T2 evaluation. An equal number of positive and negative plants within each seed batch were transplanted for evaluation in the greenhouse (i.e., for each event 40 plants, of which 20 were positives for the transgene and 20 were negative for the transgene). For the four events, a total of 160 plants were evaluated in the T2 generation. Both T1 and T2 plants were transferred to a greenhouse and evaluated for vegetative growth parameters, as described herein.

Statistical Analyses on Transgenic T1 & T2 lines

A two-factor ANOVA (analyses of variance) corrected for the unbalanced design was used as a statistical evaluation model for the numeric values of the observed plant phenotypic characteristics. The numerical values were submitted to a t-test and an F-test. The p-value was obtained by comparing the t-value to the t-distribution or, alternatively, by comparing the F-value to the F-distribution. The p-value stands for the probability that the null hypothesis (i.e., no effect of the transgene) is correct.

A t-test was performed on all the values of all plants per event. Such a t-test was repeated for each event and for each growth characteristic. The t-test was carried out to check for an effect of the gene within one transformation event, also described herein as "line-specific effect." In the t-test, the threshold for a significant line-specific effect is set at 10% probability level. Therefore, data with a p-value of the t-test under 10% means that the phenotype observed in the transgenic plants of that line was caused by the presence of the transgene. Within one

population of transformation events, some events may be under or below this threshold. This difference may be due to the difference in the position of the transgene within the rice genome (i.e., a gene might only have an effect in certain positions of the genome). Therefore, the "line-specific effect" is sometimes referred to as the "position-dependent effect."

An F-test was carried out on all the values measured for all plants of all events. An F-test was repeated for each growth characteristic. The F-test was conducted to check for an effect of the gene over all the transformation events and to verify an overall effect of the gene, also described herein as the "gene effect." In the F-test, the threshold for a significant global gene effect is set at 5% probability level. Therefore, data with a p-value of the F-test under 5% means that the observed phenotype was caused by more than just the presence of the gene, and/or the position of the transgene within the genome. A "gene effect" is an indication for the wide applicability of the gene in transgenic plants.

Vegetative Growth Measurements

The selected plants were grown in a greenhouse. Each plant received a unique barcode label to link the phenotyping data unambiguously to the corresponding plant. The selected plants were grown on soil in 10 cm diameter, clear-bottom pots under the following environmental settings: photoperiod=1 1.5 hours; daylight intensity=30,000 lux or more; daytime temperature=28°C or higher; night-time temperature=22°C; and relative humidity=60-70%. Transgenic plants and the corresponding nullizygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity (i.e., the stage were there is no more increase in biomass), the plants were passed weekly through a digital imaging cabinet. At each time point digital images (2048x1536 pixels, 16 million colors) were taken of each plant from at least 6 different angles. The parameters described herein were derived in an automated way from the digital images using image analysis software.

Plants were also passed through a root-imaging system that digitally photographs the root morphology and mass from the base of the clear-bottom pots. Plant above-ground area and root mass were determined by counting the total number of pixels from plant parts discriminated from the background. The above-ground value was averaged for the pictures taken on the same time point from the different angles and was converted to a physical surface value expressed in square mm by calibration. Experiments have shown that the above-ground plant area, which corresponds to the total maximum area, measured this way correlates with the biomass of plant parts above-ground.

In addition to digital images during the growth of the plants, when the plants reached maturity and senescence the number of panicles per plant and the total number of florets per plant were counted by hand. Dried florets were collected and those with filled seeds were mechanically separated from empty florets using an enclosed air-driven blower system. Dehusked seeds were then collected and counted using a seed counter and weighed using a standard balance. Harvest index was calculated using a ratio of the total weight of seeds produced per plant with the biomass calculated from digital images as described herein. Thousand kernel weight was calculated from the ratio of total seed weight per plant and the number of filled seeds per plant times 1000. The time to flower interval was recorded as the number of days between sowing and the emergence of the first panicle, extrapolated by the size of the panicles in the earliest imaging that a panicle was detected and the date of that imaging.

Overall Effects of ZMM28 in Rice

On the average of five events examined, pGOS2::ZMM28 transgenic plants in the T1 generation showed a statistically significant increase of 12% in the number of flowers per panicle, 22% in total seed number per plant, a 56% increase in the number of seeds filled per plant, a 53% increase in total seed weight per plant, and a 48% increase in harvest index with p-values less than 0.02, as compared to the nullizygotes. These data show that the constitutively expressed ZMM28 gene confers a strong positive effect on several important yield traits in a plant.

Example 5: Overexpression of ZMM28 Sequences in Maize

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a ZMM28 sequence (such as ZMM28/SEQ ID NO: 1) under the control of the UBI promoter and the selectable marker gene PAT (Wohlleben, *et al.,* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

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

A plasmid vector comprising the ZMM28 sequence operably linked to a ubiquitin promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows: 100 µl prepared tungsten particles in water; 10 µl (1 µg) DNA in Tris
5 EDTA buffer (1 µg total DNA); 100 µl 2.5 M $CaCl_2$; 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
10 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.
15

The sample plates are bombarded at level #4 in particle gun (U.S. Patent Number 5,240,855). All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

20 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
25 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
30 monitored and scored for an increase in nitrogen use efficiency, increase yield, or an increase in stress tolerance.

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
35 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 (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-151 1), 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 (added after bringing to volume with D-I H_2O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both
5 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)
10 *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 (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 60^0C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock
15 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 60^0C.

20 ***Example 6: Agrobacterium-mediated Transformation***

For Agrobacterium-mediated transformation of maize with a ZMM28 polynucleotide the method of Zhao is employed (U.S. 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
25 the bacteria are capable of transferring the ZMM28 polynucleotide 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
30 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.
35 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: Soybean Embryo Transformation

5 Culture Conditions

Soybean embryogenic suspension cultures (cv. Jack) are maintained in 35 ml liquid medium SB196 (see recipes below) on rotary shaker, 150 rpm, 26°C with cool white fluorescent lights on 16:8 hr day/night photoperiod at light intensity of 60-85 µE/m2/s. Cultures are subcultured every 7 days to two weeks by inoculating approximately 35 mg of tissue into 35 ml of fresh
10 liquid SB196 (the preferred subculture interval is every 7 days).

Soybean embryogenic suspension cultures are transformed with the plasmids and DNA fragments described in the following examples by the method of particle gun bombardment (Klein, *et al.,* (1987) *Nature* 327:70).

15 Soybean Embrvogenic Suspension Culture Initiation

Soybean cultures are initiated twice each month with 5-7 days between each initiation.

Pods with immature seeds from available soybean plants 45-55 days after planting are picked, removed from their shells and placed into a sterilized magenta box. The soybean seeds are sterilized by shaking them for 15 minutes in a 5% Clorox solution with 1 drop of ivory soap (95
20 ml of autoclaved distilled water plus 5 ml Clorox and 1 drop of soap). Mix well. Seeds are rinsed using 2 1-liter bottles of sterile distilled water and those less than 4 mm are placed on individual microscope slides. The small end of the seeds are cut and the cotyledons pressed out of the seed coat. Cotyledons are transferred to plates containing SB1 medium (25-30 cotyledons per plate). Plates are wrapped with fiber tape and stored for 8 weeks. After this
25 time secondary embryos are cut and placed into SB196 liquid media for 7 days.

Preparation of DNA for Bombardment

Either an intact plasmid or a DNA plasmid fragment containing the genes of interest and the selectable marker gene are used for bombardment. Plasmid DNA for bombardment are
30 routinely prepared and purified using the method described in the Promega™ Protocols and Applications Guide, Second Edition (page 106). Fragments of the plasmids carrying a ZMM28 polynucleotide are obtained by gel isolation of double digested plasmids. In each case, 100 µg of plasmid DNA is digested in 0.5 ml of the specific enzyme mix that is appropriate for the plasmid of interest. The resulting DNA fragments are separated by gel electrophoresis on 1%
35 SeaPlaque GTG agarose (BioWhitaker Molecular Applications) and the DNA fragments containing the ZMM28 polynucleotide are cut from the agarose gel. DNA is purified from the agarose using the GELase digesting enzyme following the manufacturer's protocol.

A 50 µl aliquot of sterile distilled water containing 3 mg of gold particles (3 mg gold) is added to 5 µl of a 1 µg/µl DNA solution (either intact plasmid or DNA fragment prepared as described above), 50 µl 2.5M $CaCl_2$ and 20 µl of 0.1 M spermidine. The mixture is shaken 3 min on level 3 of a vortex shaker and spun for 10 sec in a bench microfuge. After a wash with 400 µl 100% ethanol the pellet is suspended by sonication in 40 µl of 100% ethanol. Five µl of DNA suspension is dispensed to each flying disk of the Biolistic PDS1000/HE instrument disk. Each 5 µl aliquot contains approximately 0.375 mg gold per bombardment (i.e., per disk).

Tissue Preparation and Bombardment with DNA

Approximately 150-200 mg of 7 day old embryonic suspension cultures are placed in an empty, sterile 60 x 15 mm petri dish and the dish covered with plastic mesh. Tissue is bombarded 1 or 2 shots per plate with membrane rupture pressure set at 1100 PSI and the chamber evacuated to a vacuum of 27-28 inches of mercury. Tissue is placed approximately 3.5 inches from the retaining/stopping screen.

Selection of Transformed Embryos

Transformed embryos were selected either using hygromycin (when the hygromycin phosphotransferase, HPT, gene was used as the selectable marker) or chlorsulfuron (when the acetolactate synthase, ALS, gene was used as the selectable marker).

Hvgromvcin (HPT) Selection

Following bombardment, the tissue is placed into fresh SB196 media and cultured as described above. Six days post-bombardment, the SB196 is exchanged with fresh SB196 containing a selection agent of 30 mg/L hygromycin. The selection media is refreshed weekly. Four to six weeks post selection, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated, green tissue is removed and inoculated into multiwell plates to generate new, clonally propagated, transformed embryogenic suspension cultures.

Chlorsulfuron (ALS) Selection

Following bombardment, the tissue is divided between 2 flasks with fresh SB196 media and cultured as described above. Six to seven days post-bombardment, the SB196 is exchanged with fresh SB196 containing selection agent of 100 ng/ml Chlorsulfuron. The selection media is refreshed weekly. Four to six weeks post selection, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated, green tissue

is removed and inoculated into multiwell plates containing SB196 to generate new, clonally propagated, transformed embryogenic suspension cultures.

Regeneration of Soybean Somatic Embryos into Plants

5 In order to obtain whole plants from embryogenic suspension cultures, the tissue must be regenerated.

Embryo Maturation

Embryos are cultured for 4-6 weeks at 26°C in SB196 under cool white fluorescent (Phillips 10 cool white Econowatt F40/CW/RS/EW) and Agro (Phillips F40 Agro) bulbs (40 watt) on a 16:8 hr photoperiod with light intensity of 90-120 uE/m2s. After this time embryo clusters are removed to a solid agar media, SB166, for 1-2 weeks. Clusters are then subcultured to medium SB103 for 3 weeks. During this period, individual embryos can be removed from the clusters and screened for levels of ZMM28 expression and/or activity.

15

Embryo Desiccation and Germination

Matured individual embryos are desiccated by placing them into an empty, small petri dish (35 x 10 mm) for approximately 4-7 days. The plates are sealed with fiber tape (creating a small humidity chamber). Desiccated embryos are planted into SB71-4 medium where they were left 20 to germinate under the same culture conditions described above. Germinated plantlets are removed from germination medium and rinsed thoroughly with water and then planted in Redi-Earth in 24-cell pack tray, covered with clear plastic dome. After 2 weeks the dome is removed and plants hardened off for a further week. If plantlets looked hardy they are transplanted to 10" pot of Redi-Earth with up to 3 plantlets per pot. After 10 to 16 weeks, 25 mature seeds are harvested, chipped and analyzed for proteins.

Media Recipes

SB 196 - FN Lite liquid proliferation medium (per liter) -

MS FeEDTA - 100x Stock 1	10 ml
30 MS Sulfate - 100x Stock 2	10 ml
FN Lite Halides - 100x Stock 3	10 ml
FN Lite P, B, Mo - 10Ox Stock 4	10 ml
B5 vitamins (1 ml/L)	1.0 ml
2,4-D (10 mg/L final concentration)	1.0 ml
35 KNO_3	2.83 gm
$(NhU)_2SO_4$	0.463 gm
Asparagine	1.0 gm

Sucrose (1%)	10 gm
pH 5.8	

FN Lite Stock Solutions

Stock #			1000 ml	500 ml
1	MS Fe EDTA IOOx Stock			
		Na_2 EDTA*	3.724 g	1.862 g
		$FeSO_4$ - $7H_2O$	2.784 g	1.392 g
	* Add first, dissolve in dark bottle while stirring			
2	MS Sulfate 10Ox stock			
		$MgSO_4$ - $7H_2O$	37.O g	18.5 g
		$MnSO_4$ - H_2O	1.69 g	0.845 g
		$ZnSO_4$ - $7H_2O$	0.86 g	0.43 g
		$CuSO_4$ - $5H_2O$	0.0025 g	0.00125 g
3	FN Lite Halides 10Ox Stock			
		$CaCl_2$ - $2H_2O$	30.O g	15.Og
		KI	0.083 g	0.0715 g
		$CoCl_2$ - $6H_2O$	0.0025 g	0.00125 g
4	FN Lite P, B, Mo 10Ox Stock			
		KH_2PO_4	18.5 g	9.25 g
		H_3BO_3	0.62 g	0.31 g
		Na_2MoO_4 - $2H_2O$	0.025 g	0.0125 g

SB1 solid medium (per liter) comprises: 1 pkg. MS salts (GIBCO/BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 31.5 g sucrose; 2 ml 2,4-D (20 mg/L final concentration); pH 5.7; and, 8 g TC agar.

SB 166 solid medium (per liter) comprises: 1 pkg. MS salts (GIBCO/BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 60 g maltose; 750 mg $MgCl_2$ hexahydrate; 5 g activated charcoal; pH 5.7; and, 2 g gelrite.

SB 103 solid medium (per liter) comprises: 1 pkg. MS salts (GIBCO/BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 60 g maltose; 750 mg MgCl2 hexahydrate; pH 5.7; and, 2 g gelrite.

SB 71-4 solid medium (per liter) comprises: 1 bottle Gamborg's B5 salts w/ sucrose (GIBCO/BRL - Cat# 21153-036); pH 5.7; and, 5 g TC agar.

2,4-D stock is obtained premade from Phytotech cat# D 295 - concentration is 1 mg/ml.

B5 Vitamins Stock (per 100 ml) which is stored in aliquots at -20C comprises: 10 g myo-inositol; 100 mg nicotinic acid; 100 mg pyridoxine HCl; and, 1 g thiamine. If the solution does not dissolve quickly enough, apply a low level of heat via the hot stir plate.

Chlorsulfuron Stock comprises: 1 mg / ml in 0.01 N Ammonium Hydroxide.

Example 8: Variants of ZMM28 Sequences

A. Variant Nucleotide Sequences of ZMM28 That Do Not Alter the Encoded Amino Acid Sequence

The ZMM28 nucleotide sequences are used to generate variant nucleotide sequences having the nucleotide sequence of the open reading frame with about 70%, 75%, 80%, 85%, 90% and 95% nucleotide sequence identity when compared to the starting unaltered ORF nucleotide sequence of the corresponding SEQ ID NO. These functional variants are generated using a standard codon table. While the nucleotide sequence of the variants are altered, the amino acid sequence encoded by the open reading frames do not change.

B. Variant Amino Acid Sequences of ZMM28 Polypeptides

Variant amino acid sequences of the ZMM28 polypeptides are generated. In this example, one amino acid is altered. Specifically, the open reading frames are reviewed to determine the appropriate amino acid alteration. The selection of the amino acid to change is made by consulting the protein alignment (with the other orthologs and other gene family members from various species). 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). Using the protein alignment set forth in Figure 1, an appropriate amino acid can be changed. Once the targeted amino acid is identified, the procedure outlined in the following section C is followed. Variants having about 70%, 75%, 80%, 85%, 90% and 95% sequence identity are generated using this method.

C. Additional Variant Amino Acid Sequences of ZMM28 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
5 conserved regions among ZMM28 protein or among the other ZMM28 polypeptides. Based on the sequence alignment, the various regions of the ZMM28 polypeptide that can likely be altered are represented in lower case letters, while the conserved regions are represented by capital letters. It is recognized that conservative substitutions can be made in the conserved regions below without altering function. In addition, one of skill will understand that functional
10 variants of the ZMM28 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,
15 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.

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

First, any conserved amino acids in the protein that should not be changed is 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.

5

H, C, and P are not changed in any circumstance. 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 it 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
10 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
15 percent identities. Using this procedure, variants of the ZMM28 polypeptides are generating having about 80%, 85%, 90% and 95% amino acid identity to the starting unaltered ORF nucleotide sequence of SEQ ID NO: 1.

D. Disruption of Targeted Domains or Sequences of ZMM28 Polypeptides

20 Disrupted amino acid sequences of the ZMM28 polypeptides are generated. In this example, particular domains are disrupted or excluded from final polypeptide. If disrupting the N-terminal domain(s) or motif(s), the DNA codon for the starting ATG is altered by insertion, deletion or base substitution to prevent the translation of the first methionine. Generally the next available methionine will dominate the start of translation thus skipping the N-terminal
25 portion of the polypeptide. For ZMM28 gene, the first ATG can be altered to effectively prevent translation starting at this ATG and initiating downstream at amino acid position 63 thus removing the first 62 amino acids of SEQ ID NO: 2. If disrupting a C-terminal domain, a stop codon at the desired site is created by insertion, deletion or base substitution or more commonly by PCR as described below. Premature stops may lead to translation of
30 polypeptides missing the C-terminal domain(s).

An alternative method for selectively isolating a targeted domain(s) for expression is to design primers to PCR amplify the desired domain(s) with either a naturally occurring or engineered

ATG sequence at the 5' end of the clone and a naturally occurring or engineered stop codon at the 3' end of the clone. The resulting fragment will have the desired domain(s) to be cloned into expression vectors (see, Example 2). Variants of the isolated polypeptide domain(s) or motif(s) generated as described in Examples 8A, B, or C having about 70%, 75%, 80%, 85%, 5 90% and 95% sequence identity are generated using these methods.

The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

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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 15 by reference.

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

That which is claimed

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:
   (a) the nucleotide sequence set forth in SEQ ID NO: 1;
   (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2;
   (c) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1, wherein said nucleotide sequence encodes a polypeptide having ZMM28 protein activity;
   (d) a nucleotide sequence comprising at least 50 consecutive nucleotides of SEQ ID NO: 1 or a complement thereof; and,
   (e) a nucleotide sequence encoding an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 2, wherein said nucleotide sequence encodes a polypeptide having ZMM28 protein activity.
2. An expression cassette comprising the polynucleotide of claim 1.
3. The expression cassette of claim 2, wherein said polynucleotide is operably linked to a promoter that drives expression in a plant, preferably wherein said polynucleotide is operably linked to a constitutive promoter.
4. A plant comprising the expression cassette of claim 2 or claim 3, preferably wherein said plant is a monocot, further preferably wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.
5. The plant of claim 4, wherein said plant has an increased level of a polypeptide selected from the group consisting of:
   (a) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2;
   (b) a polypeptide having at least 90% sequence identity to SEQ ID NO: 2, wherein said polypeptide has ZMM28 protein activity; and
   (c) a polypeptide comprising a ZMM28 MADS domain set forth in SEQ ID NO: 8.
6. The plant of claim 4 or 5, wherein said plant has a phenotype selected from the group consisting of:
   (a) an increased total seed number;
   (b) an increased total seed weight;
   (c) an increased harvest index; and
   (d) an increased root biomass.
7. A method of increasing the level of a polypeptide in a plant comprising introducing into said plant the expression cassette of claim 2 or claim 3.
8. The method of claim 7, wherein the yield of the plant is increased.
9. The method of claim 7 or 8, wherein increasing the level of said polypeptide produces a phenotype in the plant selected from the group consisting of:

(a) an increased total seed number;

(b) an increased total seed weight;

(c) an increased harvest index; and

(d) an increased root biomass.

5 10. The method of any one of claims 7 to 9, wherein said expression cassette is stably integrated into the genome of the plant, preferably wherein said plant is a monocot, further preferably wherein said monocot is maize, wheat, rice, barley, sorghum, or rye.

11. A method of increasing yield in a plant comprising increasing expression of a ZMM28 polypeptide in said plant, wherein said ZMM28 polypeptide has ZMM28 protein activity
10 and is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having at least 80% sequence identity to the sequence set forth in SEQ ID NO: 2; and

(b) a polypeptide comprising a ZMM28 MADS domain set forth in SEQ ID NO: 8.

12. The method of claim 11, wherein said polypeptide comprises an amino acid sequence
15 having at least 95% sequence identity with the sequence set forth in SEQ ID NO: 2 or wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2.

13. The method of any one of claims 7 through 12, comprising introducing into said plant an expression cassette comprising a polynucleotide encoding said ZMM28 polypeptide operably linked to a promoter that drives expression in a plant cell, wherein said
20 polynucleotide comprises a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding the polypeptide of SEQ ID NO: 2;

(c) a nucleotide sequence comprising at least 95% sequence identity to the sequence set forth in SEQ ID NO: 1;

25 (d) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2; and,

(e) a nucleotide sequence encoding an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO: 2.

14. The method of claim 13, comprising:

30 (a) transforming a plant cell with said expression cassette; and

(b) regenerating a transformed plant from the transformed plant cell of step (a).

15. The method of claim 13 or claim 14, wherein said expression cassette is stably incorporated into the sequence of the plant.

16. The method of claim 13, wherein said promoter is a constitutive promoter.

35 17. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence comprising SEQ ID NO: 2;

(b) the amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 2, wherein said polypeptide has the ability to modulate transcription; and,

(c) the amino acid sequence comprising at least 30 consecutive amino acids of SEQ ID NO: 2, wherein said polypeptide retains the ability to modulate transcription.

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                           1                                                50
SEQ ID NO: 3     (1)   MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAHEISVLCDADVALIVFST
SEQ ID NO: 4     (1)   MGRGRVQLRRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVALIVFST
SEQ ID NO: 5     (1)   MGRGRVQLRRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEVALIVFST
SEQ ID NO: 6     (1)   MGRGRVQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSH
SEQ ID NO: 7     (1)   MGRGKVQLKRIENKINRQVTFSKRRGGLLKKAHELSVLCDAEVALIVFSN
SEQ ID NO: 2     (1)   MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAHEISVLCDAEVALIVFST

                           51                                              100
SEQ ID NO: 3    (51)   KGKLYEFSSHSS-MEGILERYQRYSFDERAVLEPNTEDQENWGDEYGILK
SEQ ID NO: 4    (51)   KGKLYEYSSQDSSMDVILERYQRYSFEERAVLDPSIGNQANWGDEYGSLK
SEQ ID NO: 5    (51)   KGKLYEYSSQDSSMDVILERYQRYSFEERAVLDPSTGDQANWGDEYGSLK
SEQ ID NO: 6    (51)   KGKLFEYSTDSC-MEKILERYERYSYAERQLIAPESDVNTNWSMEYNRLK
SEQ ID NO: 7    (51)   KGKLFEYSTDSC-MDRILEKYERYSFAERQLVSNEPQSPANWTLEYSKLK
SEQ ID NO: 2    (51)   KGKLYEYSSHSS-MEGILERYQRYSFEERAVLNPSIEDQANWGDEYVRLK

                           101                                             150
SEQ ID NO: 3   (100)   SKLDALQKSQRQLLGEQLDTLTIKELQQLEHQLEYSLKHIRSKKNQLLFE
SEQ ID NO: 4   (101)   IKLDAFQKSQRQLLGEQLGPLTTKELQQLEQQLDSSLKHIRSRKNQLLFE
SEQ ID NO: 5   (101)   IKLDALQKSQRQLLGEQLDPLTTKELQQLEQQLDSSLKHIRSRKNQLLFE
SEQ ID NO: 6   (100)   AKIELLERNQRHYLGEDLQAMSPKELQNLEQQLDTALKHIRTRKNQLMYE
SEQ ID NO: 7   (100)   ARIELLQRNHRHYMGEDLDSMSLKEIQSLEQQLDTALKNIRTRKNQLLYD
SEQ ID NO: 2   (100)   SKLDALQKSQRQLLGEQLSSLTIKELQQLEQQLDSSLKHIRSRKNQLMFD

                           151                                             200
SEQ ID NO: 3   (150)   SISELQKKEKSLKNQNNVLQK-LMETEKEKN------------NAIINTN
SEQ ID NO: 4   (151)   SISELQKKEKSLKDQNGVLQKHLVETEKEKNNVLSNIHHQEQLNGATNIN
SEQ ID NO: 5   (151)   SISELQKKEKSLKDQNGVLQKHLVETEKEKNNVLSNIHHREQLNEATNIH
SEQ ID NO: 6   (150)   SINELQKKEKAIQEQNSMLSKQIKEREKILRAQQE------------QWD
SEQ ID NO: 7   (150)   SISELQHKEKAIQEQNTMLAKKIKEKEKEIAQQP-------------QWE
SEQ ID NO: 2   (150)   SISALQKKEKALTDQNGVLQK-FMEAEKEKN------------KALMNAQ

                           201                                             250
SEQ ID NO: 3   (187)   R-EEQNGATPSTSSP------TPVTAPDPIPTTNNSQSQPR--GSGESEA
SEQ ID NO: 4   (201)   HQEQLNGATTSSPSP------TPATAQDSMATPNIGPYQSSESGGNPEP
SEQ ID NO: 5   (201)   HQEQLSGATTSSPSP------TPPTAQDSMAPPNIGPYQSR--GGGDPEP
SEQ ID NO: 6   (188)   QQNQGHNMPPPLPPQQHQIQHPYMLSHQPSPFLNMGGLYQE-----DDPM
SEQ ID NO: 7   (187)   HHRHHTNASIMPPP-------PQYSMAPQFPCINVGNTYEG-----EGAN
SEQ ID NO: 2   (187)   LREQQNGASTSSPSL------SPPIVPDSMPTLNIGPCQHR—GAAESES

                           251                      275
SEQ ID NO: 3   (228)   QPS--PAQAGNSKLPPWMLRTSHT-
SEQ ID NO: 4   (245)   QPS--PAQANNSNLPPWMLSTISSR
SEQ ID NO: 5   (243)   QPS--PAQANNSNLPPWMLRTIGNR
SEQ ID NO: 6   (233)   AMR-NDLELTLEPVYNCNLGCFAA-
SEQ ID NO: 7   (225)   EDRRNELDLTLDSLYSCHLGCFAA-
SEQ ID NO: 2   (229)   EPSPAPAQANRGNLPPWMLRTVK--
```

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/057076

A CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/415 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N AOIH

Documentation searched other than minimum documentation to the extent that such documents are Included in the fields searched

Electronic data base consulted during the international search (name of data base and where practical search terms used)

EPO-Internal , Sequence Search, WPI Data, BIOSIS, EMBASE

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category"	Citation of document with indication where appropriate of the relevant passages	Relevant to claim No
X	WO 2006/056590 A (CROPDESIGN NV [BE]; FRANKARD VALERIE [BE]) 1 June 2006 (2006-06-01) See SEQ ID NO: 3 and 4 the whole document -----	1-17

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents

'A' document defining the general state of the art which is not considered to be of particular relevance

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'X' document of particular relevance the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents such combination being obvious to a person skilled in the art

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2008	30/09/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040 Tx 31 651 epo nl Fax (+31-70) 340-3016	Oderwald, Harald

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/057076

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006056590 A	01-06-2006	AU 2005308768 A1	01-06-2006
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