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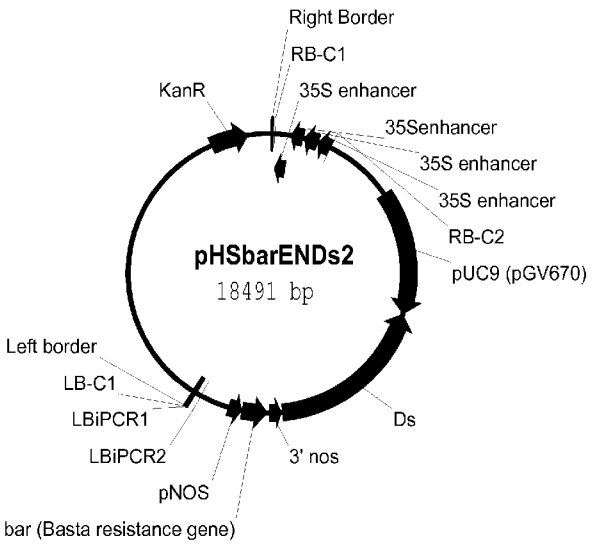
(54) Title: DROUGHT TOLERANT PLANTS AND METHODS INVOLVING GENES ENCODING TYPE C3HC4 RING FINGER ZINC-FINGER FAMILY POLYPEPTIDES

(57) Abstract: Isolated polynucleotides and polypeptides and recombinant DNA constructs useful for conferring drought tolerance, compositions (such as plants or seeds) comprising these recombinant DNA constructs, and methods utilizing these recombinant DNA constructs. The recombinant DNA construct comprises a polynucleotide operably linked to a promoter that is functional in a plant, wherein said polynucleotide encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide.

FIG. 1



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TITLE

DROUGHT TOLERANT PLANTS AND METHODS INVOLVING GENES ENCODING TYPE C3HC4 RING FINGER ZINC-FINGER FAMILY POLYPEPTIDES

This application claims the benefit of U.S. Provisional Application No. 61/158457, filed March 9, 2009, the entire content of which is herein incorporated by reference.

FIELD OF THE INVENTION

The field of invention relates to plant breeding and genetics and, in particular, relates to recombinant DNA constructs useful in plants for conferring tolerance to drought.

BACKGROUND OF THE INVENTION

Abiotic stressors significantly limit crop production worldwide. Cumulatively, these factors are estimated to be responsible for an average 70% reduction in agricultural production (Bresson, 1999).

Drought stress, in particular, not only causes a reduction in the average yield for crops but also causes yield instability through high interannual yield variation. Globally, about 35-40% of arable land falls under arid or semiarid classification. Even in non-arid regions where soils are nutrient-rich, drought stress occurs regularly for brief periods or at moderate levels. Moreover, it has been predicted that in the coming years rainfall patterns will shift and become more variable due to increased global temperatures.

U.S. studies have shown that the ten most important kinds of cultivated plants (corn, soybeans, wheat, tomatoes, etc.) produced only about 50% of the genetically possible yields on average per year; two thirds of the losses were due to the frequent combination of heat stress and water shortage (G. Schütte, S. Stirn, and V. Beusmann, Transgene Pflanzen - Sicherheitsforschung, Risikoabschätzung und Nachzulassungs-Monitoring. Birkhäuser Verlag AG, Basel-Boston-Berlin, 2001).

Plants are sessile and have to adjust to the prevailing environmental conditions of their surroundings. This has led to their development of a great

plasticity in gene regulation, morphogenesis, and metabolism. Adaptation and defense strategies involve the activation of genes encoding proteins important in the acclimation or defense towards the different stressors. Some of the molecular responses to abiotic stress factors such as drought are specific, but it has also been shown that similar genes are activated by several stressors (Royal Society of London, *Transgenic Plants and World Agriculture*, 2000, National Academy Press, Washington, DC). It is believed that about 15 percent of a plant's genome is devoted to stress perception and adaptation (see e.g., Cushman and Bohnert, 2000).

Earlier work on molecular aspects of abiotic stress responses was accomplished by differential and/or subtractive analysis (e.g., see Bray, 1993, Shinozaki and Yamaguchi-Shinozaki, 1997, Zhu et al., 1997, Thomashow, 1999). Other methods include selection of candidate genes (e.g., selection of genes from a particular known module and analyzing expression of such a gene or its active product under stresses, or by functional complementation in a stressor system that is well defined, see Xiong and Zhu, 2001). Additionally, forward and reverse genetic studies involving the identification and isolation of mutations in regulatory genes have also been used to provide evidence for observed changes in gene expression under stress or exposure (Xiong and Zhu, 2001).

Activation tagging can be utilized to identify genes with the ability to affect a trait. This approach has been used in the model plant species *Arabidopsis thaliana* (Weigel et al., *Plant Physiol.* 122:1003-1013 (2000)). Insertions of transcriptional enhancer elements can dominantly activate and/or elevate the expression of nearby endogenous genes. This method can be used to select genes involved in agronomically important phenotypes, including stress tolerance.

SUMMARY OF THE INVENTION

The present invention includes:

In one embodiment, a plant comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method

of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, and wherein said plant exhibits increased drought tolerance when compared to a control plant not comprising said recombinant DNA construct.

In another embodiment, a method of increasing drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct; and optionally, (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct.

In another embodiment, a method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) evaluating the transgenic plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct; and optionally, (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and optionally, (e) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

In another embodiment, a method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; (c) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (d) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

In another embodiment, the present invention includes any of the methods of the present invention wherein the plant is a maize plant or a soybean plant.

In another embodiment, the present invention includes an isolated polynucleotide comprising: (a) a nucleotide sequence encoding a Zinc-Finger (C3HC4-type RING finger) family polypeptide, wherein the polypeptide has an amino acid sequence of at least 90% or 95% sequence identity, based on the Clustal V method of alignment, when compared to one of SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33 or (b) a full complement of the nucleotide sequence, wherein the full complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary. The polypeptide may comprise the amino acid sequence of SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33. The nucleotide sequence may comprise the nucleotide sequence of SEQ ID NO: 16, 18, 26, 28, 30 or 32.

In another embodiment, the present invention concerns a recombinant DNA construct comprising any of the isolated polynucleotides of the present invention operably linked to at least one regulatory sequence, and a cell, a plant, and a seed comprising the recombinant DNA construct.

In another embodiment, the present invention includes a vector comprising any of the isolated polynucleotides of the present invention.

In another embodiment, the present invention concerns a cell, plant or seed comprising any of the recombinant DNA constructs of the present invention. The cell may be eukaryotic, e.g., a yeast, insect or plant cell, or prokaryotic, e.g., a bacterium.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTING

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

Figure 1 shows a schematic of the pHSbarENDs2 activation tagging construct (SEQ ID NO:1) used to make the *Arabidopsis* populations.

Figure 2 shows a map of the vector pDONR™/Zeo (SEQ ID NO:2). The attP1 site is at nucleotides 570-801; the attP2 site is at nucleotides 2754-2985 (complementary strand).

Figure 3 shows a map of the vector pDONR™221 (SEQ ID NO:3). The attP1 site is at nucleotides 570-801; the attP2 site is at nucleotides 2754-2985 (complementary strand).

Figure 4 shows a map of the vector pBC-yellow (SEQ ID NO:4), a destination vector for use in construction of expression vectors for *Arabidopsis*. The attR1 site is at nucleotides 11276-11399 (complementary strand); the attR2 site is at nucleotides 9695-9819 (complementary strand).

Figure 5 shows a map of PHP27840 (SEQ ID NO:5), a destination vector for use in construction of expression vectors for soybean. The attR1 site is at nucleotides 7310-7434; the attR2 site is at nucleotides 8890-9014.

Figure 6 shows a map of PHP23236 (SEQ ID NO:6), a destination vector for use in construction of expression vectors for Gaspe Flint derived maize lines. The attR1 site is at nucleotides 2006-2130; the attR2 site is at nucleotides 2899-3023.

Figure 7 shows a map of PHP10523 (SEQ ID NO:7), a plasmid DNA present in *Agrobacterium* strain LBA4404 (Komari et al., *Plant J.* 10:165-174 (1996); NCBI General Identifier No. 59797027).

Figure 8 shows a map of PHP23235 (SEQ ID NO:8), a vector used to construct the destination vector PHP23236.

Figure 9 shows a map of PHP28647 (SEQ ID NO:9), a destination vector for use with maize inbred-derived lines. The attR1 site is at nucleotides 2289-2413; the attR2 site is at nucleotides 3869-3993.

Figure 10 shows the evaluation of individual maize lines transformed with PHP31373.

Figure 11 shows the ABA sensitivity during seed germination of wild-type (Col, Columbine) and T2 transgenic Arabidopsis seeds containing the Zinc-Finger (C3HC4-type RING finger) family polypeptide (#27, At2g01150) (left panel). ABA inhibition of postgermination growth was significantly increased in transgenic lines over-expressing At2g01150 (right panel). Col = Columbine; #27 = At2g01150.

Figure 12 shows the treatment schedule for screening plants with enhanced drought tolerance.

Figures 13A-13B show the multiple alignment of the amino acid sequences of the Zinc-Finger (C3HC4-type RING finger) family polypeptide of SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33. Residues that are identical to the residue of SEQ ID NO:17 at a given position are enclosed in a box.

Figure 14 shows the percent sequence identity and the divergence values for each pair of amino acids sequences of Zinc-Finger (C3HC4-type RING finger) family polypeptide displayed in Figures 13A-13B.

Figure 15 shows wild type (WT) and transgenic Arabidopsis plant containing the Zinc-Finger (C3HC4-type RING finger) family polypeptide (RHA2b) at 0, 2, 4 hrs and overnight (ON) after rewatering the plants.

Figure 16 shows the average yield of field grown transgenic maize plants versus their bulk null (control) plants exposed to a gradual drought stress (left panel) or a rapid drought stress (right panel).

SEQ ID NO:1 is the nucleotide sequence of the pHSbarENDs2 activation tagging vector.

SEQ ID NO:2 is the nucleotide sequence of the GATEWAY® donor vector pDONR™/Zeo.

SEQ ID NO:3 is the nucleotide sequence of the GATEWAY® donor vector pDONR™221.

SEQ ID NO:4 is the nucleotide sequence of pBC-yellow, a destination vector for use with *Arabidopsis*.

SEQ ID NO:5 is the nucleotide sequence of PHP27840, a destination vector for use with soybean.

SEQ ID NO:6 is the nucleotide sequence of PHP23236, a destination vector for use with Gaspe Flint derived maize lines.

SEQ ID NO:7 is the nucleotide sequence of PHP10523 (Komari et al., *Plant J.* 10:165-174 (1996); NCBI General Identifier No. 59797027).

SEQ ID NO:8 is the nucleotide sequence of PHP23235, a destination vector for use with Gaspe Flint derived lines.

SEQ ID NO:9 is the nucleotide sequence of PHP28647, a destination vector for use with maize inbred-derived lines.

SEQ ID NO:10 is the nucleotide sequence of the attB1 site.

SEQ ID NO:11 is the nucleotide sequence of the attB2 site.

SEQ ID NO:12 is the nucleotide sequence of the At2g01150-5'attB forward primer, containing the attB1 sequence, used to amplify the At2g01150 protein-coding region.

SEQ ID NO:13 is the nucleotide sequence of the At2g01150-3'attB reverse primer, containing the attB2 sequence, used to amplify the At2g01150 protein-coding region.

SEQ ID NO:14 is the nucleotide sequence of the VC062 primer, containing the T3 promoter and attB1 site, useful to amplify cDNA inserts cloned into a BLUESCRIPT® II SK(+) vector (Stratagene).

SEQ ID NO:15 is the nucleotide sequence of the VC063 primer, containing the T7 promoter and attB2 site, useful to amplify cDNA inserts cloned into a BLUESCRIPT® II SK(+) vector (Stratagene).

SEQ ID NO:16 corresponds to NCBI GI No. 98960889, which is the cDNA nucleotide sequence of locus At2g01150 encoding an *Arabidopsis* Zinc-Finger (C3HC4-type RING finger) family polypeptide (RHA2B). This sequence was obtained by PCR amplification of Arabidopsis cDNA using SEQ ID NO:12 and SEQ ID NO:13 as PCR primers.

SEQ ID NO:17 corresponds to the amino acid sequence encoded by SEQ ID NO:16 .

SEQ ID NO:18 corresponds to NCBI GI NO: 156896364 (Bra#S40765917; gb=EX097840;ug=Bra.5893) , which is a cDNA nucleotide sequence of *Brassica rapa subsp. Pekinensis*.

SEQ ID NO:19 corresponds to the predicted amino acid sequence encoded by SEQ ID NO:34 (Bra#S40765917).

SEQ ID NO:20 corresponds to a predicted polypeptide sequence (jgi_Araly1_484022) from *Arabidopsis lyrata* genomic DNA assembled by the Joint Genome Institute (JGI) using FGENESH predictions.

SEQ ID NO:21 corresponds to a predicted polypeptide sequence (Glyma10g41480.1) assembled by the Joint Genome Institute (JGI) from soybean genomic DNA, locus Glyma10g41480.1.

SEQ ID NO:22 corresponds to NCBI GI No. 225424108, which is the amino acid sequence of a hypothetical protein from *Vitis vinifera.*

SEQ ID NO:23 corresponds to NCBI GI No. 224101783, which is the amino acid sequence of a predicted protein from *Populus trichocarpa.*

SEQ ID NO:24 corresponds to NCBI GI No. 224108389, which is the amino acid sequence of a predicted protein from *Populus trichocarpa.*

SEQ ID NO:25 corresponds to NCBI GI No. 255570699, which is the amino acid sequence of a conserved hypothetical protein from *Ricinus communis*.

SEQ ID NO:26 corresponds to NCBI GI NO: 158947513 (Rsa#S42024301; gb=EX909789 ; ug=Rsa.15663) , which is the cDNA nucleotide sequence of RS3CT64JQ RS3(RT) from *Raphanus sativus*.

SEQ ID NO:27 corresponds to the predicted amino acid sequence encoded by SEQ ID NO:26.

SEQ ID NO:28 corresponds to NCBI GI NO: 167441352 (Rsa#S43018457; gb=FD946889; ug=Rsa.25923) , which is the cDNA nucleotide sequence of RS2GG53TF RS2(RS) from *Raphanus sativus*.

SEQ ID NO:29 corresponds to the predicted amino acid sequence encoded by SEQ ID NO:28 (Rsa#S43018457).

SEQ ID NO:30 corresponds to NCBI GI NO: 151207511 (Bna#S39191941; gb=EV120552; ug=Bna.9890) , which is a cDNA nucleotide sequence of *Brassica napus*.

SEQ ID NO:31 corresponds to the predicted amino acid sequence encoded by SEQ ID NO:30 (Bna#S39191941).

SEQ ID NO:32 corresponds to NCBI GI NO: 156952314 (Bra#S40797032; gb=EX127956; ug=Bra.5294) , which is a cDNA nucleotide sequence of *Brassica rapa*.

SEQ ID NO:33 corresponds to the predicted amino acid sequence encoded by SEQ ID NO:32 (Bra_S40797032).

SEQ ID NO:34 corresponds to NCBI GI NO: 30684171, which is the cDNA nucleotide sequence of Arabidopsis thaliana encoding a protein binding / ubiquitin-protein ligase/ zinc ion binding (RHA2A) polypeptide.

SEQ ID NO:35 corresponds to the amino acid sequence encoded by SEQ ID NO:34.

The sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Res. 13*:3021-3030 (1985) and in the *Biochemical J. 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

DETAILED DESCRIPTION

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

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

As used herein:

The following terms are used interchangeably herein: “Zinc-Finger (C3HC4-type RING finger) family polypeptide”, “RHA2B”, “RHA2b”, “RING-H2 FINGER PROTEIN 2B”,” At-C3HC4_ZF”

“Zinc-Finger (C3HC4-type RING finger) family polypeptide” refers to a C3HC4 type zinc-finger (RING finger) which is a cysteine-rich domain of 40 to 60 residues that coordinates two zinc ions, and has the consensus sequence: C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-C-X2-C-X(4-48)-C-X2-C where X is any amino acid (Borden KL and Freemont PS. 1996 Curr. Opin. Struct. Biol. 6:395-401). Many proteins containing a RING finger play a key role in the ubiquitination pathway (Lorick KL, et al. 1999. Proc. Natl. Acad. Sci. 96:11364-11369).

Zinc finger (Znf) domains are relatively small protein motifs that bind one or more zinc atoms, and which usually contain multiple finger-like protrusions that make tandem contacts with their target molecule.

A RING-finger (Really Interesting New Gene) refers to a specialized type of Zinc-finger of 40 to 60 residues that binds two atoms of zinc; defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)- H-X(2-3)-(N/C/H)-X2-C-X(4-48)C-X2-C; probably involved in mediating protein-protein interactions ((Borden KL and Freemont PS. 1996 Curr Opin Struct Biol. 1996 Jun;6(3):395-401). A RING –finger has two variants, the C3HC4-type and a C3H2C3-type (RING-H2 finger), which have different cysteine/histidine pattern.

An “Expressed Sequence Tag” (“EST”) is a DNA sequence derived from a cDNA library and therefore is a sequence which has been transcribed. An EST is typically obtained by a single sequencing pass of a cDNA insert. The sequence of an entire cDNA insert is termed the “Full-Insert Sequence” (“FIS”). A “Contig” sequence is a sequence assembled from two or more sequences that can be selected from, but not limited to, the group consisting of an EST, FIS and PCR sequence. A sequence encoding an entire or functional protein is termed a “Complete Gene Sequence” (“CGS”) and can be derived from an FIS or a contig.

A "trait” refers to a physiological, morphological, biochemical, or physical characteristic of a plant or particular plant material or cell. In some instances, this characteristic is visible to the human eye, such as seed or plant size, or can be measured by biochemical techniques, such as detecting the protein, starch,

or oil content of seed or leaves, or by observation of a metabolic or physiological process, e.g. by measuring tolerance to water deprivation or particular salt or sugar concentrations, or by the observation of the expression level of a gene or genes, or by agricultural observations such as osmotic stress tolerance or yield.

"Agronomic characteristic" is a measurable parameter including but not limited to, greenness, yield, growth rate, biomass, fresh weight at maturation, dry weight at maturation, fruit yield, seed yield, total plant nitrogen content, fruit nitrogen content, seed nitrogen content, nitrogen content in a vegetative tissue, total plant free amino acid content, fruit free amino acid content, seed free amino acid content, free amino acid content in a vegetative tissue, total plant protein content, fruit protein content, seed protein content, protein content in a vegetative tissue, drought tolerance, nitrogen uptake, root lodging, harvest index, stalk lodging, plant height, ear height , ear length salt tolerance, early seedling vigor and seedling emergence under low temperature stress.

Increased biomass can be measured, for example, as an increase in plant height, plant total leaf area, plant fresh weight, plant dry weight or plant seed yield, as compared with control plants.

The ability to increase the biomass or size of a plant would have several important commercial applications. Crop species may be generated that produce larger cultivars, generating higher yield in, for example, plants in which the vegetative portion of the plant is useful as food, biofuel or both.

Increased leaf size may be of particular interest. Increasing leaf biomass can be used to increase production of plant-derived pharmaceutical or industrial products. An increase in total plant photosynthesis is typically achieved by increasing leaf area of the plant. Additional photosynthetic capacity may be used to increase the yield derived from particular plant tissue, including the leaves, roots, fruits or seed, or permit the growth of a plant under decreased light intensity or under high light intensity.

Modification of the biomass of another tissue, such as root tissue, may be useful to improve a plant's ability to grow under harsh environmental conditions, including drought or nutrient deprivation, because larger roots may better reach water or nutrients or take up water or nutrients.

For some ornamental plants, the ability to provide larger varieties would be highly desirable. For many plants, including fruit-bearing trees, trees that are used for lumber production, or trees and shrubs that serve as view or wind screens, increased stature provides improved benefits in the forms of greater yield or improved screening.

"Transgenic" refers to any cell, cell line, callus, tissue, plant part or plant, the genome of which has been altered by the presence of a heterologous nucleic acid, such as a recombinant DNA construct, including those initial transgenic events as well as those created by sexual crosses or asexual propagation from the initial transgenic event. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

“Genome” as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondrial, plastid) of the cell.

“Plant" includes reference to whole plants, plant organs, plant tissues, seeds and plant cells and progeny of same. Plant cells include, without limitation, cells from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

"Progeny" comprises any subsequent generation of a plant.

"Transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. For example, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct.

"Heterologous" with respect to sequence means a sequence that originates from a foreign species, or, if from the same species, is substantially

modified from its native form in composition and/or genomic locus by deliberate human intervention.

"Polynucleotide", "nucleic acid sequence", "nucleotide sequence", or "nucleic acid fragment" are used interchangeably and is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

"Polypeptide", "peptide", "amino acid sequence" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms "polypeptide", "peptide", "amino acid sequence", and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation.

"Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell.

"cDNA" refers to a DNA that is complementary to and synthesized from a mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded form using the Klenow fragment of DNA polymerase I.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or pro-peptides present in the primary translation product have been removed.

"Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and pro-peptides still present. Pre- and pro-peptides may be and are not limited to intracellular localization signals.

"Isolated" refers to materials, such as nucleic acid molecules and/or proteins, which are substantially free or otherwise removed from components that normally accompany or interact with the materials in a naturally occurring environment. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

"Recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. "Recombinant" also includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or a cell derived from a cell so modified, but does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

"Recombinant DNA construct" refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that normally found in nature.

The terms "entry clone" and "entry vector" are used interchangeably herein.

"Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences. The terms "regulatory sequence" and "regulatory element" are used interchangeably herein.

"Promoter" refers to a nucleic acid fragment capable of controlling transcription of another nucleic acid fragment.

"Promoter functional in a plant" is a promoter capable of controlling transcription in plant cells whether or not its origin is from a plant cell.

"Tissue-specific promoter" and "tissue-preferred promoter" are used interchangeably, and refer to a promoter that is expressed predominantly but not necessarily exclusively in one tissue or organ, but that may also be expressed in one specific cell.

"Developmentally regulated promoter" refers to a promoter whose activity is determined by developmental events.

"Operably linked" refers to the association of nucleic acid fragments in a single fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a nucleic acid fragment when it is capable of regulating the transcription of that nucleic acid fragment.

"Expression" refers to the production of a functional product. For example, expression of a nucleic acid fragment may refer to transcription of the nucleic acid fragment (e.g., transcription resulting in mRNA or functional RNA) and/or translation of mRNA into a precursor or mature protein.

"Phenotype" means the detectable characteristics of a cell or organism.

"Introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

A "transformed cell" is any cell into which a nucleic acid fragment (e.g., a recombinant DNA construct) has been introduced.

"Transformation" as used herein refers to both stable transformation and transient transformation.

"Stable transformation" refers to the introduction of a nucleic acid fragment into a genome of a host organism resulting in genetically stable

inheritance. Once stably transformed, the nucleic acid fragment is stably integrated in the genome of the host organism and any subsequent generation.

"Transient transformation" refers to the introduction of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host organism resulting in gene expression without genetically stable inheritance.

"Allele" is one of several alternative forms of a gene occupying a given locus on a chromosome. When the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant are the same that plant is homozygous at that locus. If the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant differ that plant is heterozygous at that locus. If a transgene is present on one of a pair of homologous chromosomes in a diploid plant that plant is hemizygous at that locus.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys. 100*:1627-1632). A "mitochondrial signal peptide" is an amino acid sequence which directs a precursor protein into the mitochondria (Zhang and Glaser (2002) *Trends Plant Sci 7*:14-21).

Sequence alignments and percent identity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the Megalign® program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, WI). Unless stated otherwise, multiple alignment of the sequences provided herein were performed using the Clustal V method of alignment (Higgins and

Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences, using the Clustal V program, it is possible to obtain "percent identity" and "divergence" values by viewing the "sequence distances" table on the same program; unless stated otherwise, percent identities and divergences provided and claimed herein were calculated in this manner.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Sambrook").

Turning now to the embodiments:

Embodiments include isolated polynucleotides and polypeptides, recombinant DNA constructs useful for conferring drought tolerance, compositions (such as plants or seeds) comprising these recombinant DNA constructs, and methods utilizing these recombinant DNA constructs.

Isolated Polynucleotides and Polypeptides:

The present invention includes the following isolated polynucleotides and polypeptides:

An isolated polynucleotide comprising: (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, 33; or (ii) a full complement of the nucleic acid sequence of (i), wherein the full complement and the nucleic acid sequence of (i) consist of the same number of nucleotides and are 100% complementary. Any

of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs (including suppression DNA constructs) of the present invention. The polypeptide is preferably a Zinc-Finger (C3HC4-type RING finger) family polypeptide. The polypeptide preferably has drought tolerance activity.

An isolated polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, OR 33. The polypeptide is preferably a Zinc-Finger (C3HC4-type RING finger) family polypeptide. The polypeptide preferably has drought tolerance activity.

An isolated polynucleotide comprising (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 16, 18, 26, 28, 30 or 32.; or (ii) a full complement of the nucleic acid sequence of (i). Any of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs (including suppression DNA constructs) of the present invention. The isolated polynucleotide preferably encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide. The Zinc-Finger (C3HC4-type RING finger) family polypeptide preferably has drought tolerance activity.

Recombinant DNA Constructs and Suppression DNA Constructs:

In one aspect, the present invention includes recombinant DNA constructs (including suppression DNA constructs).

In one embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein the polynucleotide comprises (i) a nucleic acid sequence encoding an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%,

66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide comprises (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 16, 18, 26, 28, 30 or 32; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide. The Zinc-Finger (C3HC4-type RING finger) family polypeptide preferably has drought tolerance activity . The Zinc-Finger (C3HC4-type RING finger) family polypeptide may be from *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* and *Glycine tomentella*.

In another aspect, the present invention includes suppression DNA constructs.

A suppression DNA construct may comprise at least one regulatory sequence (e.g., a promoter functional in a plant) operably linked to (a) all or part of: (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%

sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (a)(i); or (b) a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; or (c) all or part of: (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 16, 18, 26, 28, 30 or 32, or (ii) a full complement of the nucleic acid sequence of (c)(i). The suppression DNA construct may comprise a cosuppression construct, antisense construct, viral-suppression construct, hairpin suppression construct, stem-loop suppression construct, double-stranded RNA-producing construct, RNAi construct, or small RNA construct (e.g., an siRNA construct or an miRNA construct).

It is understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences. Alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged

residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

"Suppression DNA construct" is a recombinant DNA construct which when transformed or stably integrated into the genome of the plant, results in "silencing" of a target gene in the plant. The target gene may be endogenous or transgenic to the plant. "Silencing," as used herein with respect to the target gene, refers generally to the suppression of levels of mRNA or protein/enzyme expressed by the target gene, and/or the level of the enzyme activity or protein functionality. The terms "suppression", "suppressing" and "silencing", used interchangeably herein, include lowering, reducing, declining, decreasing, inhibiting, eliminating or preventing. "Silencing" or "gene silencing" does not specify mechanism and is inclusive, and not limited to, anti-sense, cosuppression, viral-suppression, hairpin suppression, stem-loop suppression, RNAi-based approaches, and small RNA-based approaches.

A suppression DNA construct may comprise a region derived from a target gene of interest and may comprise all or part of the nucleic acid sequence of the sense strand (or antisense strand) of the target gene of interest. Depending upon the approach to be utilized, the region may be 100% identical or less than 100% identical (e.g., at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) to all or part of the sense strand (or antisense strand) of the gene of interest.

Suppression DNA constructs are well-known in the art, are readily constructed once the target gene of interest is selected, and include, without limitation, cosuppression constructs, antisense constructs, viral-suppression constructs, hairpin suppression constructs, stem-loop suppression constructs, double-stranded RNA-producing constructs, and more generally, RNAi (RNA

interference) constructs and small RNA constructs such as siRNA (short interfering RNA) constructs and miRNA (microRNA) constructs.

"Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target gene or gene product. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target isolated nucleic acid fragment (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence.

"Cosuppression" refers to the production of sense RNA transcripts capable of suppressing the expression of the target gene or gene product. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. Cosuppression constructs in plants have been previously designed by focusing on overexpression of a nucleic acid sequence having homology to a native mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the overexpressed sequence (see Vaucheret et al., *Plant J.* 16:651-659 (1998); and Gura, *Nature* 404:804-808 (2000)).

Another variation describes the use of plant viral sequences to direct the suppression of proximal mRNA encoding sequences (PCT Publication No. WO 98/36083 published on August 20, 1998).

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., *Nature* 391:806 (1998)). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing (PTGS) or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire et al., *Trends Genet.* 15:358 (1999)).

Small RNAs play an important role in controlling gene expression. Regulation of many developmental processes, including flowering, is controlled

by small RNAs. It is now possible to engineer changes in gene expression of plant genes by using transgenic constructs which produce small RNAs in the plant.

Small RNAs appear to function by base-pairing to complementary RNA or DNA target sequences. When bound to RNA, small RNAs trigger either RNA cleavage or translational inhibition of the target sequence. When bound to DNA target sequences, it is thought that small RNAs can mediate DNA methylation of the target sequence. The consequence of these events, regardless of the specific mechanism, is that gene expression is inhibited.

MicroRNAs (miRNAs) are noncoding RNAs of about 19 to about 24 nucleotides (nt) in length that have been identified in both animals and plants (Lagos-Quintana et al., *Science* 294:853-858 (2001), Lagos-Quintana et al., *Curr. Biol.* 12:735-739 (2002); Lau et al., *Science* 294:858-862 (2001); Lee and Ambros, *Science* 294:862-864 (2001); Llave et al., *Plant Cell* 14:1605-1619 (2002); Mourelatos et al., *Genes. Dev.* 16:720-728 (2002); Park et al., *Curr. Biol.* 12:1484-1495 (2002); Reinhart et al., *Genes. Dev.* 16:1616-1626 (2002)). They are processed from longer precursor transcripts that range in size from approximately 70 to 200 nt, and these precursor transcripts have the ability to form stable hairpin structures.

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Regulatory Sequences:

A recombinant DNA construct (including a suppression DNA construct) of the present invention may comprise at least one regulatory sequence.

A regulatory sequence may be a promoter.

A number of promoters can be used in recombinant DNA constructs of the present invention. The promoters can be selected based on the desired outcome, and may include constitutive, tissue-specific, inducible, or other promoters for expression in the host organism.

Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

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

Suitable constitutive promoters for use in a plant host cell include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell et al., Nature 313:810-812 (1985)); rice actin (McElroy et al., Plant Cell 2:163-171 (1990)); ubiquitin (Christensen et al., Plant Mol. Biol. 12:619-632 (1989) and Christensen et al., Plant Mol. Biol. 18:675-689 (1992)); pEMU (Last et al., Theor. Appl. Genet. 81:581-588 (1991)); MAS (Velten et al., EMBO J. 3:2723-2730 (1984)); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those discussed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

In choosing a promoter to use in the methods of the invention, it may be desirable to use a tissue-specific or developmentally regulated promoter.

A tissue-specific or developmentally regulated promoter is a DNA sequence which regulates the expression of a DNA sequence selectively in the cells/tissues of a plant critical to tassel development, seed set, or both, and limits the expression of such a DNA sequence to the period of tassel development or seed maturation in the plant. Any identifiable promoter may be used in the methods of the present invention which causes the desired temporal and spatial expression.

Promoters which are seed or embryo-specific and may be useful in the invention include soybean Kunitz trypsin inhibitor (Kti3, Jofuku and Goldberg, Plant Cell 1:1079-1093 (1989)), patatin (potato tubers) (Rocha-Sosa, M., et al. (1989) EMBO J. 8:23-29), convicilin, vicilin, and legumin (pea cotyledons) (Rerie, W.G., et al. (1991) Mol. Gen. Genet. 259:149-157; Newbigin, E.J., et al. (1990) Planta 180:461-470; Higgins, T.J.V., et al. (1988) Plant. Mol. Biol. 11:683-695), zein (maize endosperm) (Schemthaner, J.P., et al. (1988) EMBO J. 7:1249-1255), phaseolin (bean cotyledon) (Segupta-Gopalan, C., et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:3320-3324), phytohemagglutinin (bean cotyledon) (Voelker, T. et al. (1987) EMBO J. 6:3571-3577), B-conglycinin and glycinin (soybean cotyledon) (Chen, Z-L, et al. (1988) EMBO J. 7:297- 302), glutelin (rice endosperm), hordein (barley endosperm) (Marris, C., et al. (1988) Plant Mol. Biol. 10:359-366), glutenin and gliadin (wheat endosperm) (Colot, V., et al. (1987) EMBO J. 6:3559-3564), and sporamin (sweet potato tuberous root) (Hattori, T., et al. (1990) Plant Mol. Biol. 14:595-604). Promoters of seed-specific genes operably linked to heterologous coding regions in chimeric gene constructions maintain their temporal and spatial expression pattern in transgenic plants. Such examples include *Arabidopsis thaliana* 2S seed storage protein gene promoter to express enkephalin peptides in *Arabidopsis* and *Brassica napus* seeds (Vanderkerckhove et al., Bio/Technology 7:L929-932 (1989)), bean lectin and bean beta-phaseolin promoters to express luciferase (Riggs et al., Plant Sci. 63:47-57 (1989)), and wheat glutenin promoters to express chloramphenicol acetyl transferase (Colot et al., EMBO J 6:3559- 3564 (1987)).

Inducible promoters selectively express an operably linked DNA sequence in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters regulated by light, heat, stress, flooding or drought, phytohormones, wounding, or chemicals such as ethanol, jasmonate, salicylic acid, or safeners.

Promoters for use in the current invention include the following: 1) the stress-inducible RD29A promoter (Kasuga et al. (1999) Nature Biotechnol.

17:287-91); 2) the barley promoter, B22E; expression of B22E is specific to the pedicel in developing maize kernels ("Primary Structure of a Novel Barley Gene Differentially Expressed in Immature Aleurone Layers". Klemsdal, S.S. et al., Mol. Gen. Genet. 228(1/2):9-16 (1991)); and 3) maize promoter, Zag2 ("Identification and molecular characterization of ZAG1, the maize homolog of the *Arabidopsis* floral homeotic gene AGAMOUS", Schmidt, R.J. et al., Plant Cell 5(7):729-737 (1993); "Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of *AGAMOUS*-like *MADS-box* genes from maize", Theissen et al. Gene 156(2):155-166 (1995); NCBI GenBank Accession No. X80206)). Zag2 transcripts can be detected 5 days prior to pollination to 7 to 8 days after pollination ("DAP"), and directs expression in the carpel of developing female inflorescences and Ciml which is specific to the nucleus of developing maize kernels. Ciml transcript is detected 4 to 5 days before pollination to 6 to 8 DAP. Other useful promoters include any promoter which can be derived from a gene whose expression is maternally associated with developing female florets.

Additional promoters for regulating the expression of the nucleotide sequences of the present invention in plants are stalk-specific promoters. Such stalk-specific promoters include the alfalfa S2A promoter (GenBank Accession No. EF030816; Abrahams et al., Plant Mol. Biol. 27:513-528 (1995)) and S2B promoter (GenBank Accession No. EF030817) and the like, herein incorporated by reference.

Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples

may be found in the compilation by Okamuro, J. K., and Goldberg, R. B., Biochemistry of Plants 15:1-82 (1989).

Promoters for use in the current invention may include: RIP2, mLIP15, ZmCOR1, Rab17, CaMV 35S, RD29A, B22E, Zag2, SAM synthetase, ubiquitin, CaMV 19S, nos, Adh, sucrose synthase, R-allele, the vascular tissue preferred promoters S2A (Genbank accession number EF030816) and S2B (Genbank accession number EF030817), and the constitutive promoter GOS2 from *Zea mays.* Other promoters include root preferred promoters, such as the maize NAS2 promoter, the maize Cyclo promoter (US 2006/0156439, published July 13, 2006), the maize ROOTMET2 promoter (WO05063998, published July 14, 2005), the CR1BIO promoter (WO06055487, published May 26, 2006), the CRWAQ81 (WO05035770, published April 21, 2005) and the maize ZRP2.47 promoter (NCBI accession number: U38790; GI No. 1063664),

Recombinant DNA constructs of the present invention may also include other regulatory sequences, including but not limited to, translation leader sequences, introns, and polyadenylation recognition sequences. In another embodiment of the present invention, a recombinant DNA construct of the present invention further comprises an enhancer or silencer.

An intron sequence can be added to the 5' untranslated region, the protein-coding region or the 3' untranslated region to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold. Buchman and Berg, *Mol. Cell Biol.* 8:4395-4405 (1988); Callis et al., *Genes Dev*. 1:1183-1200 (1987).

Any plant can be selected for the identification of regulatory sequences and Zinc-Finger (C3HC4-type RING finger) family polypeptide genes to be used in recombinant DNA constructs of the present invention. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, *Arabidopsis*, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines,

clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini.

Compositions:

A composition of the present invention is a plant comprising in its genome any of the recombinant DNA constructs (including any of the suppression DNA constructs) of the present invention (such as any of the constructs discussed above). Compositions also include any progeny of the plant, and any seed obtained from the plant or its progeny, wherein the progeny or seed comprises within its genome the recombinant DNA construct (or suppression DNA construct). Progeny includes subsequent generations obtained by self-pollination or out-crossing of a plant. Progeny also includes hybrids and inbreds.

In hybrid seed propagated crops, mature transgenic plants can be self-pollinated to produce a homozygous inbred plant. The inbred plant produces seed containing the newly introduced recombinant DNA construct (or suppression DNA construct). These seeds can be grown to produce plants that would exhibit an altered agronomic characteristic (e.g., an increased agronomic characteristic optionally under water limiting conditions), or used in a breeding program to produce hybrid seed, which can be grown to produce plants that would exhibit such an altered agronomic characteristic. The seeds may be maize seeds.

The plant may be a monocotyledonous or dicotyledonous plant, for example, a maize or soybean plant, such as a maize hybrid plant or a maize inbred plant. The plant may also be sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet.

The recombinant DNA construct may be stably integrated into the genome of the plant.

Particularly embodiments include but are not limited to the following:

1. A plant (for example, a maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, and wherein said plant exhibits increased drought tolerance when compared to a control plant not comprising said recombinant DNA construct. The plant may further exhibit an alteration of at least one agronomic characteristic when compared to the control plant.

2. A plant (for example, a maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide, and wherein said plant exhibits increased drought tolerance when compared to a control plant not comprising said recombinant DNA construct. The plant may further exhibit an alteration of at least one agronomic characteristic when compared to the control plant.

3. A plant (for example, a maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant not comprising said recombinant DNA construct.

4. A plant (for example, a maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes a

polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant not comprising said recombinant DNA construct.

5. A plant (for example, a maize or soybean plant) comprising in its genome a suppression DNA construct comprising at least one regulatory element operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant not comprising said suppression DNA construct.

6. A plant (for example, a maize or soybean plant) comprising in its genome a suppression DNA construct comprising at least one regulatory element operably linked to all or part of (a) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (b) a full complement of the nucleic acid sequence of (a),

and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant not comprising said suppression DNA construct.

7. Any progeny of the above plants in embodiments 1-6, any seeds of the above plants in embodiments 1-6, any seeds of progeny of the above plants in embodiments 1-6, and cells from any of the above plants in embodiments 1-6 and progeny thereof.

In any of the foregoing embodiments 1-7 or any other embodiments of the present invention, the Zinc-Finger (C3HC4-type RING finger) family polypeptide may be from *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.

In any of the foregoing embodiments 1-7 or any other embodiments of the present invention, the recombinant DNA construct (or suppression DNA construct) may comprise at least a promoter functional in a plant as a regulatory sequence.

In any of the foregoing embodiments 1-7 or any other embodiments of the present invention, the alteration of at least one agronomic characteristic is either an increase or decrease.

In any of the foregoing embodiments 1-7 or any other embodiments of the present invention, the at least one agronomic characteristic may be selected from the group consisting of greenness, yield, growth rate, biomass, fresh weight at maturation, dry weight at maturation, fruit yield, seed yield, total plant nitrogen content, fruit nitrogen content, seed nitrogen content, nitrogen content in a vegetative tissue, total plant free amino acid content, fruit free amino acid content, seed free amino acid content, free amino acid content in a vegetative tissue, total plant protein content, fruit protein content, seed protein content, protein content in a vegetative tissue, drought tolerance, nitrogen uptake, root lodging, harvest index, stalk lodging, plant height, ear height, ear length, salt tolerance, early seedling vigor and seedling emergence under low temperature stress. . For example, the alteration of at least one agronomic characteristic may be an increase in yield, greenness or biomass.

In any of the foregoing embodiments 1-7 or any other embodiments of the present invention, the plant may exhibit the alteration of at least one agronomic

characteristic when compared, under water limiting conditions, to a control plant not comprising said recombinant DNA construct (or said suppression DNA construct).

"Drought" refers to a decrease in water availability to a plant that, especially when prolonged, can cause damage to the plant or prevent its successful growth (e.g., limiting plant growth or seed yield).

"Drought tolerance" is a trait of a plant to survive under drought conditions over prolonged periods of time without exhibiting substantial physiological or physical deterioration.

"Drought tolerance activity" of a polypeptide indicates that over-expression of the polypeptide in a transgenic plant confers increased drought tolerance to the transgenic plant relative to a reference or control plant.

"Increased drought tolerance" of a plant is measured relative to a reference or control plant, and is a trait of the plant to survive under drought conditions over prolonged periods of time, without exhibiting the same degree of physiological or physical deterioration relative to the reference or control plant grown under similar drought conditions. Typically, when a transgenic plant comprising a recombinant DNA construct or suppression DNA construct in its genome exhibits increased drought tolerance relative to a reference or control plant, the reference or control plant does not comprise in its genome the recombinant DNA construct or suppression DNA construct.

One of ordinary skill in the art is familiar with protocols for simulating drought conditions and for evaluating drought tolerance of plants that have been subjected to simulated or naturally-occurring drought conditions. For example, one can simulate drought conditions by giving plants less water than normally required or no water over a period of time, and one can evaluate drought tolerance by looking for differences in physiological and/or physical condition, including (but not limited to) vigor, growth, size, or root length, or in particular, leaf color or leaf area size. Other techniques for evaluating drought tolerance include measuring chlorophyll fluorescence, photosynthetic rates and gas exchange rates.

A drought stress experiment may involve a chronic stress (i.e., slow dry down) and/or may involve two acute stresses (i.e., abrupt removal of water)

separated by a day or two of recovery. Chronic stress may last 8 – 10 days. Acute stress may last 3 – 5 days. The following variables may be measured during drought stress and well watered treatments of transgenic plants and relevant control plants:

The variable "% area chg_start chronic - acute2" is a measure of the percent change in total area determined by remote visible spectrum imaging between the first day of chronic stress and the day of the second acute stress

The variable "% area chg_start chronic - end chronic" is a measure of the percent change in total area determined by remote visible spectrum imaging between the first day of chronic stress and the last day of chronic stress

The variable "% area chg_start chronic – harvest" is a measure of the percent change in total area determined by remote visible spectrum imaging between the first day of chronic stress and the day of harvest

The variable "% area chg_start chronic - recovery24hr" is a measure of the percent change in total area determined by remote visible spectrum imaging between the first day of chronic stress and 24 hrs into the recovery (24hrs after acute stress 2)

The variable "psii_acute1" is a measure of Photosystem II (PSII) efficiency at the end of the first acute stress period. It provides an estimate of the efficiency at which light is absorbed by PSII antennae and is directly related to carbon dioxide assimilation within the leaf.

The variable "psii_acute2" is a measure of Photosystem II (PSII) efficiency at the end of the second acute stress period. It provides an estimate of the efficiency at which light is absorbed by PSII antennae and is directly related to carbon dioxide assimilation within the leaf.

The variable "fv/fm_acute1" is a measure of the optimum quantum yield (Fv'/Fm') at the end of the first acute stress - (variable fluorescence difference between the maximum and minimum fluorescence / maximum fluorescence)

The variable "fv/fm_acute2" is a measure of the optimum quantum yield (Fv'/Fm') at the end of the second acute stress - (variable flourescence difference between the maximum and minimum fluorescence / maximum fluorescence)

The variable "leaf rolling_harvest" is a measure of the ratio of top image to side image on the day of harvest.

The variable "leaf rolling_recovery24hr" is a measure of the ratio of top image to side image 24 hours into the recovery.

The variable "Specific Growth Rate (SGR)" represents the change in total plant surface area (as measured by Lemna Tec Instrument) over a single day ($Y(t) = Y0*e^{r*t}$). $Y(t) = Y0*e^{r*t}$ is equivalent to % change in $Y/\Delta t$ where the individual terms are as follows: Y(t) = Total surface area at t; Y0 = Initial total surface area (estimated); r = Specific Growth Rate day^{-1}, and t = Days After Planting ("DAP")

The variable "shoot dry weight" is a measure of the shoot weight 96 hours after being placed into a 104 ºC oven

The variable "shoot fresh weight" is a measure of the shoot weight immediately after being cut from the plant

The Examples below describe some representative protocols and techniques for simulating drought conditions and/or evaluating drought tolerance.

One can also evaluate drought tolerance by the ability of a plant to maintain sufficient yield (at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% yield) in field testing under simulated or naturally-occurring drought conditions (e.g., by measuring for substantially equivalent yield under drought conditions compared to non-drought conditions, or by measuring for less yield loss under drought conditions compared to a control or reference plant).

One of ordinary skill in the art would readily recognize a suitable control or reference plant to be utilized when assessing or measuring an agronomic characteristic or phenotype of a transgenic plant in any embodiment of the present invention in which a control plant is utilized (e.g., compositions or methods as described herein). For example, by way of non-limiting illustrations:

1. Progeny of a transformed plant which is hemizygous with respect to a recombinant DNA construct (or suppression DNA construct), such that the progeny are segregating into plants either comprising or not comprising the

recombinant DNA construct (or suppression DNA construct): the progeny comprising the recombinant DNA construct (or suppression DNA construct) would be typically measured relative to the progeny not comprising the recombinant DNA construct (or suppression DNA construct) (i.e., the progeny not comprising the recombinant DNA construct (or the suppression DNA construct) is the control or reference plant).

2. Introgression of a recombinant DNA construct (or suppression DNA construct) into an inbred line, such as in maize, or into a variety, such as in soybean: the introgressed line would typically be measured relative to the parent inbred or variety line (i.e., the parent inbred or variety line is the control or reference plant).

3. Two hybrid lines, where the first hybrid line is produced from two parent inbred lines, and the second hybrid line is produced from the same two parent inbred lines except that one of the parent inbred lines contains a recombinant DNA construct (or suppression DNA construct): the second hybrid line would typically be measured relative to the first hybrid line (i.e., the first hybrid line is the control or reference plant).

4. A plant comprising a recombinant DNA construct (or suppression DNA construct): the plant may be assessed or measured relative to a control plant not comprising the recombinant DNA construct (or suppression DNA construct) but otherwise having a comparable genetic background to the plant (e.g., sharing at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity of nuclear genetic material compared to the plant comprising the recombinant DNA construct (or suppression DNA construct)). There are many laboratory-based techniques available for the analysis, comparison and characterization of plant genetic backgrounds; among these are Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Amplified Fragment Length Polymorphisms (AFLP®s), and Simple Sequence Repeats (SSRs) which are also referred to as Microsatellites.

Furthermore, one of ordinary skill in the art would readily recognize that a suitable control or reference plant to be utilized when assessing or measuring an agronomic characteristic or phenotype of a transgenic plant would not include a plant that had been previously selected, via mutagenesis or transformation, for the desired agronomic characteristic or phenotype.

Methods:

Methods include but are not limited to methods for increasing drought tolerance in a plant, methods for evaluating drought tolerance in a plant, methods for altering an agronomic characteristic in a plant, methods for determining an alteration of an agronomic characteristic in a plant, and methods for producing seed. The plant may be a monocotyledonous or dicotyledonous plant, for example, a maize or soybean plant. The plant may also be sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet. The seed is may be a maize or soybean seed, for example, a maize hybrid seed or maize inbred seed.

Methods include but are not limited to the following:

A method for transforming a cell comprising transforming a cell with any of the isolated polynucleotides of the present invention. The cell transformed by this method is also included. In particular embodiments, the cell is eukaryotic cell, e.g., a yeast, insect or plant cell, or prokaryotic, e.g., a bacterial cell.

A method for producing a transgenic plant comprising transforming a plant cell with any of the isolated polynucleotides or recombinant DNA constructs of the present invention and regenerating a transgenic plant from the transformed plant cell. The invention is also directed to the transgenic plant produced by this method, and transgenic seed obtained from this transgenic plant.

A method for isolating a polypeptide of the invention from a cell or culture medium of the cell, wherein the cell comprises a recombinant DNA construct comprising a polynucleotide of the invention operably linked to at least one regulatory sequence, and wherein the transformed host cell is grown under conditions that are suitable for expression of the recombinant DNA construct.

A method of altering the level of expression of a polypeptide of the invention in a host cell comprising: (a) transforming a host cell with a recombinant DNA construct of the present invention; and (b) growing the

transformed host cell under conditions that are suitable for expression of the recombinant DNA construct wherein expression of the recombinant DNA construct results in production of altered levels of the polypeptide of the invention in the transformed host cell.

A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; and (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct. The method may further comprise (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct.

A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24,

25, 27, 29, 31, OR 33, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (a)(i); and (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the suppression DNA construct. The method may further comprise (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the suppression DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the suppression DNA construct..

A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; and (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the suppression DNA construct. The method may further comprise (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the suppression DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the suppression DNA construct..

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct

comprising a polynucleotide operably linked to at least on regulatory sequence (for example, a promoter functional in a plant), wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, OR 33, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) evaluating the transgenic plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (e) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, OR 33, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (a)(i); (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; and (c) evaluating the transgenic plant for drought tolerance compared to a control plant not comprising the suppression DNA construct. The method may further comprise (d) obtaining

a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (e) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the suppression DNA construct.

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; and (c) evaluating the transgenic plant for drought tolerance compared to a control plant not comprising the suppression DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (e) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the suppression DNA construct.

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; (c) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (d) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (a)(i); (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; (c) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (d) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the suppression DNA construct.

A method of evaluating drought tolerance in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%,

76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; (c) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (d) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the suppression DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least on regulatory sequence (for example, a promoter functional in a plant), wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome said recombinant DNA construct; and (c) determining whether the transgenic plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the recombinant DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (e) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the recombinant DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (i); (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; and (c) determining whether the transgenic plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (e) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which

said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; and (c) determining whether the transgenic plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (e) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome said recombinant DNA construct; (c) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (d) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the recombinant DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA

construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or (ii) a full complement of the nucleic acid sequence of (i); (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; (c) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (d) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct.

A method of determining an alteration of an agronomic characteristic in a plant, comprising (a) introducing into a regenerable plant cell a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 56%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a Zinc-Finger (C3HC4-type RING finger) family polypeptide; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the suppression DNA construct; (c) obtaining a progeny plant derived from said transgenic plant, wherein the

progeny plant comprises in its genome the suppression DNA construct; and (d) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant not comprising the suppression DNA construct.

A method of producing seed (for example, seed that can be sold as a drought tolerant product offering) comprising any of the preceding methods, and further comprising obtaining seeds from said progeny plant, wherein said seeds comprise in their genome said recombinant DNA construct (or suppression DNA construct).

In any of the preceding methods or any other embodiments of methods of the present invention, in said introducing step said regenerable plant cell may comprise a callus cell, an embryogenic callus cell, a gametic cell, a meristematic cell, or a cell of an immature embryo. The regenerable plant cells may derive from an inbred maize plant.

In any of the preceding methods or any other embodiments of methods of the present invention, said regenerating step may comprise the following: (i) culturing said transformed plant cells in a media comprising an embryogenic promoting hormone until callus organization is observed; (ii) transferring said transformed plant cells of step (i) to a first media which includes a tissue organization promoting hormone; and (iii) subculturing said transformed plant cells after step (ii) onto a second media, to allow for shoot elongation, root development or both.

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

alteration of at least one agronomic characteristic may be an increase in yield, greenness or biomass.

In any of the preceding methods or any other embodiments of methods of the present invention, the plant may exhibit the alteration of at least one agronomic characteristic when compared, under water limiting conditions, to a control plant not comprising said recombinant DNA construct (or said suppression DNA construct).

In any of the preceding methods or any other embodiments of methods of the present invention, alternatives exist for introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence. For example, one may introduce into a regenerable plant cell a regulatory sequence (such as one or more enhancers, optionally as part of a transposable element), and then screen for an event in which the regulatory sequence is operably linked to an endogenous gene encoding a polypeptide of the instant invention.

The introduction of recombinant DNA constructs of the present invention into plants may be carried out by any suitable technique, including but not limited to direct DNA uptake, chemical treatment, electroporation, microinjection, cell fusion, infection, vector-mediated DNA transfer, bombardment, or *Agrobacterium*-mediated transformation. Techniques for plant transformation and regeneration have been described in International Patent Publication WO 2009/006276, the contents of which are herein incorporated by reference.

The development or regeneration of plants containing the foreign, exogenous isolated nucleic acid fragment that encodes a protein of interest is well known in the art. The regenerated plants may be self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

EXAMPLES

The present invention is further illustrated in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

EXAMPLE 1

Creation of an *Arabidopsis* Population with Activation-Tagged Genes

An 18.5-kb T-DNA based binary construct was created, pHSbarENDs2 (FIG. 1; SEQ ID NO:1), that contains four multimerized enhancer elements derived from the Cauliflower Mosaic Virus 35S promoter (corresponding to sequences -341 to -64, as defined by Odell et al., *Nature* 313:810-812 (1985)). The construct also contains vector sequences (pUC9) and a polylinker to allow plasmid rescue, transposon sequences (Ds) to remobilize the T-DNA, and the bar gene to allow for glufosinate selection of transgenic plants. In principle, only the 10.8-kb segment from the right border (RB) to left border (LB) inclusive will be transferred into the host plant genome. Since the enhancer elements are located near the RB, they can induce cis-activation of genomic loci following T-DNA integration.

Arabidopsis activation-tagged populations were created by whole plant *Agrobacterium* transformation. The pHSbarENDs2 construct was transformed into *Agrobacterium tumefaciens* strain C58, grown in LB at 25 °C to OD600 ~1.0. Cells were then pelleted by centrifugation and resuspended in an equal volume of 5% sucrose/0.05% Silwet L-77 (OSI Specialties, Inc). At early bolting, soil grown *Arabidopsis thaliana* ecotype Col-0 were top watered with the *Agrobacterium* suspension. A week later, the same plants were top watered again with the same *Agrobacterium* strain in sucrose/Silwet. The plants were

then allowed to set seed as normal. The resulting T1 seed were sown on soil, and transgenic seedlings were selected by spraying with glufosinate (Finale®; AgrEvo; Bayer Environmental Science). A total of 100,000 glufosinate resistant T1 seedlings were selected. T2 seed from each line was kept separate.

EXAMPLE 2

Screens to Identify Lines with Enhanced Drought Tolerance

Quantitative Drought Screen: From each of 96,000 separate T1 activation-tagged lines, nine glufosinate resistant T2 plants are sown, each in a single pot on Scotts® Metro-Mix® 200 soil. Flats are configured with 8 square pots each. Each of the square pots is filled to the top with soil. Each pot (or cell) is sown to produce 9 glufosinate resistant seedlings in a 3x3 array.

The soil is watered to saturation and then plants are grown under standard conditions (i.e., 16 hour light, 8 hour dark cycle; 22°C; ~60% relative humidity). No additional water is given.

Digital images of the plants are taken at the onset of visible drought stress symptoms. Images are taken once a day (at the same time of day), until the plants appear dessicated. Typically, four consecutive days of data is captured.

Color analysis is employed for identifying potential drought tolerant lines. Color analysis can be used to measure the increase in the percentage of leaf area that falls into a yellow color bin. Using hue, saturation and intensity data ("HSI"), the yellow color bin consists of hues 35 to 45.

Maintenance of leaf area is also used as another criterion for identifying potential drought tolerant lines, since *Arabidopsis* leaves wilt during drought stress. Maintenance of leaf area can be measured as reduction of rosette leaf area over time.

Leaf area is measured in terms of the number of green pixels obtained using the LemnaTec imaging system. Activation-tagged and control (e.g., wild-type) plants are grown side by side in flats that contain 72 plants (9 plants/pot). When wilting begins, images are measured for a number of days to monitor the wilting process. From these data wilting profiles are determined based on the green pixel counts obtained over four consecutive days for activation-tagged and accompanying control plants. The profile is selected from a series of measurements over the four day period that gives the largest degree of wilting.

The ability to withstand drought is measured by the tendency of activation-tagged plants to resist wilting compared to control plants.

LemnaTec HTSBonitUV software is used to analyze CCD images. Estimates of the leaf area of the *Arabidopsis* plants are obtained in terms of the number of green pixels. The data for each image is averaged to obtain estimates of mean and standard deviation for the green pixel counts for activation-tagged and wild-type plants. Parameters for a noise function are obtained by straight line regression of the squared deviation versus the mean pixel count using data for all images in a batch. Error estimates for the mean pixel count data are calculated using the fit parameters for the noise function. The mean pixel counts for activation-tagged and wild-type plants are summed to obtain an assessment of the overall leaf area for each image. The four-day interval with maximal wilting is obtained by selecting the interval that corresponds to the maximum difference in plant growth. The individual wilting responses of the activation-tagged and wild-type plants are obtained by normalization of the data using the value of the green pixel count of the first day in the interval. The drought tolerance of the activation-tagged plant compared to the wild-type plant is scored by summing the weighted difference between the wilting response of activation-tagged plants and wild-type plants over day two to day four; the weights are estimated by propagating the error in the data. A positive drought tolerance score corresponds to an activation-tagged plant with slower wilting compared to the wild-type plant. Significance of the difference in wilting response between activation-tagged and wild-type plants is obtained from the weighted sum of the squared deviations.

Lines with a significant delay in yellow color accumulation and/or with significant maintenance of rosette leaf area, when compared to the average of the whole flat, are designated as Phase 1 hits. Phase 1 hits are re-screened in duplicate under the same assay conditions. When either or both of the Phase 2 replicates show a significant difference (score of greater than 0.9) from the whole flat mean, the line is then considered a validated drought tolerant line.

Lines with Enhanced Drought Tolerance can also be screened using the following method (see also Figure 12 for treatment schedule):

Transgenic maize seedlings are screened for drought tolerance by measuring chlorophyll fluorescence performance, biomass accumulation, and drought survival. Transgenic plants are compared against the null tracngenic (not containing the transgene). Experimental design is a Randomized Complete Block and Replication consist of 13 positive plants from each event and a construct null (2 negatives each event).

Plant are grown at well watered (WW) conditions = 60% Field Capacity (%FC) to a three leaf stage. At the three leaf stage and under WW conditions the first fluorescence measurement is taken on the uppermost fully extended leaf at the inflection point, in the leaf margin and avoiding the mid rib.

This is followed by imposing a moderate drought stress (FIG 12. day 13, MOD DRT) by maintaining 20% FC for duration of 9 to 10 days.

During this stress treatment leaves may appear gray and rolling may occur. At the end of MOD DRT plants are recovered (MOD rec) by increasing to 25% FC. During this time, leaves will begin to unroll. This is a time sensitive step that may take up to 1 hour to occur and can be dependent upon the construct and events being tested. When plants appear to have recovered completed (leaves unrolled), the second fluorescence measurement is taken.

This is followed by imposing a severe drought stress (SEV DRT) by withholding all water until the plants collapse. Duration of severe drought stress is 8-10 days and/or when plants have collapse.

Thereafter, a recovery (REC) is imposed by watering all plants to 100% FC. Maintain 100% FC 72 hours.

Survival score (yes/no) is recorded after 24, 48 and 72 hour recovery.

The entire shoot (Fresh) is sampled and weights are recorded. (Fresh shoot weights). Fresh shoot material is then dried for 120hrs at 70 degrees at which time a Dry Shoot weight is recorded.

Measured variables are defined as follows:

The variable “Fv’/Fm’ no stress” is a measure of the optimum quantum yield (Fv’/Fm’) under optimal water conditions on the uppermost fully extended leaf (most often the third leaf) at the inflection point, in the leaf margin and avoiding the mid rib. Fv’/Fm’ provides an estimate of the maximum efficiency of

PSII photochemistry at a given PPFD, which is the PSII operating efficiency if all the PSII centers were open (Q_A oxidized) .

The variable "Fv'/Fm' stress" is a measure of the optimum quantum yield (Fv'/Fm') under water stressed conditions (25% field capacity). The measure is preceded by a moderate drought period where field capacity drops from 60% to 20%. At which time the field capacity is brought to 25% and the measure collected.

The variable "phiPSII_no stress" is a measure of Photosystem II (PSII) efficiency under optimal water conditions on the uppermost fully extended leaf (most often the third leaf) at the inflection point, in the leaf margin and avoiding the mid rib. phiPSII provides an estimate of the PSII operating efficiency, which estimates the efficiency at which light absorbed by PSII is used for Q_A reduction.

The variable "phiPSII_stress" is a measure of Photosystem II (PSII) efficiency under water stressed conditions (25% field capacity). The measure is preceded by a moderate drought period where field capacity drops from 60% to 20%. At which time the field capacity is brought to 25% and the measure collected.

EXAMPLE 3

Identification of Activation-Tagged Genes

Genes flanking the T-DNA insert in drought tolerant lines are identified using one, or both, of the following two standard procedures: (1) thermal asymmetric interlaced (TAIL) PCR (Liu et al., (1995), *Plant J. 8*:457-63); and (2) SAIFF PCR (Siebert et al., (1995) *Nucleic Acids Res. 23*:1087-1088). In lines with complex multimerized T-DNA inserts, TAIL PCR and SAIFF PCR may both prove insufficient to identify candidate genes. In these cases, other procedures, including inverse PCR, plasmid rescue and/or genomic library construction, can be employed.

A successful result is one where a single TAIL or SAIFF PCR fragment contains a T-DNA border sequence and *Arabidopsis* genomic sequence.

Once a tag of genomic sequence flanking a T-DNA insert is obtained, candidate genes are identified by alignment to publicly available *Arabidopsis* genome sequence.

Specifically, the annotated gene nearest the 35S enhancer elements/T-DNA RB are candidates for genes that are activated.

To verify that an identified gene is truly near a T-DNA and to rule out the possibility that the TAIL/SAIFF fragment is a chimeric cloning artifact, a diagnostic PCR on genomic DNA is done with one oligo in the T-DNA and one oligo specific for the candidate gene. Genomic DNA samples that give a PCR product are interpreted as representing a T-DNA insertion. This analysis also verifies a situation in which more than one insertion event occurs in the same line, e.g., if multiple differing genomic fragments are identified in TAIL and/or SAIFF PCR analyses.

EXAMPLE 4A

Identification of Activation-Tagged Zinc-Finger (C3HC4-type RING finger) family polypeptide Gene

An activation-tagged line (No. 102143) showing drought tolerance was further analyzed. DNA from the line was extracted, and genes flanking the T-DNA insert in the mutant line were identified using SAIFF PCR (Siebert et al., *Nucleic Acids Res.* 23:1087-1088 (1995)). A PCR amplified fragment was identified that contained T-DNA border sequence and *Arabidopsis* genomic sequence. Genomic sequences flanking the T-DNA insert was obtained, and the candidate gene was identified by alignment to the completed *Arabidopsis* genome. For a given T-DNA integration event, the annotated gene nearest the 35S enhancer elements/T-DNA RB was the candidate for gene that is activated in the line. In the case of line No. 102143, the gene nearest the 35S enhancers at the integration site was At2g01150 (SEQ ID NO:16; NCBI GI No. 98960889, Accession BT025510), encoding a Zinc-Finger (C3HC4-type RING finger) family polypeptide (SEQ ID NO:17; NCBI GI No. 98960889).

EXAMPLE 4B

Assay for Expression Level of Candidate Drought Tolerance Genes

A functional activation-tagged allele should result in either up-regulation of the candidate gene in tissues where it is normally expressed, ectopic expression in tissues that do not normally express that gene, or both. Expression levels of the candidate genes in the cognate mutant line vs. wild-type are compared. A standard RT-PCR procedure, such as the QuantiTect® Reverse Transcription

Kit from Qiagen®, is used. RT-PCR of the actin gene is used as a control to show that the amplification and loading of samples from the mutant line and wild-type are similar.

Assay conditions are optimized for each gene. Expression levels are checked in mature rosette leaves. If the activation-tagged allele results in ectopic expression in other tissues (e.g., roots), it is not detected by this assay. As such, a positive result is useful but a negative result does not eliminate a gene from further analysis.

EXAMPLE 5

Validation of *Arabidopsis* Candidate Gene At2g01150 (Zinc-Finger family polypeptide) via Transformation into *Arabidopsis*

Candidate genes can be transformed into *Arabidopsis* and overexpressed under the 35S promoter. If the same or similar phenotype is observed in the transgenic line as in the parent activation-tagged line, then the candidate gene is considered to be a validated "lead gene" in *Arabidopsis*.

The candidate *Arabidopsis* Zinc-Finger (C3HC4-type RING finger) family polypeptide gene (At2g01150; SEQ ID NO:16) was tested for its ability to confer drought tolerance in the following manner.

A 16.8-kb T-DNA based binary vector, called pBC-yellow (SEQ ID NO:4; FIG. 4), was constructed with a 1.3-kb 35S promoter immediately upstream of the INVITROGEN™ GATEWAY® C1 conversion insert. The vector also contains the RD29a promoter driving expression of the gene for ZS-Yellow (INVITROGEN™), which confers yellow fluorescence to transformed seed.

The At2g01150 cDNA protein-coding region was amplified by RT-PCR with the following primers:

(1) At2g01150-5'attB forward primer (SEQ ID NO:12):
TTAAACAAGTTTGTACAAAAAAGCAGGCTCAACAATGGGACTACA
AGGTCAGCTC

(2) At2g01150-3'attB reverse primer (SEQ ID NO:13):
TTAAACCACTTTGTACAAGAAAGCTGGGTTCAATGAGATGATGCA
GTAGA

The forward primer contains the attB1 sequence (ACAAGTTTGTACAAAAAAGCAGGCT; SEQ ID NO:10) and a consensus

Kozak sequence (CAACA) adjacent to the first 21 nucleotides of the protein-coding region, beginning with the ATG start codon, of said cDNA.

The reverse primer contains the attB2 sequence (ACCACTTTGTACAAGAAAGCTGGGT; SEQ ID NO:11) adjacent to the reverse complement of the last 21 nucleotides of the protein-coding region, beginning with the reverse complement of the stop codon, of said cDNA.

Using the INVITROGEN™ GATEWAY® CLONASE™ technology, a BP Recombination Reaction was performed with pDONR™/Zeo (SEQ ID NO:2; FIG. 2). This process removed the bacteria lethal ccdB gene, as well as the chloramphenicol resistance gene (CAM) from pDONR™/Zeo and directionally cloned the PCR product with flanking attB1 and attB2 sites creating an entry clone (PHP31324). This entry clone was used for a subsequent LR Recombination Reaction with a destination vector, as follows.

A 16.8-kb T-DNA based binary vector (destination vector), called pBC-yellow (SEQ ID NO:4; FIG. 4), was constructed with a 1.3-kb 35S promoter immediately upstream of the INVITROGEN™ GATEWAY® C1 conversion insert, which contains the bacterial lethal ccdB gene as well as the chloramphenicol resistance gene (CAM) flanked by attR1 and attR2 sequences. The vector also contains the RD29a promoter driving expression of the gene for ZS-Yellow (INVITROGEN™), which confers yellow fluorescence to transformed seed. Using the INVITROGEN™ GATEWAY® technology, an LR Recombination Reaction was performed on the entry clone, containing the directionally cloned PCR product, and pBC-yellow. This allowed for rapid and directional cloning of the candidate gene behind the 35S promoter in pBC-yellow to create the 35S promoter::At2g01150 expression construct, pBC-Yellow-At2g01150.

Applicants then introduced the 35S promoter::At2g01150 expression construct into wild-type *Arabidopsis* ecotype Col-0, using the same *Agrobacterium*-mediated transformation procedure described in Example 1. Transgenic T1 seeds were selected by yellow fluorescence, and T1 seeds were plated next to wild-type seeds and grown under water limiting conditions. Growth conditions and imaging analysis were as described in Example 2. It was found that the original drought tolerance phenotype from activation tagging could be recapitulated in wild-type *Arabidopsis* plants that were transformed with a

construct where At2g01150 was directly expressed by the 35S promoter. The drought tolerance score, as determined by the method of Example 2, was 2.258.

EXAMPLE 6

Preparation of cDNA Libraries and Isolation and Sequencing of cDNA Clones

cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in UNI-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). The UNI-ZAP™ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBluescript®. In addition, the cDNAs may be introduced directly into precut Bluescript® II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant pBluescript® plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al., (1991) *Science 252*:1651-1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

Full-insert sequence (FIS) data is generated utilizing a modified transposition protocol. Clones identified for FIS are recovered from archived glycerol stocks as single colonies, and plasmid DNAs are isolated via alkaline lysis. Isolated DNA templates are reacted with vector primed M13 forward and reverse oligonucleotides in a PCR-based sequencing reaction and loaded onto automated sequencers. Confirmation of clone identification is performed by sequence alignment to the original EST sequence from which the FIS request is made.

Confirmed templates are transposed via the Primer Island transposition kit (PE Applied Biosystems, Foster City, CA) which is based upon the

Saccharomyces cerevisiae Ty1 transposable element (Devine and Boeke (1994) *Nucleic Acids Res. 22*:3765-3772). The *in vitro* transposition system places unique binding sites randomly throughout a population of large DNA molecules. The transposed DNA is then used to transform DH10B electro-competent cells (Gibco BRL/Life Technologies, Rockville, MD) via electroporation. The transposable element contains an additional selectable marker (named DHFR; Fling and Richards (1983) *Nucleic Acids Res. 11*:5147-5158), allowing for dual selection on agar plates of only those subclones containing the integrated transposon. Multiple subclones are randomly selected from each transposition reaction, plasmid DNAs are prepared via alkaline lysis, and templates are sequenced (ABI Prism® dye-terminator ReadyReaction mix) outward from the transposition event site, utilizing unique primers specific to the binding sites within the transposon.

Sequence data is collected (ABI Prism® Collections) and assembled using Phred and Phrap (Ewing et al. (1998) *Genome Res. 8*:175-185; Ewing and Green (1998) *Genome Res. 8*:186-194). Phred is a public domain software program which re-reads the ABI sequence data, re-calls the bases, assigns quality values, and writes the base calls and quality values into editable output files. The Phrap sequence assembly program uses these quality values to increase the accuracy of the assembled sequence contigs. Assemblies are viewed by the Consed sequence editor (Gordon et al. (1998) *Genome Res. 8*:195-202).

In some of the clones the cDNA fragment may correspond to a portion of the 3'-terminus of the gene and does not cover the entire open reading frame. In order to obtain the upstream information one of two different protocols is used. The first of these methods results in the production of a fragment of DNA containing a portion of the desired gene sequence while the second method results in the production of a fragment containing the entire open reading frame. Both of these methods use two rounds of PCR amplification to obtain fragments from one or more libraries. The libraries some times are chosen based on previous knowledge that the specific gene should be found in a certain tissue and some times are randomly-chosen. Reactions to obtain the same gene may be performed on several libraries in parallel or on a pool of libraries. Library

pools are normally prepared using from 3 to 5 different libraries and normalized to a uniform dilution. In the first round of amplification both methods use a vector-specific (forward) primer corresponding to a portion of the vector located at the 5'-terminus of the clone coupled with a gene-specific (reverse) primer. The first method uses a sequence that is complementary to a portion of the already known gene sequence while the second method uses a gene-specific primer complementary to a portion of the 3'-untranslated region (also referred to as UTR). In the second round of amplification a nested set of primers is used for both methods. The resulting DNA fragment is ligated into a pBluescript® vector using a commercial kit and following the manufacturer's protocol. This kit is selected from many available from several vendors including INVITROGEN™ (Carlsbad, CA), Promega Biotech (Madison, WI), and Gibco-BRL (Gaithersburg, MD). The plasmid DNA is isolated by alkaline lysis method and submitted for sequencing and assembly using Phred/Phrap, as above.

EXAMPLE 7

Identification of cDNA Clones

cDNA clones encoding Zinc-Finger (C3HC4-type RING finger) family polypeptides can be identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol. 215*:403-410; see also the explanation of the BLAST algorithm on the world wide web site for the National Center for Biotechnology Information at the National Library of Medicine of the National Institutes of Health) searches for similarity to amino acid sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The DNA sequences from clones can be translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nat. Genet. 3*:266-272) provided by the NCBI. The polypeptides encoded by the cDNA sequences can be analyzed for similarity to all publicly available amino acid sequences contained in the "nr" database using the BLASTP algorithm provided by the National Center for Biotechnology Information (NCBI). For convenience,

the P-value (probability) or the E-value (expectation) of observing a match of a cDNA-encoded sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value or E-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA-encoded sequence and the BLAST "hit" represent homologous proteins.

ESTs sequences can be compared to the Genbank database as described above. ESTs that contain sequences more 5- or 3-prime can be found by using the BLASTn algorithm (Altschul et al (1997) *Nucleic Acids Res.* *25*:3389-3402.) against the Du Pont proprietary database comparing nucleotide sequences that share common or overlapping regions of sequence homology. Where common or overlapping sequences exist between two or more nucleic acid fragments, the sequences can be assembled into a single contiguous nucleotide sequence, thus extending the original fragment in either the 5 or 3 prime direction. Once the most 5-prime EST is identified, its complete sequence can be determined by Full Insert Sequencing as described above. Homologous genes belonging to different species can be found by comparing the amino acid sequence of a known gene (from either a proprietary source or a public database) against an EST database using the tBLASTn algorithm. The tBLASTn algorithm searches an amino acid query against a nucleotide database that is translated in all 6 reading frames. This search allows for differences in nucleotide codon usage between different species, and for codon degeneracy.

EXAMPLE 8

Characterization of cDNA Clones Encoding Zinc-Finger (C3HC4-type RING finger) family polypeptides

cDNA libraries representing mRNAs from various tissues of Sugar Beet, Canola, Maize, Rice, Soybean, Wheat and Catmint can be prepared and cDNA clones encoding Zinc-Finger (C3HC4-type RING finger) family polypeptides can be identified.

EXAMPLE 9

Preparation of a Plant Expression Vector Containing a Homolog to the *Arabidopsis* Lead Gene

Sequences homologous to the *Arabidopsis* EXPA10 polypeptide can be identified using sequence comparison algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al., J. Mol. Biol. 215:403-410 (1993); see also the explanation of the BLAST algorithm on the world wide web site for the National Center for Biotechnology Information at the National Library of Medicine of the National Institutes of Health). Sequences encoding homologous EXPA10 polypeptides can be PCR-amplified by any of the following methods.

Method 1 (RNA-based): If the 5' and 3' sequence information for the protein-coding region of a gene encoding a EXPA10 polypeptide homolog is available, gene-specific primers can be designed as outlined in Example 5. RT-PCR can be used with plant RNA to obtain a nucleic acid fragment containing the protein-coding region flanked by attB1 (SEQ ID NO: 10) and attB2 (SEQ ID NO:11) sequences. The primer may contain a consensus Kozak sequence (CAACA) upstream of the start codon.

Method 2 (DNA-based): Alternatively, if a cDNA clone is available for a gene encoding a EXPA10 polypeptide homolog, the entire cDNA insert (containing 5' and 3' non-coding regions) can be PCR amplified. Forward and reverse primers can be designed that contain either the attB1 sequence and vector-specific sequence that precedes the cDNA insert or the attB2 sequence and vector-specific sequence that follows the cDNA insert, respectively. For a cDNA insert cloned into the vector pBulescript SK+, the forward primer VC062 (SEQ ID NO: 14) and the reverse primer VC063 (SEQ ID NO: 15) can be used.

Method 3 (genomic DNA): Genomic sequences can be obtained using long range genomic PCR capture. Primers can be designed based on the sequence of the genomic locus and the resulting PCR product can be sequenced. The sequence can be analyzed using the FGENESH (Salamov, A. and Solovyev, V. (2000) *Genome Res.*, 10: 516-522) program, and optionally, can be aligned with homologous sequences from other species to assist in identification of putative introns.

Methods 1, 2, and 3 can be modified according to procedures known by one skilled in the art. For example, the primers of Method 1 may contain restriction sites instead of attB1 and attB2 sites, for subsequent cloning of the PCR product into a vector containing attB1 and attB2 sites. Additionally, Method 2 can involve amplification from a cDNA clone, a lambda clone, a BAC clone or genomic DNA.

A PCR product obtained by either method above can be combined with the GATEWAY® donor vector, such as pDONR™/Zeo (INVITROGEN™; FIG. 2; SEQ ID NO:2) or pDONR™221 (INVITROGEN™; FIG. 3; SEQ ID NO:3), using a BP Recombination Reaction. This process removes the bacteria lethal ccdB gene, as well as the chloramphenicol resistance gene (CAM) from pDONR™221 and directionally clones the PCR product with flanking attB1 and attB2 sites to create an entry clone. Using the INVITROGEN™ GATEWAY® CLONASE™ technology, the sequence encoding the homologous EXPA10 polypeptide from the entry clone can then be transferred to a suitable destination vector, such as pBC-Yellow (FIG. 4; SEQ ID NO:4), PHP27840 (FIG. 5; SEQ ID NO:5) or PHP23236 (FIG. 6; SEQ ID NO:6), to obtain a plant expression vector for use with *Arabidopsis*, soybean and corn, respectively.

The attP1 and attP2 sites of donor vectors pDONR™/Zeo or pDONR™221are shown in Figures 2 and 3, respectively. The attR1 and attR2 sites of destination vectors pBC-Yellow, PHP27840 and PHP23236 are shown in Figures 4, 5 and 6, respectively.

Alternatively a MultiSite GATEWAY® LR recombination reaction between multiple entry clones and a suitable destination vector can be performed to create an expression vector.

EXAMPLE 10

Preparation of Soybean Expression Vectors and Transformation of Soybean with Validated *Arabidopsis* Lead Genes

Soybean plants can be transformed to overexpress a validated *Arabidopsis* lead gene or the corresponding homologs from various species in order to examine the resulting phenotype.

The same GATEWAY® entry clone described in Example 5 can be used to directionally clone each gene into the PHP27840 vector (SEQ ID NO:5; FIG. 5) such that expression of the gene is under control of the SCP1 promoter.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides.

To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26 °C on an appropriate agar medium for 6-10 weeks. Somatic embryos, which produce secondary embryos, are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiply as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26 ºC with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium. Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A DUPONT™ BIOLISTIC™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from cauliflower mosaic virus (Odell et al. (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene 25*:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. Another selectable marker gene which can be used to facilitate soybean transformation is an herbicide-resistant acetolactate synthase (ALS) gene from soybean or *Arabidopsis.* ALS is the first common enzyme in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine. Mutations in ALS have been identified that convey resistance to some or all of three classes of inhibitors of ALS (US Patent No. 5,013,659; the entire contents of which are herein incorporated by reference). Expression of the herbicide-resistant ALS

gene can be under the control of a SAM synthetase promoter (U.S. Patent Application No. US-2003-0226166-A1; the entire contents of which are herein incorporated by reference).

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

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

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

T1 plants can be subjected to a soil-based drought stress. Using image analysis, plant area, volume, growth rate and color analysis can be taken at multiple times before and during drought stress. Overexpression constructs that

result in a significant delay in wilting or leaf area reduction, yellow color accumulation and/or increased growth rate during drought stress will be considered evidence that the *Arabidopsis* gene functions in soybean to enhance drought tolerance.

Soybean plants transformed with validated genes can then be assayed under more vigorous field-based studies to study yield enhancement and/or stability under well-watered and water-limiting conditions.

EXAMPLE 11

Transformation of Maize with Validated *Arabidopsis* Lead Genes Using Particle Bombardment

Maize plants can be transformed to overexpress a validated *Arabidopsis* lead gene or the corresponding homologs from various species in order to examine the resulting phenotype.

The same GATEWAY® entry clone described in Example 5 can be used to directionally clone each gene into a maize transformation vector. Expression of the gene in the maize transformation vector can be under control of a constitutive promoter such as the maize ubiquitin promoter (Christensen et al., (1989) *Plant Mol. Biol.* 12:619-632 and Christensen et al., (1992) *Plant Mol. Biol.* 18:675-689)

The recombinant DNA construct described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to

provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from cauliflower mosaic virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 μm in diameter) are coated with DNA using the following technique. Ten μg of plasmid DNAs are added to 50 μL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 μL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 μL of ethanol. An aliquot (5 μL) of the DNA-coated gold particles can be placed in the center of a KAPTON™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a DUPONT™ BIOLISTIC™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covers a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains bialaphos (5 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing bialaphos. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the bialaphos-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology 8*:833-839). Transgenic T0 plants can be regenerated and their phenotype determined following high throughput ("HTP") procedures. T1 seed can be collected.

T1 plants can be subjected to a soil-based drought stress. Using image analysis, plant area, volume, growth rate and color analysis can be taken at multiple times before and during drought stress. Overexpression constructs that result in a significant delay in wilting or leaf area reduction, yellow color accumulation and/or increased growth rate during drought stress will be considered evidence that the *Arabidopsis* gene functions in maize to enhance drought tolerance.

EXAMPLE 12

Electroporation of *Agrobacterium tumefaciens* LBA4404

Electroporation competent cells (40 μL), such as *Agrobacterium tumefaciens* LBA4404 containing PHP10523 (FIG. 7; SEQ ID NO:7), are thawed on ice (20-30 min). PHP10523 contains VIR genes for T-DNA transfer, an *Agrobacterium* low copy number plasmid origin of replication, a tetracycline resistance gene, and a Cos site for in vivo DNA bimolecular recombination. Meanwhile the electroporation cuvette is chilled on ice. The electroporator settings are adjusted to 2.1 kV. A DNA aliquot (0.5 μL parental DNA at a concentration of 0.2 μg -1.0 μg in low salt buffer or twice distilled H_2O) is mixed with the thawed *Agrobacterium tumefaciens* LBA4404 cells while still on ice. The mixture is transferred to the bottom of electroporation cuvette and kept at rest on ice for 1-2 min. The cells are electroporated (Eppendorf electroporator

2510) by pushing the "pulse" button twice (ideally achieving a 4.0 millisecond pulse). Subsequently, 0.5 mL of room temperature 2xYT medium (or SOC medium) are added to the cuvette and transferred to a 15 mL snap-cap tube (e.g., FALCON™ tube). The cells are incubated at 28-30 °C, 200-250 rpm for 3 h.

Aliquots of 250 μL are spread onto plates containing YM medium and 50 μg/mL spectinomycin and incubated three days at 28-30 °C. To increase the number of transformants one of two optional steps can be performed:

Option 1: Overlay plates with 30 μL of 15 mg/mL rifampicin. LBA4404 has a chromosomal resistance gene for rifampicin. This additional selection eliminates some contaminating colonies observed when using poorer preparations of LBA4404 competent cells.

Option 2: Perform two replicates of the electroporation to compensate for poorer electrocompetent cells.

Identification of transformants:

Four independent colonies are picked and streaked on plates containing AB minimal medium and 50 μg/mL spectinomycin for isolation of single colonies. The plates are incubated at 28 °C for two to three days. A single colony for each putative co-integrate is picked and inoculated with 4 mL of 10 g/L bactopeptone, 10 g/L yeast extract, 5 g/L sodium chloride and 50 mg/L spectinomycin. The mixture is incubated for 24 h at 28 °C with shaking. Plasmid DNA from 4 mL of culture is isolated using Qiagen® Miniprep and an optional Buffer PB wash. The DNA is eluted in 30 μL. Aliquots of 2 μL are used to electroporate 20 μL of DH10b + 20 μL of twice distilled H_2O as per above. Optionally a 15 μL aliquot can be used to transform 75-100 μL of INVITROGEN™ Library Efficiency DH5α. The cells are spread on plates containing LB medium and 50 μg/mL spectinomycin and incubated at 37 °C overnight.

Three to four independent colonies are picked for each putative co-integrate and inoculated 4 mL of 2xYT medium (10 g/L bactopeptone, 10 g/L yeast extract, 5 g/L sodium chloride) with 50 μg/mL spectinomycin. The cells are incubated at 37 °C overnight with shaking. Next, isolate the plasmid DNA from 4 mL of culture using QIAprep® Miniprep with optional Buffer PB wash (elute in 50 μL). Use 8 μL for digestion with SalI (using parental DNA and PHP10523 as

controls). Three more digestions using restriction enzymes BamHI, EcoRI, and HindIII are performed for 4 plasmids that represent 2 putative co-integrates with correct SalI digestion pattern (using parental DNA and PHP10523 as controls). Electronic gels are recommended for comparison.

EXAMPLE 13

Transformation of Maize Using *Agrobacterium*

Maize plants can be transformed to overexpress a validated *Arabidopsis* lead gene or the corresponding homologs from various species in order to examine the resulting phenotype.

Agrobacterium-mediated transformation of maize is performed essentially as described by Zhao et al. in *Meth. Mol. Biol.* 318:315-323 (2006) (see also Zhao et al., *Mol. Breed.* 8:323-333 (2001) and U.S. Patent No. 5,981,840 issued November 9, 1999, incorporated herein by reference). The transformation process involves bacterium innoculation, co-cultivation, resting, selection and plant regeneration.

1. Immature Embryo Preparation:

Immature maize embryos are dissected from caryopses and placed in a 2 mL microtube containing 2 mL PHI-A medium.

2. *Agrobacterium* Infection and Co-Cultivation of Immature Embryos:

2.1 Infection Step:

PHI-A medium of (1) is removed with 1 mL micropipettor, and 1 mL of *Agrobacterium* suspension is added. The tube is gently inverted to mix. The mixture is incubated for 5 min at room temperature.

2.2 Co-culture Step:

The *Agrobacterium* suspension is removed from the infection step with a 1 mL micropipettor. Using a sterile spatula the embryos are scraped from the tube and transferred to a plate of PHI-B medium in a 100x15 mm Petri dish. The embryos are oriented with the embryonic axis down on the surface of the medium. Plates with the embryos are cultured at 20 °C, in darkness, for three days. L-Cysteine can be used in the co-cultivation phase. With the standard binary vector, the co-cultivation medium supplied with 100-400 mg/L L-cysteine is critical for recovering stable transgenic events.

3. Selection of Putative Transgenic Events:

To each plate of PHI-D medium in a 100x15 mm Petri dish, 10 embryos are transferred, maintaining orientation and the dishes are sealed with parafilm. The plates are incubated in darkness at 28 °C. Actively growing putative events, as pale yellow embryonic tissue, are expected to be visible in six to to eight weeks. Embryos that produce no events may be brown and necrotic, and little friable tissue growth is evident. Putative transgenic embryonic tissue is subcultured to fresh PHI-D plates at two-three week intervals, depending on growth rate. The events are recorded.

4. Regeneration of T0 plants:

Embryonic tissue propagated on PHI-D medium is subcultured to PHI-E medium (somatic embryo maturation medium), in 100x25 mm Petri dishes and incubated at 28 °C, in darkness, until somatic embryos mature, for about ten to eighteen days. Individual, matured somatic embryos with well-defined scutellum and coleoptile are transferred to PHI-F embryo germination medium and incubated at 28 °C in the light (about 80 µE from cool white or equivalent fluorescent lamps). In seven to ten days, regenerated plants, about 10 cm tall, are potted in horticultural mix and hardened-off using standard horticultural methods.

Media for Plant Transformation:

1. PHI-A: 4g/L CHU basal salts, 1.0 mL/L 1000X Eriksson's vitamin mix, 0.5 mg/L thiamin HCl, 1.5 mg/L 2,4-D, 0.69 g/L L-proline, 68.5 g/L sucrose, 36 g/L glucose, pH 5.2. Add 100 µM acetosyringone (filter-sterilized).
2. PHI-B: PHI-A without glucose, increase 2,4-D to 2 mg/L, reduce sucrose to 30 g/L and supplemente with 0.85 mg/L silver nitrate (filter-sterilized), 3.0 g/L Gelrite®, 100 µM acetosyringone (filter-sterilized), pH 5.8.
3. PHI-C: PHI-B without Gelrite® and acetosyringonee, reduce 2,4-D to 1.5 mg/L and supplemente with 8.0 g/L agar, 0.5 g/L 2-[N-morpholino]ethane-sulfonic acid (MES) buffer, 100 mg/L carbenicillin (filter-sterilized).
4. PHI-D: PHI-C supplemented with 3 mg/L bialaphos (filter-sterilized).

5. PHI-E: 4.3 g/L of Murashige and Skoog (MS) salts, (Gibco, BRL 11117-074), 0.5 mg/L nicotinic acid, 0.1 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 2.0 mg/L glycine, 0.1 g/L myo-inositol, 0.5 mg/L zeatin (Sigma, Cat. No. Z-0164), 1 mg/L indole acetic acid (IAA), 26.4 µg/L abscisic acid (ABA), 60 g/L sucrose, 3 mg/L bialaphos (filter-sterilized), 100 mg/L carbenicillin (filter-sterilized), 8 g/L agar, pH 5.6.

6. PHI-F: PHI-E without zeatin, IAA, ABA; reduce sucrose to 40 g/L; replacing agar with 1.5 g/L Gelrite®; pH 5.6.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., *Bio/Technology* 8:833-839 (1990)).

Transgenic T0 plants can be regenerated and their phenotype determined. T1 seed can be collected.

Furthermore, a recombinant DNA construct containing a validated *Arabidopsis* gene can be introduced into an elite maize inbred line either by direct transformation or introgression from a separately transformed line.

Transgenic plants, either inbred or hybrid, can undergo more vigorous field-based experiments to study yield enhancement and/or stability under water limiting and water non-limiting conditions.

Subsequent yield analysis can be done to determine whether plants that contain the validated *Arabidopsis* lead gene have an improvement in yield performance (under water limiting or non-limiting conditions), when compared to the control (or reference) plants that do not contain the validated *Arabidopsis* lead gene. Specifically, water limiting conditions can be imposed during the flowering and/or grain fill period for plants that contain the validated *Arabidopsis* lead gene and the control plants. Plants containing the validated *Arabidopsis* lead gene would have less yield loss relative to the control plants, for example, at least 25% less yield loss, under water limiting conditions, or would have increased yield relative to the control plants under water non-limiting conditions.

EXAMPLE 14A

Preparation of *Arabidopsis* Lead Gene (At2g01150)

Expression Vector for Transformation of Maize

Using INVITROGEN's™ GATEWAY® technology, an LR Recombination Reaction was performed with an entry clone (PHP31324) and a destination vector (PHP28647) to create the precursor plasmid PHP31363. The vector PHP31363 contains the following expression cassettes:

1. Ubiquitin promoter::moPAT::PinII terminator; cassette expressing the PAT herbicide resistance gene used for selection during the transformation process.

2. LTP2 promoter::DS-RED2::PinII terminator; cassette expressing the DS-RED color marker gene used for seed sorting.

3. Ubiquitin promoter::At2g01150::PinII terminator; cassette overexpressing the gene of interest, *Arabidopsis* Zinc-Finger (C3HC4-type RING finger) family polypeptide.

EXAMPLE 14B

Transformation of Maize with the *Arabidopsis* Lead Gene (At2g01150) Using *Agrobacterium*

The Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette present in vector PHP31363 can be introduced into a maize inbred line, or a transformable maize line derived from an elite maize inbred line, using *Agrobacterium*-mediated transformation as described in Examples 12 and 13.

Vector PHP31363 can be electroporated into the LBA4404 *Agrobacterium* strain containing vector PHP10523 (FIG. 7; SEQ ID NO:7) to create the co-integrate vector PHP31373. The co-integrate vector is formed by recombination of the 2 plasmids, PHP31363 and PHP10523, through the COS recombination sites contained on each vector. The co-integrate vector PHP31373 contains the same 3 expression cassettes as above (Example 14A) in addition to other genes (TET, TET, TRFA, ORI terminator, CTL, ORI V, VIR C1, VIR C2, VIR G, VIR B) needed for the *Agrobacterium* strain and the *Agrobacterium*-mediated transformation.

EXAMPLE 15

Preparation of the Destination Vector PHP23236 for Transformation Into Gaspe Flint Derived Maize Lines

Destination vector PHP23236 (FIG. 6 , SEQ ID NO:6) was obtained by transformation of *Agrobacterium* strain LBA4404 containing plasmid PHP10523 (FIG. 7, SEQ ID NO:7) with plasmid PHP23235 (FIG. 8, SEQ ID NO:8) and isolation of the resulting co-integration product. Destination vector PHP23236, can be used in a recombination reaction with an entry clone as described in Example 16 to create a maize expression vector for transformation of Gaspe Flint-derived maize lines.

EXAMPLE 16

Preparation of Plasmids for Transformation into Gaspe Flint Derived Maize Lines

Using the INVITROGEN™ GATEWAY® LR Recombination technology, the same entry clone described in Example 5A, was directionally cloned into the destination vector PHP23236 (SEQ ID NO:6; FIG. 6) to create an expression vector, PHP30775. This expression vector contains the cDNA of interest, encoding the *Arabidopsis* Zinc-Finger (C3HC4-type RING finger) family polypeptide under control of the UBI promoter and is a T-DNA binary vector for *Agrobacterium*-mediated transformation into corn as described, but not limited to, the examples described herein.

EXAMPLE 17

Transformation of Gaspe Flint Derived Maize Lines with a Validated *Arabidopsis* Lead Gene

Maize plants can be transformed to overexpress the *Arabidopsis* lead gene or the corresponding homologs from other species in order to examine the resulting phenotype.

Recipient Plants:

Recipient plant cells can be from a uniform maize line having a short life cycle ("fast cycling"), a reduced size, and high transformation potential. Typical of these plant cells for maize are plant cells from any of the publicly available Gaspe Flint (GBF) line varieties. One possible candidate plant line variety is the F1 hybrid of GBF x QTM (Quick Turnaround Maize, a publicly available form of

Gaspe Flint selected for growth under greenhouse conditions) disclosed in Tomes et al. U.S. Patent Application Publication No. 2003/0221212. Transgenic plants obtained from this line are of such a reduced size that they can be grown in four inch pots (1/4 the space needed for a normal sized maize plant) and mature in less than 2.5 months. (Traditionally 3.5 months is required to obtain transgenic T0 seed once the transgenic plants are acclimated to the greenhouse.) Another suitable line is a double haploid line of GS3 (a highly transformable line) X Gaspe Flint. Yet another suitable line is a transformable elite inbred line carrying a transgene which causes early flowering, reduced stature, or both.

Transformation Protocol:

Any suitable method may be used to introduce the transgenes into the maize cells, including but not limited to inoculation type procedures using *Agrobacterium* based vectors. Transformation may be performed on immature embryos of the recipient (target) plant.

Precision Growth and Plant Tracking:

The event population of transgenic (T0) plants resulting from the transformed maize embryos is grown in a controlled greenhouse environment using a modified randomized block design to reduce or eliminate environmental error. A randomized block design is a plant layout in which the experimental plants are divided into groups (e.g., thirty plants per group), referred to as blocks, and each plant is randomly assigned a location with the block.

For a group of thirty plants, twenty-four transformed, experimental plants and six control plants (plants with a set phenotype) (collectively, a "replicate group") are placed in pots which are arranged in an array (a.k.a. a replicate group or block) on a table located inside a greenhouse. Each plant, control or experimental, is randomly assigned to a location with the block which is mapped to a unique, physical greenhouse location as well as to the replicate group. Multiple replicate groups of thirty plants each may be grown in the same greenhouse in a single experiment. The layout (arrangement) of the replicate groups should be determined to minimize space requirements as well as environmental effects within the greenhouse. Such a layout may be referred to as a compressed greenhouse layout.

An alternative to the addition of a specific control group is to identify those transgenic plants that do not express the gene of interest. A variety of techniques such as RT-PCR can be applied to quantitatively assess the expression level of the introduced gene. T0 plants that do not express the transgene can be compared to those which do.

Each plant in the event population is identified and tracked throughout the evaluation process, and the data gathered from that plant is automatically associated with that plant so that the gathered data can be associated with the transgene carried by the plant. For example, each plant container can have a machine readable label (such as a Universal Product Code (UPC) bar code) which includes information about the plant identity, which in turn is correlated to a greenhouse location so that data obtained from the plant can be automatically associated with that plant.

Alternatively any efficient, machine readable, plant identification system can be used, such as two-dimensional matrix codes or even radio frequency identification tags (RFID) in which the data is received and interpreted by a radio frequency receiver/processor. See U.S. Published Patent Application No. 2004/0122592, incorporated herein by reference.

Phenotypic Analysis Using Three-Dimensional Imaging:

Each greenhouse plant in the T0 event population, including any control plants, is analyzed for agronomic characteristics of interest, and the agronomic data for each plant is recorded or stored in a manner so that it is associated with the identifying data (see above) for that plant. Confirmation of a phenotype (gene effect) can be accomplished in the T1 generation with a similar experimental design to that described above.

The T0 plants are analyzed at the phenotypic level using quantitative, non-destructive imaging technology throughout the plant's entire greenhouse life cycle to assess the traits of interest. A digital imaging analyzer may be used for automatic multi-dimensional analyzing of total plants. The imaging may be done inside the greenhouse. Two camera systems, located at the top and side, and an apparatus to rotate the plant, are used to view and image plants from all sides. Images are acquired from the top, front and side of each plant. All three

images together provide sufficient information to evaluate the biomass, size and morphology of each plant.

Due to the change in size of the plants from the time the first leaf appears from the soil to the time the plants are at the end of their development, the early stages of plant development are best documented with a higher magnification from the top. This may be accomplished by using a motorized zoom lens system that is fully controlled by the imaging software.

In a single imaging analysis operation, the following events occur: (1) the plant is conveyed inside the analyzer area, rotated 360 degrees so its machine readable label can be read, and left at rest until its leaves stop moving; (2) the side image is taken and entered into a database; (3) the plant is rotated 90 degrees and again left at rest until its leaves stop moving, and (4) the plant is transported out of the analyzer.

Plants are allowed at least six hours of darkness per twenty four hour period in order to have a normal day/night cycle.

Imaging Instrumentation:

Any suitable imaging instrumentation may be used, including but not limited to light spectrum digital imaging instrumentation commercially available from LemnaTec GmbH of Wurselen, Germany. The images are taken and analyzed with a LemnaTec Scanalyzer HTS LT-0001-2 having a 1/2" IT Progressive Scan IEE CCD imaging device. The imaging cameras may be equipped with a motor zoom, motor aperture and motor focus. All camera settings may be made using LemnaTec software. For example, the instrumental variance of the imaging analyzer is less than about 5% for major components and less than about 10% for minor components.

Software:

The imaging analysis system comprises a LemnaTec HTS Bonit software program for color and architecture analysis and a server database for storing data from about 500,000 analyses, including the analysis dates. The original images and the analyzed images are stored together to allow the user to do as much reanalyzing as desired. The database can be connected to the imaging hardware for automatic data collection and storage. A variety of commercially available software systems (e.g. Matlab, others) can be used for quantitative

interpretation of the imaging data, and any of these software systems can be applied to the image data set.

Conveyor System:

A conveyor system with a plant rotating device may be used to transport the plants to the imaging area and rotate them during imaging. For example, up to four plants, each with a maximum height of 1.5 m, are loaded onto cars that travel over the circulating conveyor system and through the imaging measurement area. In this case the total footprint of the unit (imaging analyzer and conveyor loop) is about 5 m x 5 m.

The conveyor system can be enlarged to accommodate more plants at a time. The plants are transported along the conveyor loop to the imaging area and are analyzed for up to 50 seconds per plant. Three views of the plant are taken. The conveyor system, as well as the imaging equipment, should be capable of being used in greenhouse environmental conditions.

Illumination:

Any suitable mode of illumination may be used for the image acquisition. For example, a top light above a black background can be used. Alternatively, a combination of top- and backlight using a white background can be used. The illuminated area should be housed to ensure constant illumination conditions. The housing should be longer than the measurement area so that constant light conditions prevail without requiring the opening and closing or doors. Alternatively, the illumination can be varied to cause excitation of either transgene (e.g., green fluorescent protein (GFP), red fluorescent protein (RFP)) or endogenous (e.g. Chlorophyll) fluorophores.

Biomass Estimation Based on Three-Dimensional Imaging:

For best estimation of biomass the plant images should be taken from at least three axes, for example, the top and two side (sides 1 and 2) views. These images are then analyzed to separate the plant from the background, pot and pollen control bag (if applicable). The total area of the plant can be estimated by the calculation:

Estimated Total Plant Area(pixels) = Top Area(pixels) + Side1Area(pixels) + Side2Area(pixels)

In the equation above the units of area are "arbitrary units". Arbitrary units are entirely sufficient to detect gene effects on plant size and growth in this system because what is desired is to detect differences (both positive-larger and negative-smaller) from the experimental mean, or control mean. The arbitrary units of size (e.g. area) may be trivially converted to physical measurements by the addition of a physical reference to the imaging process. For instance, a physical reference of known area can be included in both top and side imaging processes. Based on the area of these physical references a conversion factor can be determined to allow conversion from pixels to a unit of area such as square centimeters (cm^2). The physical reference may or may not be an independent sample. For instance, the pot, with a known diameter and height, could serve as an adequate physical reference.

Color Classification:

The imaging technology may also be used to determine plant color and to assign plant colors to various color classes. The assignment of image colors to color classes is an inherent feature of the LemnaTec software. With other image analysis software systems color classification may be determined by a variety of computational approaches.

For the determination of plant size and growth parameters, a useful classification scheme is to define a simple color scheme including two or three shades of green and, in addition, a color class for chlorosis, necrosis and bleaching, should these conditions occur. A background color class which includes non plant colors in the image (for example pot and soil colors) is also used and these pixels are specifically excluded from the determination of size. The plants are analyzed under controlled constant illumination so that any change within one plant over time, or between plants or different batches of plants (e.g. seasonal differences) can be quantified.

In addition to its usefulness in determining plant size growth, color classification can be used to assess other yield component traits. For these other yield component traits additional color classification schemes may be used. For instance, the trait known as "staygreen", which has been associated with improvements in yield, may be assessed by a color classification that separates

shades of green from shades of yellow and brown (which are indicative of senescing tissues). By applying this color classification to images taken toward the end of the T0 or T1 plants' life cycle, plants that have increased amounts of green colors relative to yellow and brown colors (expressed, for instance, as Green/Yellow Ratio) may be identified. Plants with a significant difference in this Green/Yellow ratio can be identified as carrying transgenes which impact this important agronomic trait.

The skilled plant biologist will recognize that other plant colors arise which can indicate plant health or stress response (for instance anthocyanins), and that other color classification schemes can provide further measures of gene action in traits related to these responses.

Plant Architecture Analysis:

Transgenes which modify plant architecture parameters may also be identified using the present invention, including such parameters as maximum height and width, internodal distances, angle between leaves and stem, number of leaves starting at nodes and leaf length. The LemnaTec system software may be used to determine plant architecture as follows. The plant is reduced to its main geometric architecture in a first imaging step and then, based on this image, parameterized identification of the different architecture parameters can be performed. Transgenes that modify any of these architecture parameters either singly or in combination can be identified by applying the statistical approaches previously described.

Pollen Shed Date:

Pollen shed date is an important parameter to be analyzed in a transformed plant, and may be determined by the first appearance on the plant of an active male flower. To find the male flower object, the upper end of the stem is classified by color to detect yellow or violet anthers. This color classification analysis is then used to define an active flower, which in turn can be used to calculate pollen shed date.

Alternatively, pollen shed date and other easily visually detected plant attributes (e.g. pollination date, first silk date) can be recorded by the personnel responsible for performing plant care. To maximize data integrity and process efficiency this data is tracked by utilizing the same barcodes utilized by the

LemnaTec light spectrum digital analyzing device. A computer with a barcode reader, a palm device, or a notebook PC may be used for ease of data capture recording time of observation, plant identifier, and the operator who captured the data.

Orientation of the Plants:

Mature maize plants grown at densities approximating commercial planting often have a planar architecture. That is, the plant has a clearly discernable broad side, and a narrow side. The image of the plant from the broadside is determined. To each plant a well defined basic orientation is assigned to obtain the maximum difference between the broadside and edgewise images. The top image is used to determine the main axis of the plant, and an additional rotating device is used to turn the plant to the appropriate orientation prior to starting the main image acquisition.

EXAMPLE 18 A

Screening of Gaspe Flint Derived Maize Lines for Drought Tolerance

Transgenic Gaspe Flint derived maize lines containing the candidate gene can be screened for tolerance to drought stress in the following manner.

Transgenic maize plants are subjected to well-watered conditions (control) and to drought-stressed conditions. Transgenic maize plants are screened at the T1 stage or later.

Stress is imposed starting at 10 to 14 days after sowing (DAS) or 7 days after transplanting, and is continued through to silking. Pots are watered by an automated system fitted to timers to provide watering at 25 or 50% of field capacity during the entire period of drought-stress treatment. The intensity and duration of this stress will allow identification of the impact on vegetative growth as well as on the anthesis-silking interval.

Potting mixture: A mixture of 1/3 TURFACE® (Profile Products LLC, IL, USA), 1/3 sand and 1/3 *SB300* (Sun Gro Horticulture, WA, USA) can be used. The *SB300* can be replaced with *Fafard Fine-Germ* (Conrad Fafard, Inc., MA, USA) and the proportion of sand in the mixture can be reduced. Thus, a final potting mixture can be 3/8 (37.5%) TURFACE®, 3/8 (37.5%) *Fafard* and ¼ (25%) sand.

Field Capacity Determination: The weight of the soil mixture (w1) to be used in one S200 pot (minus the pot weight) is measured. If all components of the soil mix are not dry, the soil is dried at 100°C to constant weight before determining w1. The soil in the pot is watered to full saturation and all the gravitational water is allowed to drain out. The weight of the soil (w2) after all gravitational water has seeped out (minus the pot weight) is determined. Field capacity is the weight of the water remaining in the soil obtained as w2-w1. It can be written as a percentage of the oven-dry soil weight.

Stress Treatment: During the early part of plant growth (10 DAS to 21 DAS), the well-watered control has a daily watering of 75% field capacity and the drought-stress treatment has a daily watering of 25% field capacity, both as a single daily dose at or around 10 AM. As the plants grow bigger, by 21 DAS, it will become necessary to increase the daily watering of the well-watered control to full field capacity and the drought stress treatment to 50% field capacity.

Nutrient Solution: A modified Hoagland's solution at 1/16 dilution with tap water is used for irrigation (Table 1, Table2).

TABLE 1

Preparation of 20 L of Modified Hoagland's Solution Using the Following Recipe:

Component	Amount/20 L
10X Micronutrient Solution	16 mL
KH_2PO_4 (MW: 136.02)	22 g
$MgSO_4$ (MW: 120.36)	77 g
KNO_3 (MW: 101.2)	129.5 g
$Ca(NO_3)_2 \cdot 4H_20$ (MW: 236.15)	151 g
NH_4NO_3 (MW: 80.04)	25.6 g
Sprint 330 (Iron chelate)	32 g

TABLE 2

Preparation of 1L of 10X Micronutrient Solution Using the Following Recipe:

Component	mg/L	Concentration
H_3BO_3	1854	30 mM
$MnCl_2 \cdot 4H_20$	1980	10 mM

$ZnSO_4 \cdot 7H_20$	2874	10 mM
$CuSO_4 \cdot 5H_20$	250	1 mM
$H_2MoO_4 \cdot H_20$	242	1 mM

Fertilizer grade KNO_3 is used.

It is useful to add half a teaspoon of OSMOCOTE® (NPK 15:9:12) to the pot at the time of transplanting or after emergence (The Scotts Miracle-Gro Company, OH, USA).

Border plants: Place a row of border plants on bench-edges adjacent to the glass walls of the greenhouse or adjacent to other potential causes of microenvironment variability such as a cooler fan.

Automation: Watering can be done using PVC pipes with drilled holes to supply water to systematically positioned pots using a siphoning device. Irrigation scheduling can be done using timers.

Statistical analysis: Mean values for plant size, color and chlorophyll fluorescence recorded on transgenic events under different stress treatments will be exported to Spotfire (Spotfire, Inc., MA, USA). Treatment means will be evaluated for differences using Analysis of Variance.

Replications: Eight to ten individual plants are used per treatment per event.

Observations Made: Lemnatec measurements are made three times a week throughout growth to capture plant-growth rate. Leaf color determinations are made three times a week throughout the stress period using Lemnatec. Chlorophyll fluorescence is recorded as PhiPSII (which is indicative of the operating quantum efficiency of photosystem II photochemistry; also referred to as δF/Fm' or F'q/Fm') and Fv'/Fm' (which is the maximum efficiency of photosystem II) two to four times during the experimental period, starting at 11 AM on the measurement days, using the Hansatech FMS2 instrument (LemnaTec GmbH, Wurselen, Germany). Measurements are started during the stress period at the beginning of visible drought stress symptoms, namely, leaf greying and the start of leaf rolling until the end of the experiment and measurements are recorded on the youngest most fully expanded leaf. The

dates of tasseling and silking on individual plants are recorded, and the ASI is computed.

The above methods may be used to select transgenic plants with increased drought tolerance when compared to a control plant not comprising said recombinant DNA construct.

EXAMPLE 18B

Transformation and Evaluation of Maize Lines transformed with PHP31373

The Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette present in co-integrate vector PHP31373 was introduced into a transformable maize line derived from an elite maize inbred line as described in Examples 14A and 14B.

T1 seeds were obtained for nine transformation events and evaluated for drought tolerance activity essentially as described in Example 18 A. Figure 10 shows the variables for each transgenic event that were significantly altered, as compared to the segregant nulls. A "positive effect" was defined as statistically significant improvement in that variable for the transgenic event relative to the null control. A "negative effect" was defined as a statistically significant improvement in that variable for the null control relative to the transgenic event.

For the construct evaluated, PHP31373, the statistical value associated with each improved variable is presented in Figure 10. A significant positive result had a P-value of less than or equal to 0.1. The results for individual transformed maize lines are presented in Figure 10.

Figure 10 indicates that in experiment 1, seven out of nine events and in a replicate experiment 2, five out of nine events, showed a positive effect on at least one variable (such as shoot weight) when plants were grown under reduced water (drought stress) conditions.

EXAMPLE 19 A

Yield Analysis of Maize Lines with the

Arabidopsis Lead Gene

A recombinant DNA construct containing a validated *Arabidopsis* gene can be introduced into an elite maize inbred line either by direct transformation or introgression from a separately transformed line.

Transgenic plants, either inbred or hybrid, can undergo more vigorous field-based experiments to study yield enhancement and/or stability under well-watered and water-limiting conditions.

Subsequent yield analysis can be done to determine whether plants that contain the validated *Arabidopsis* lead gene have an improvement in yield performance under water-limiting conditions, when compared to the control plants that do not contain the validated *Arabidopsis* lead gene. Specifically, drought conditions can be imposed during the flowering and/or grain fill period for plants that contain the validated *Arabidopsis* lead gene and the control plants. Reduction in yield can be measured for both. Plants containing the validated *Arabidopsis* lead gene have less yield loss relative to the control plants, for example, at least 25% less yield loss.

The above method may be used to select transgenic plants with increased yield, under water-limiting conditions and/or well-watered conditions, when compared to a control plant not comprising said recombinant DNA construct.

EXAMPLE 19 B

Yield Analysis of Maize Lines transformed with PHP31373 encoding the *Arabidopsis* Lead Gene At2g01150

The Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette present in the co-integrate vector PHP31373 was introduced into a transformable maize line derived from an elite maize inbred line as described in Examples 14A and 14B.

Nine transgenic events were field tested in 2009 at Johnston, IA ("JH"), York, NE ("YK"), and Woodland, CA ("WO"). At the Woodland, CA, location, drought conditions were imposed during flowering ("FS"; flowering stress) and during the grain fill period ("GFS"; grain fill stress). The JH location was well-watered, and the YK location experienced mild drought during the grain-filling period. Yield data (bushel/ acre; bu/ac) of the 9 transgenic events is shown in Table 3 together with the wild type control (WT) and bulk null control (BN). Statistical significance is reported at $P<0.1$ for a two-tailed test. Three events had positive effects in the YK environment and one event had positive effects in the WO_GFS location when compared to the WT or BN control. One event had

positive effects in the WO_GFS location. Three events had a significant negative effect in two of the four locations. Event E7899. 44.1.4 showed a higher yield in three of the four locations when compared to the WT control, with a significant increase of 15 to 25 bu/ac in the WO-GFS location when compared to the BN or WT control , respectively.

TABLE 3

2009 Field Test of Maize Transformed with PHP31373

	Yield (bu/ac)			
EVENT	YK	JH	WO-FS	WO-GFS
E7899.44.1.10	169	178	94 **	56 **
E7899.44.1.4	174	181	113	100 *
E7899.44.1.8	177 *	183	103	60 **
E7899.44.2.1	170	174 **	108 **	75
E7899.44.2.3	177	183	98 **	66 **
E7899.44.3.10	178 *	175 **	105 **	53 **
E7899.44.3.11	173	189	103 **	76
E7899.44.7.2	176	174 **	122	66 **
E7899.44.8.4	178 *	183	119	73
WT	170	189	109	75
BN	170	189	130	85

* Significant gain in yield
** Significant loss in yield

Figure 16 shows the average yield of 8 transgenic maize events (containing the Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette) vs. the bulk null yield. The gray line (labeled 1:1 ratio) marks where the ratio of yield between transgenic and bulk null is the 1:1, representing equal yield. It can be observed that when overall yield levels are low, the transgenic plants show a yield advantage over non- transgenic nulls (see areas circled where transgenic yield is higher than the 1: 1 ratio.

Furthermore, when maize plants are exposed to a gradual stress (Figure 16, left panel) a cross over point (at around 80 bu/acre) can be observed at a higher yield level when compared to plants that are exposed to a rapid stress (at around 50 bu/acre) right panel).

EXAMPLE 20A

Preparation of Maize Zinc-Finger (C3HC4-type RING finger) family polypeptide Lead Gene Expression Vector for Transformation of Maize

Clones encoding a maize Zinc-Finger (C3HC4-type RING finger) family polypeptide-can be identified. The protein-coding region of these clones can be introduced into the INVITROGEN™ vector pENTR/D-TOPO® to create entry clones.

EXAMPLE 20B

Transformation of Maize with Maize Zinc-Finger (C3HC4-type RING finger) family polypeptide Lead Gene Using *Agrobacterium*

A maize Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette can be introduced into a maize inbred line, or a transformable maize line derived from an elite maize inbred line, using *Agrobacterium*-mediated transformation as described in Examples 12 and 13.

EXAMPLE 21

Preparation of Maize Expression Plasmids for Transformation into Gaspe Flint Derived Maize Lines

Clones encoding a complete maize Zinc-Finger (C3HC4-type RING finger) family polypeptide homolog can be identified.

Using the INVITROGEN™ GATEWAY® Recombination technology described in Example 9, the clones encoding maize Zinc-Finger (C3HC4-type RING finger) family polypeptide homologs can be directionally cloned into a destination vector to create expression vectors. Each expression vector can contains the cDNA of interest under control of the UBI promoter and is a T-DNA binary vector for *Agrobacterium*-mediated transformation into corn as described, but not limited to, the examples described herein.

EXAMPLE 22

Transformation and Evaluation of Soybean with Soybean Homologs of Validated Lead Genes

Based on homology searches, one or several candidate soybean homologs of validated *Arabidopsis* lead genes can be identified and also be assessed for their ability to enhance drought tolerance in soybean. Vector

construction, plant transformation and phenotypic analysis will be similar to that in previously described Examples.

EXAMPLE 23

Transformation and Evaluation of Maize with Maize Homologs of Validated Lead Genes

Based on homology searches, one or several candidate maize homologs of validated *Arabidopsis* lead genes can be identified and also be assessed for their ability to enhance drought tolerance in maize. Vector construction, plant transformation and phenotypic analysis will be similar to that in previously described Examples.

EXAMPLE 24

Transformation of *Arabidopsis* with Maize and Soybean Homologs of Validated Lead Genes

Soybean and maize homologs to validated *Arabidopsis* lead genes can be transformed into *Arabidopsis* under control of the 35S promoter and assessed for their ability to enhance drought tolerance in *Arabidopsis*. Vector construction, plant transformation and phenotypic analysis will be similar to that in previously described Examples.

EXAMPLE 25

Further characterization of *Arabidopsis* Candidate Gene At2g01150 (Zinc-Finger family polypeptide; RHA2B)

The candidate *Arabidopsis* Zinc-Finger (C3HC4-type RING finger) family polypeptide gene (At2g01150; SEQ ID NO:16) was tested for its ability to confer drought tolerance as described in Example 5.

To further characterize the Arabidopsis Candidate Gene At2g01150, experiments were conducted to test if the Arabidopsis Candidate Gene At2g01150 plays a role in ABA responses. A ring finger E3 Ligase (RHA2a) of Arabidopsis AtRHA2a has been reported to regulate ABA-mediated control of seed germination (Bu, Q. et al. Plant Physiology Vol150, pp 463-481).

ABA sensitivity was examined during the germination of wild-type (Wt) and T2 transgenic Arabidopsis seeds containing the Zinc-Finger (C3HC4-type RING finger) family polypeptide (referred to as 35S- At2g01150 seeds) (Figure 11, left panel). Wt and pooled T2 transgenic seeds (~100 seeds) were plated

and kept for 4 days at 4°C to break dormancy. Petri dishes were than incubated at 22°C under 16 hr light/8 hr dark. Germination was scored everyday. Two replicate plates were scored for each treatment. On ½ MS-agarose plates, both wt and 35-At2g01150 had similar germination percentages. In the presence ABA, 35S-At2g01150 seeds showed increased ABA sensitivity in comparison to wt plants. Similarly, the ratio of the ABA inhibition of postgermination growth was significantly increased in transgenic lines over-expressing At2g01150 (see #27, Figure 11, right panel).

Consistent with ABA hypersensitivity, 35S-At2g1150 transgenic plants displays enhanced drought tolerance in the drought/rewatering assay. The drought/rewatering assay measures the difference in recovery after dehydration with plants grown in soil. The plants were grown under 16 hr illumination at 22°C and 40% relative humidity. Drought stress was imposed on 3-week old plants by withholding water for two weeks, then plants were rewatered. The recovery rate of 35-At2g01150 plants was markedly increased in comparison of wt plants.

Drought/rewatering experiments indicated that the transgenic Arabidopisis plants over-expressing At2g01150 (RHA2B) performed better and showed drought tolerance activity when compared to than wild type plants not containing the transgene (Figure 15).

EXAMPLE 26

Stay Green Analysis of Maize Lines transformed with PHP31373 encoding the *Arabidopsis* Lead Gene At2g01150

The Zinc-Finger (C3HC4-type RING finger) family polypeptide expression cassette present in co-integrate vector PHP31373 was introduced into a transformable maize line derived from an elite maize inbred line as described in Examples 14A and 14B.

Stay-green is a visual estimate of the proportion of the canopy that is green on the date the observation was made. When used for drought experiments, scoring was done when differences in drought-induced senescence were observed. Stay green scores reflect proportion of canopy that was still green at the date scores were taken (9=90% green, 1=10% green).

Nine transgenic events were field tested in 2009 at the Woodland, CA.. Drought conditions were imposed during flowering ("FS"; flowering stress) and during the grain fill period ("GFS"; grain fill stress). Stay green phenotype of the 9 transgenic events is shown in Table 4 together with the wild type control (WT) and bulk null control (BN). Statistical significance is reported at P<0.1 for a two-tailed test.

TABLE 4

Stay Green Phenotype of Maize Transformed with PHP31373

EVENT	WO_FS	WO-GFS
E7899.44.1.10	7.7	5.3 *
E7899.44.1.4	5.8	4.6 *
E7899.44.1.8	7.4	4.3
E7899.44.2.1	6.6	4.7 *
E7899.44.2.3	7.4	4.7 *
E7899.44.3.10	7.7	5.3 *
E7899.44.3.11	5.7	4.0
E7899.44.7.2	7.1	4.0
E7899.44.8.4	7.7	4.6 *
WT	**5.9**	**3.3**
BN	**6.9**	**3.7**

* Significant gain in stay green

CLAIMS

What is claimed is:

1. A plant comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, and wherein said plant exhibits increased drought tolerance when compared to a control plant not comprising said recombinant DNA construct.

2. The plant of Claim 1, wherein the plant is a maize plant or a soybean plant.

3. A method of increasing drought tolerance in a plant, comprising:

(a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33; and

(b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct.

4. The method of Claim 3, further comprising:

(c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant not comprising the recombinant DNA construct.

5. A method of evaluating drought tolerance in a plant, comprising:

(a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one

regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33;

(b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and

(c) evaluating the transgenic plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

6. The method of Claim 5, further comprising:

(d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and

(e) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

7. A method of evaluating drought tolerance in a plant, comprising:

(a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33;

(b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct;

(c) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and

(d) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

8. The method of Claim 3, wherein the plant is a maize plant or a soybean plant.

9. The method of Claim 4, wherein the plant is a maize plant or a soybean plant.

10. The method of Claim 5, wherein the plant is a maize plant or a soybean plant.

11. The method of Claim 6, wherein the plant is a maize plant or a soybean plant.

12. The method of Claim 7, wherein the plant is a maize plant or a soybean plant.

13. An isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a Zinc-Finger (C3HC4-type RING finger) family polypeptide, wherein the polypeptide has an amino acid sequence of at least 90% sequence identity, based on the Clustal V method of alignment with pairwise alignment default parameters of KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33, or

(b) the full complement of the nucleotide sequence of (a).

14. The polynucleotide of Claim 13, wherein the polypeptide has an amino acid sequence of at least 95% sequence identity, based on the Clustal V method of alignment with the pairwise alignment default parameters, when compared to SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33.

15. The polynucleotide of Claim 28, wherein the amino acid sequence of the polypeptide comprises SEQ ID NO: 17, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, or 33.

16. The polynucleotide of Claim 13 wherein the nucleotide sequence comprises SEQ ID NO: 16.

17. A vector comprising the polynucleotide of Claim 13.

18. A recombinant DNA construct comprising the isolated polynucleotide of Claim 13 operably linked to at least one regulatory sequence.

19. A cell comprising the recombinant DNA construct of Claim 18, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, and insect cell and a plant cell.

20. A plant or seed comprising the recombinant DNA construct of Claim 18.

FIG. 1

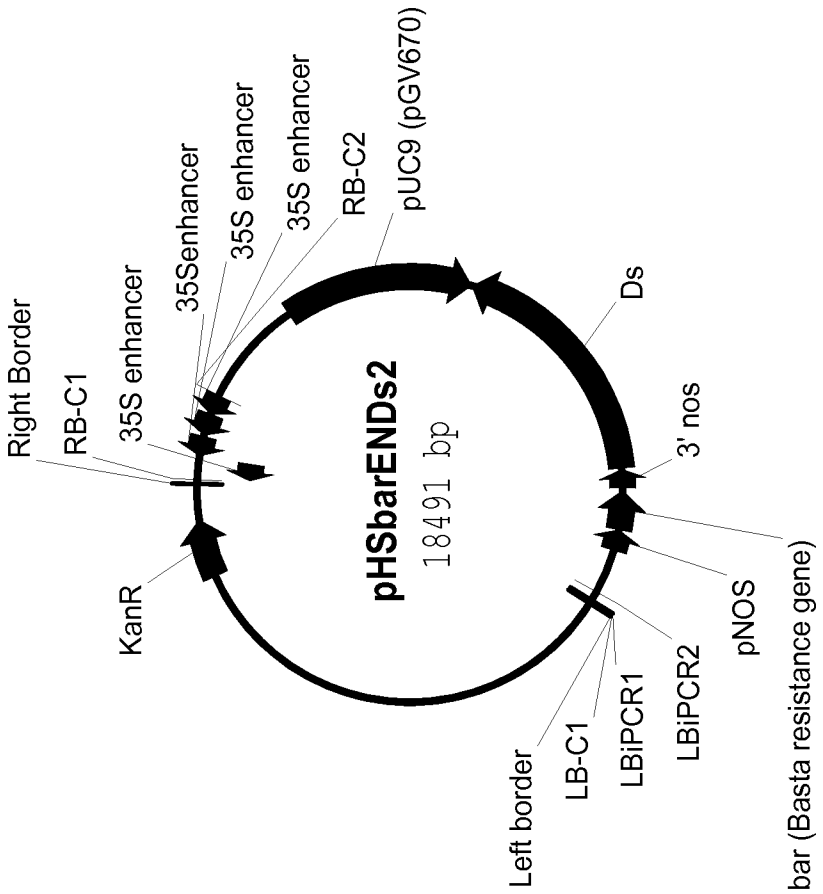

pHSbarENDs2
18491 bp
Right Border
RB-C1
35S enhancer
35Senhancer
35S enhancer
35S enhancer
RB-C2
pUC9 (pGV670)
Ds
3' nos
pNOS
bar (Basta resistance gene)
LBiPCR2
LBiPCR1
LB-C1
Left border
KanR

FIG. 2

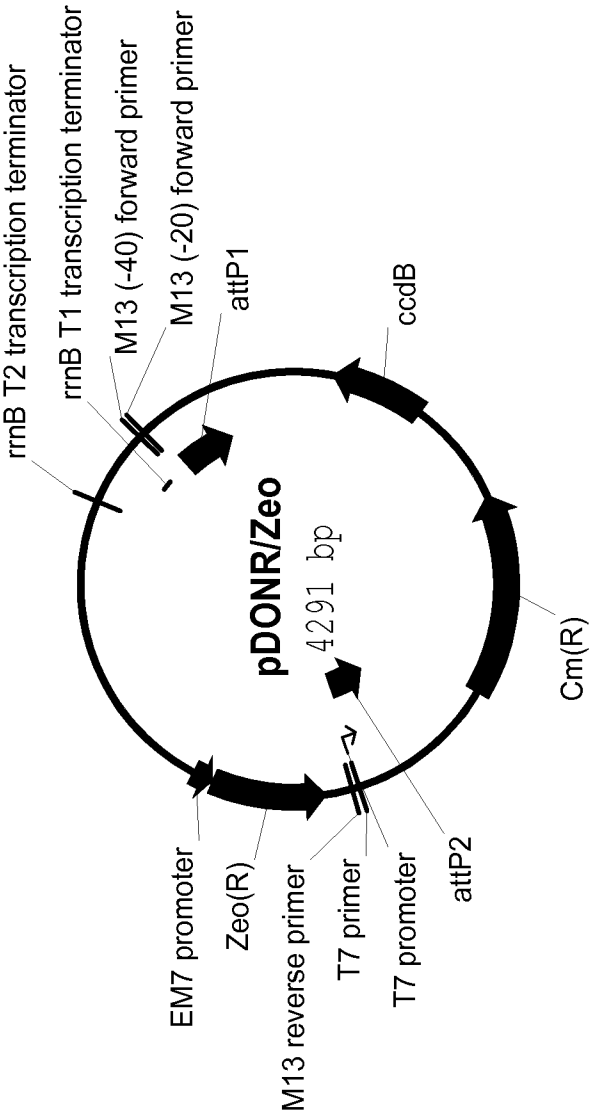

rrnB T2 transcription terminator
rrnB T1 transcription terminator
M13 (-40) forward primer
M13 (-20) forward primer
attP1
ccdB
Cm(R)
attP2
T7 promoter
T7 primer
M13 reverse primer
Zeo(R)
EM7 promoter
pDONR/Zeo
4291 bp

FIG. 3

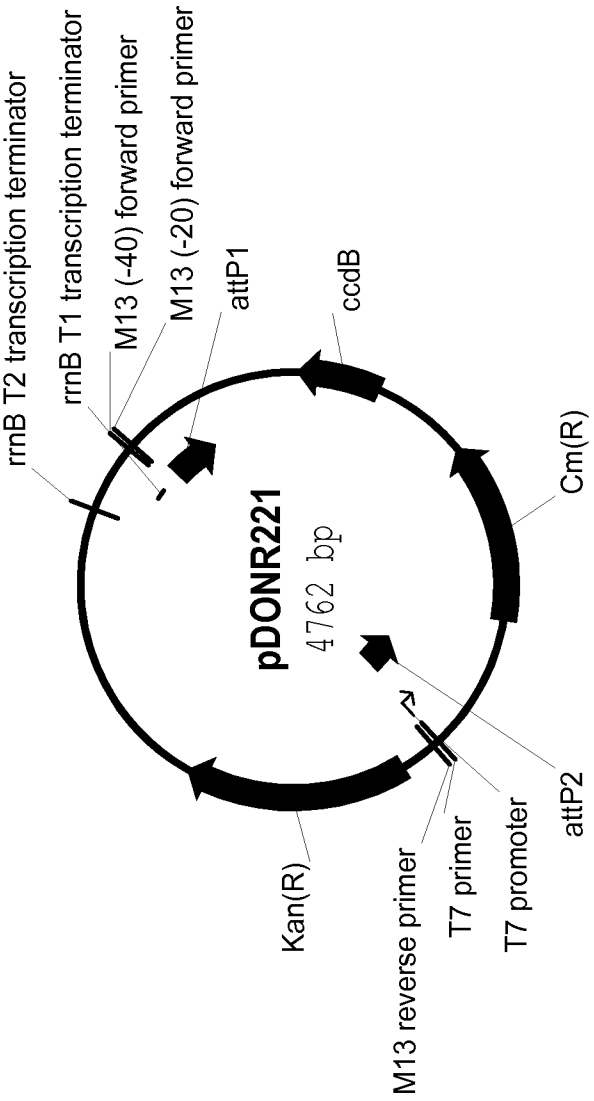

rrnB T2 transcription terminator
rrnB T1 transcription terminator
M13 (-40) forward primer
M13 (-20) forward primer
attP1
ccdB
Cm(R)
attP2
T7 promoter
T7 primer
M13 reverse primer
Kan(R)
pDONR221
4762 bp

FIG. 4

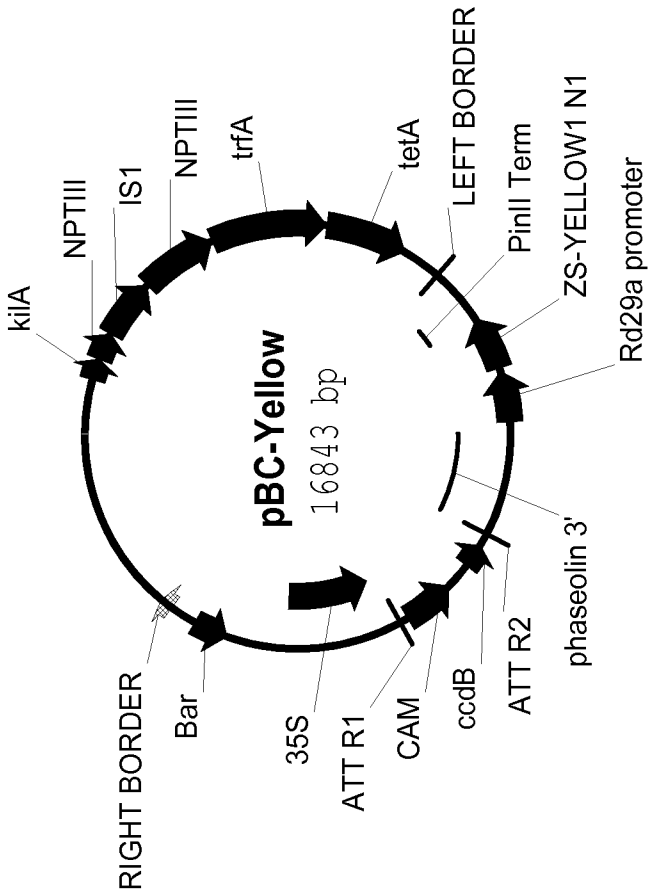

pBC-Yellow
16843 bp
kilA
NPTIII
IS1
NPTIII
trfA
tetA
LEFT BORDER
PinII Term
ZS-YELLOW1 N1
Rd29a promoter
phaseolin 3'
ATT R2
ccdB
CAM
ATT R1
35S
Bar
RIGHT BORDER

FIG. 5

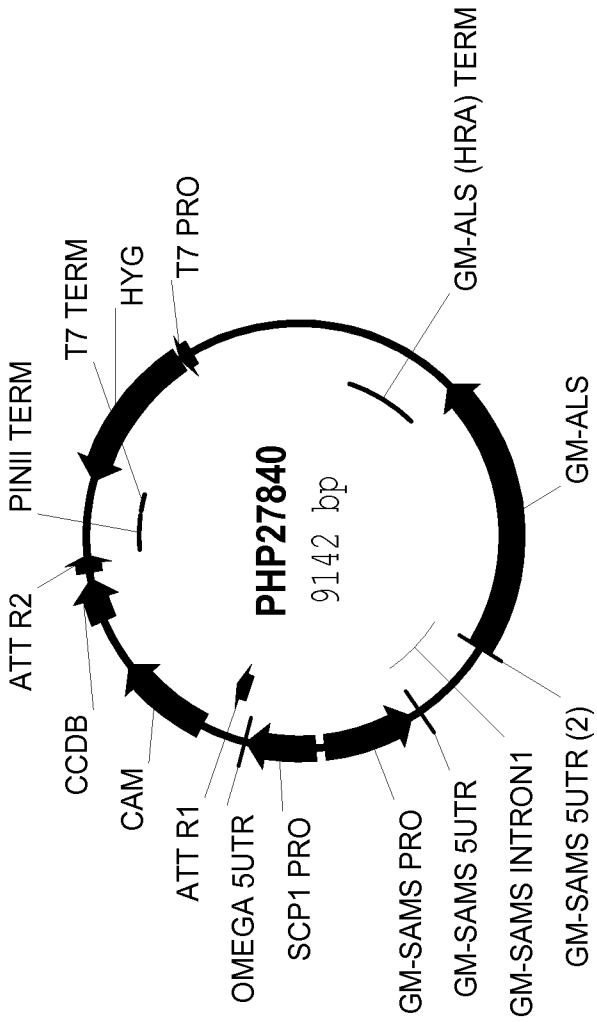

PHP27840
9142 bp
GM-ALS (HRA) TERM
T7 PRO
HYG
T7 TERM
PINII TERM
ATT R2
CCDB
CAM
ATT R1
OMEGA 5UTR
SCP1 PRO
GM-SAMS PRO
GM-SAMS 5UTR
GM-SAMS INTRON1
GM-SAMS 5UTR (2)
GM-ALS

FIG. 6

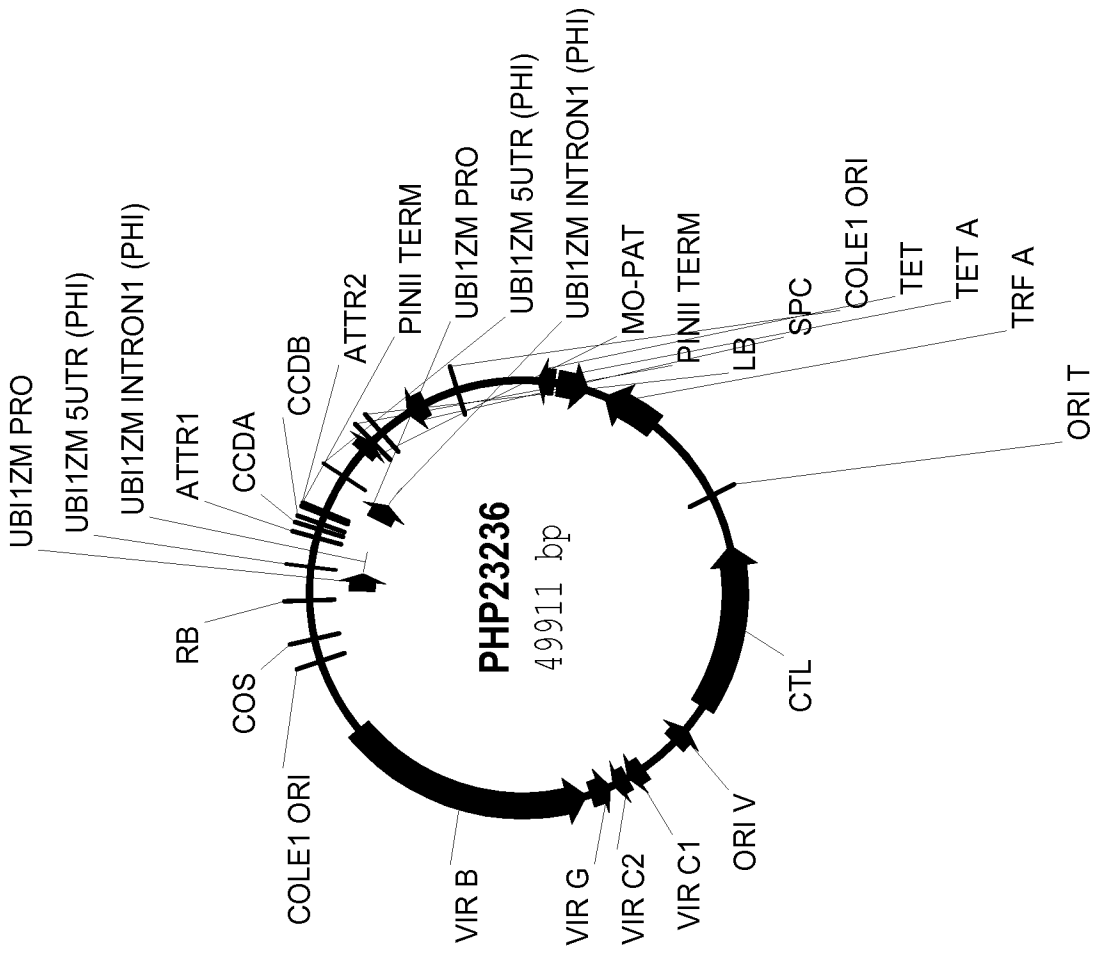

PHP23236
49911 bp
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM INTRON1 (PHI)
ATTR1
CCDA
CCDB
ATTR2
PINII TERM
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM INTRON1 (PHI)
MO-PAT
PINII TERM
LB
SPC
COLE1 ORI
TET
TET A
TRF A
ORI T
CTL
ORI V
VIR C1
VIR C2
VIR G
VIR B
COLE1 ORI
COS
RB

FIG. 7

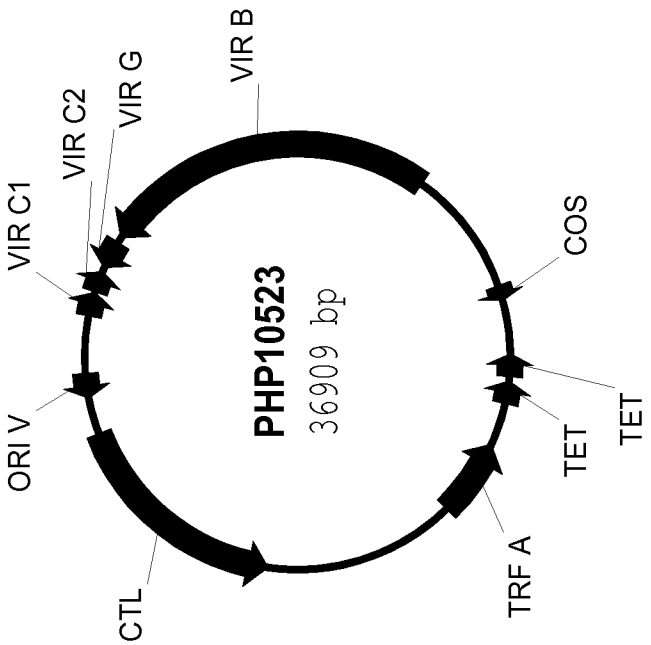

VIR B
VIR G
VIR C2
VIR C1
COS
PHP10523
36909 bp
ORI V
TET
TET
CTL
TRF A

FIG. 8

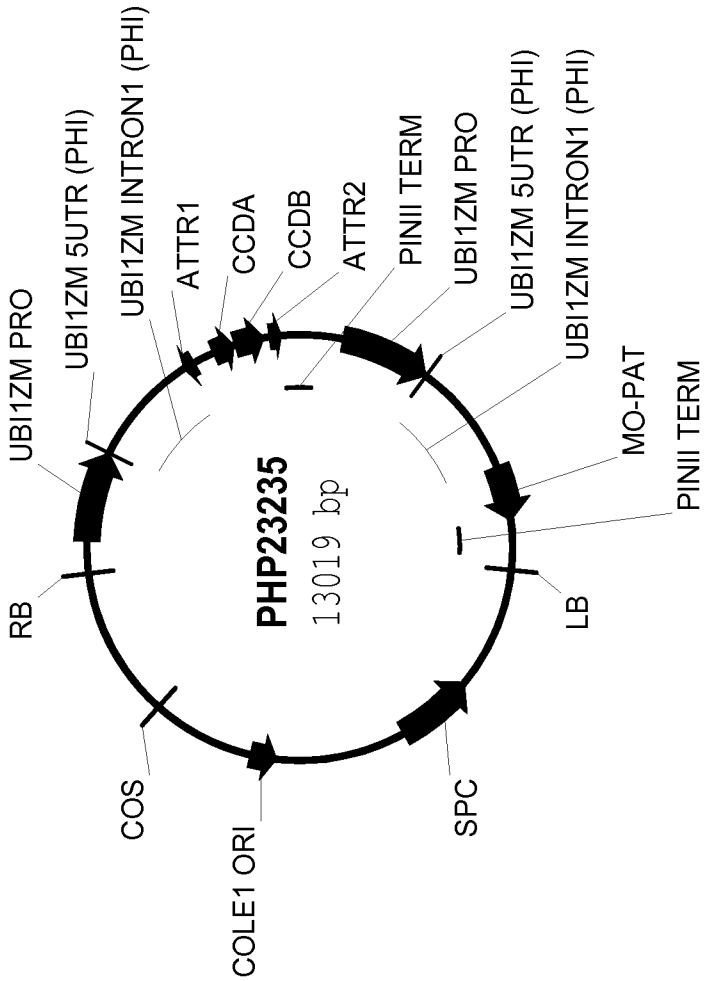

PHP23235
13019 bp
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM INTRON1 (PHI)
ATTR1
CCDA
CCDB
ATTR2
PINII TERM
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM INTRON1 (PHI)
MO-PAT
PINII TERM
LB
SPC
COLE1 ORI
COS
RB

FIG. 9

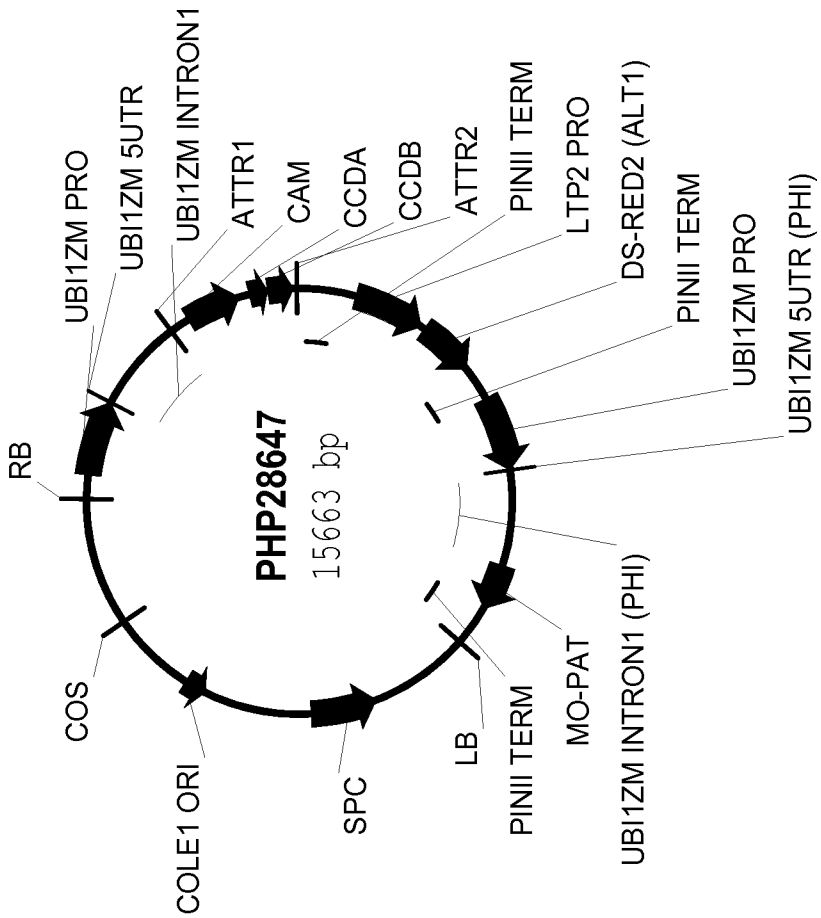

PHP28647
15663 bp
RB
UBI1ZM PRO
UBI1ZM 5UTR
UBI1ZM INTRON1
ATTR1
CAM
CCDA
CCDB
ATTR2
PINII TERM
LTP2 PRO
DS-RED2 (ALT1)
PINII TERM
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM INTRON1 (PHI)
MO-PAT
PINII TERM
LB
SPC
COLE1 ORI
COS

FIG. 10

Evaluation of Individual Maize Lines Transformed with PHP31373*

Exp.	Treatment	PHPName	Event	Fv'/Fm' no stress	Fv'/Fm' stress	phiPSII no stress	phiPSII stress	Shoot Dwt (g)	Shoot Fwt (g)
1	reduce water	PHP31373	E7899.44.1.10		0.0021		0.0014		0.0554
			E7899.44.1.4		0.0988				
			E7899.44.1.8	(0.0157)	0.0263		0.0597		0.0019
			E7899.44.2.1		0.0030		0.0046		0.0121
			E7899.44.2.3		0.0384		0.0077		0.0396
			E7899.44.3.10		0.0003		0.0002		0.0008
			E7899.44.3.11		0.0739				
			E7899.44.7.2	(0.0805)		(0.0781)			0.0732
			E7899.44.8.4					0.0541	0.0006
2	reduce water	PHP31373	E7899.44.1.10				(0.0527)		0.0194
			E7899.44.1.4						
			E7899.44.1.8						
			E7899.44.2.1					0.0273	0.0013
			E7899.44.2.3					0.0204	0.0006
			E7899.44.3.10						0.0003
			E7899.44.3.11						
			E7899.44.7.2	(0.0241)		(0.0569)			
			E7899.44.8.4						0.0116

* The P-values shown reflects the performance of the event against the reference. Significant positive effect has P-value less than or equal to 0.1; significant negative effect is in parenthesis; blank when difference not significant

FIG. 11

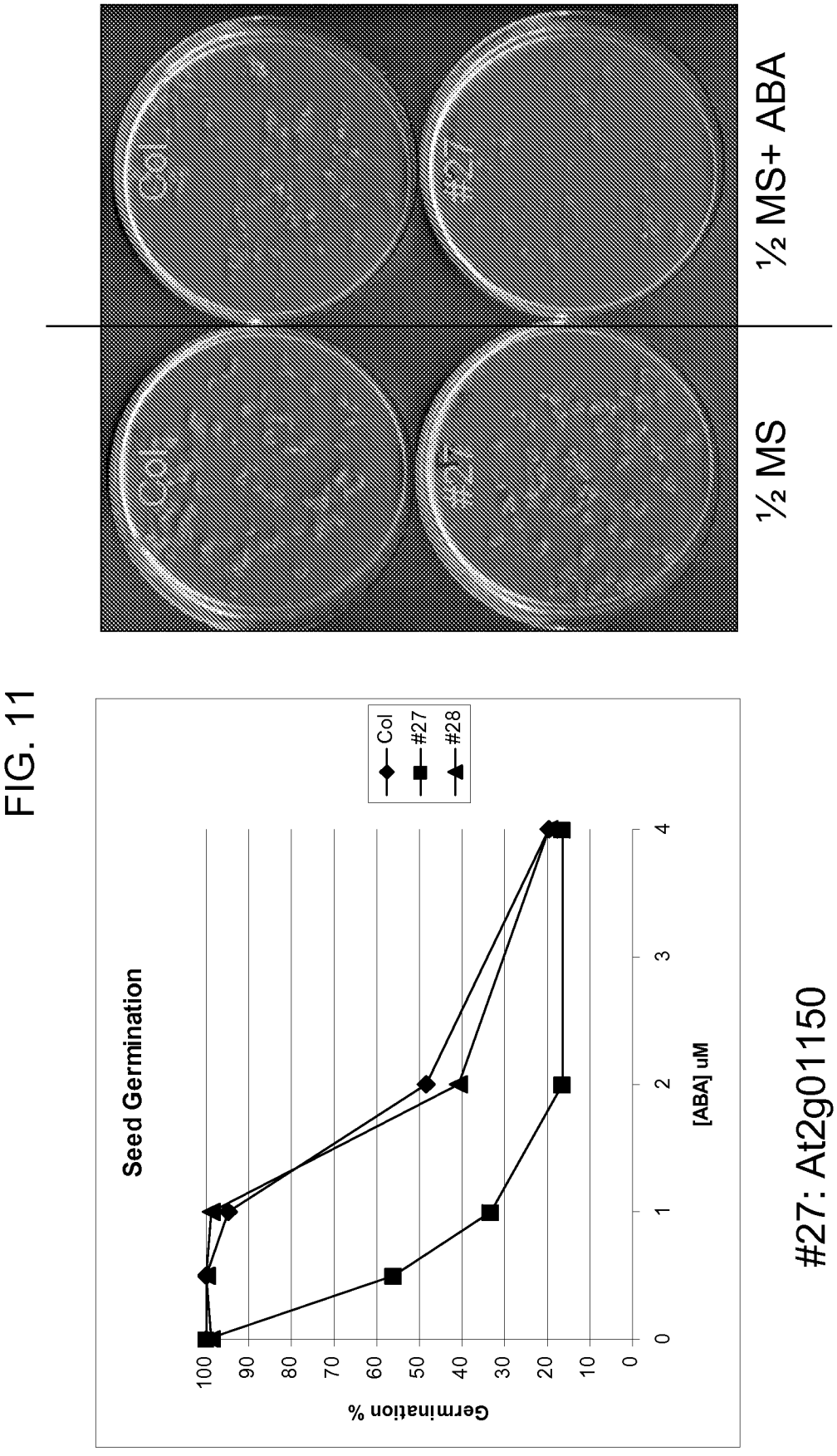

Col
#27
½ MS
½ MS+ ABA
Seed Germination
Germination %
[ABA] uM
Col
#27
#28
#27: At2g01150
Col: columbine control

FIG. 12

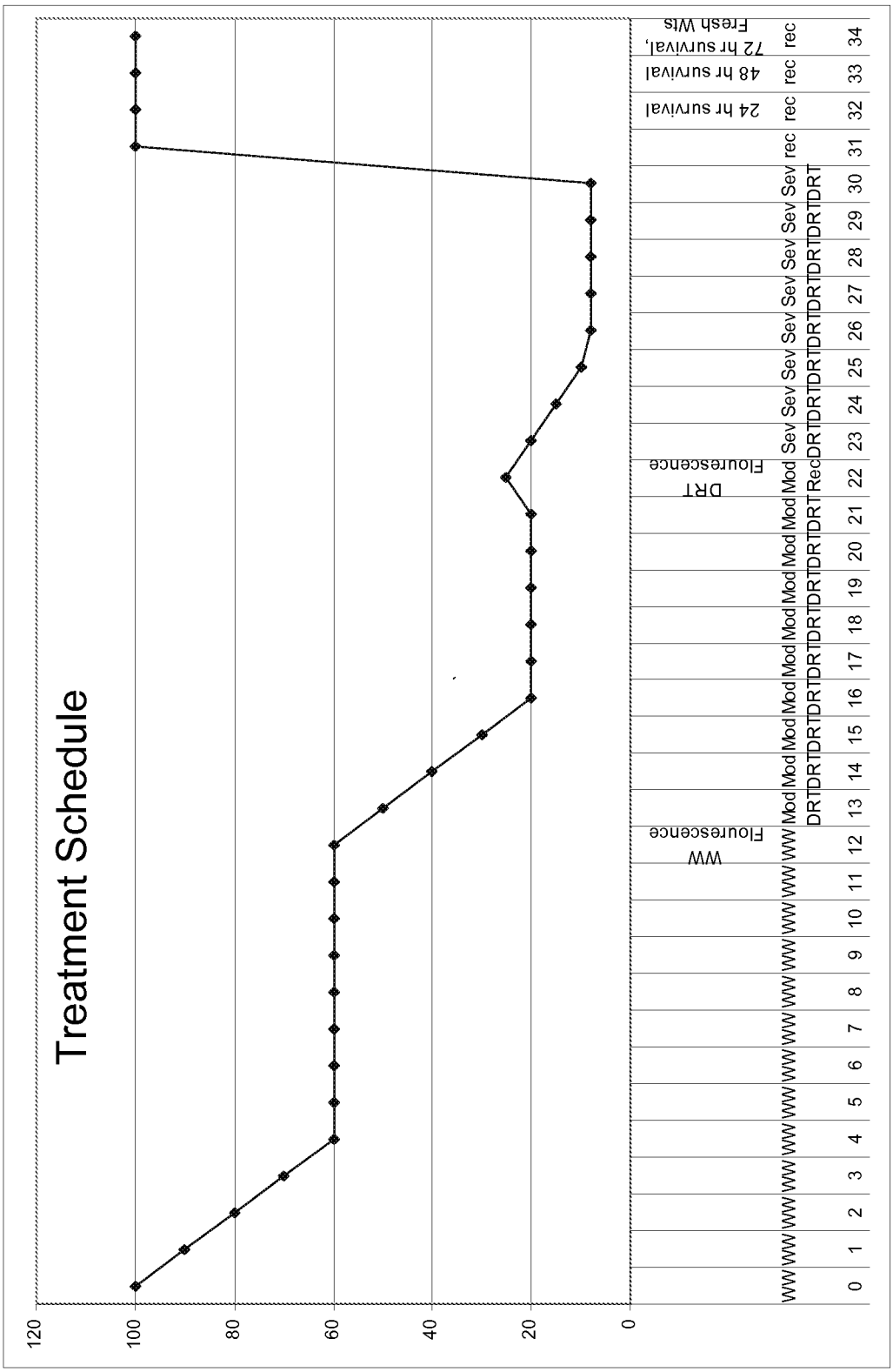

Treatment Schedule
% Field capacity
days
120
100
80
60
40
20
0
WW Flourescence
DRT Flourescence
24 hr survival
48 hr survival
72 hr survival, Fresh Wts
rec
Sev
Mod
WW
DRT

FIG. 13 A

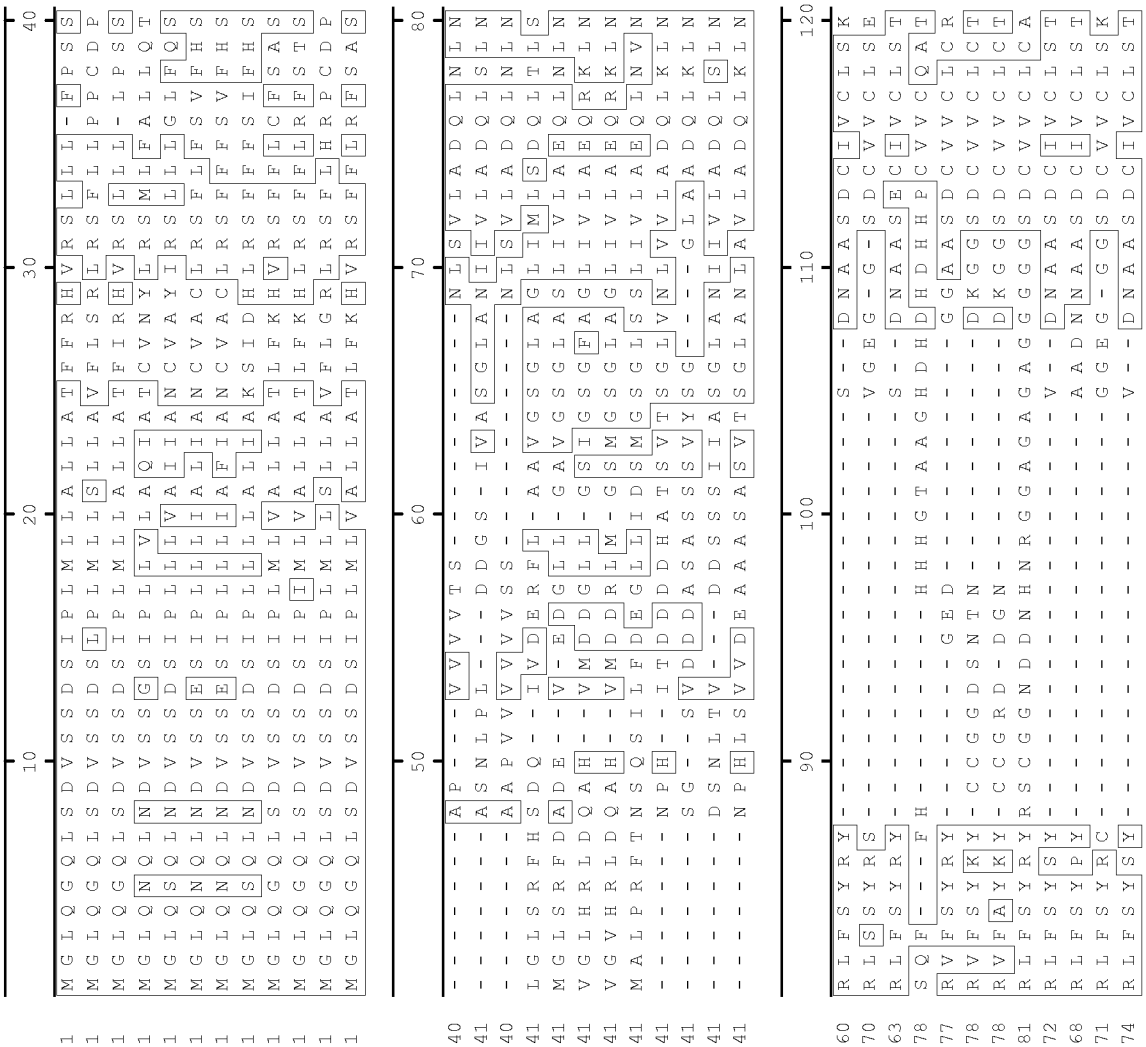


FIG. 13 B

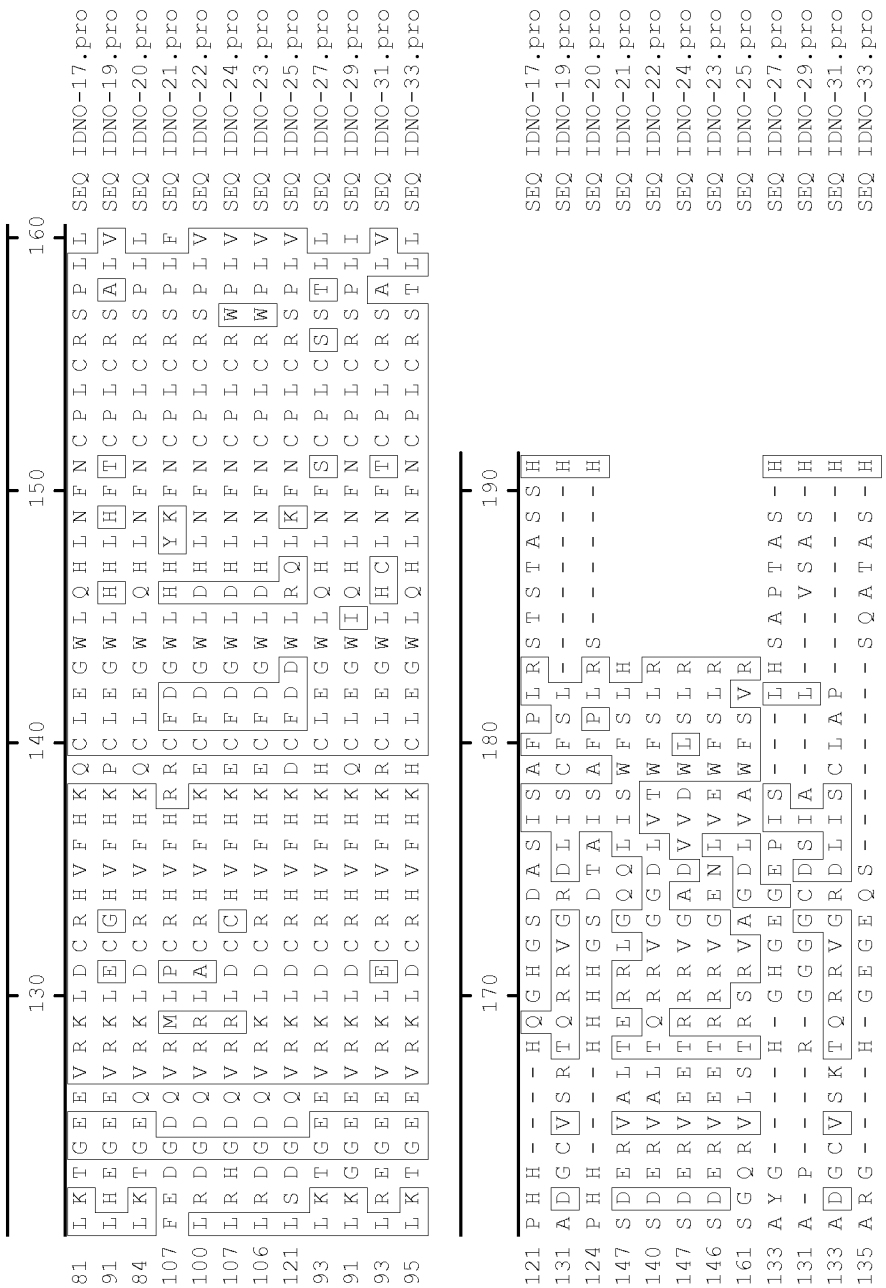
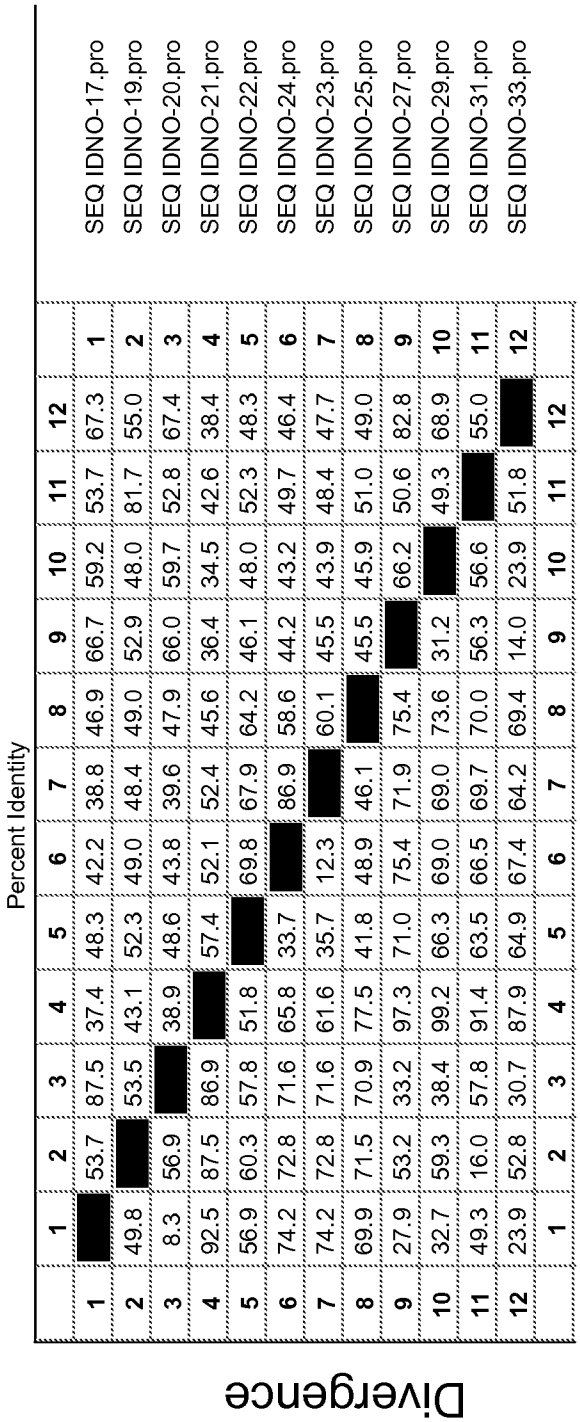

130
140
150
160
170
180
190
SEQ IDNO-17.pro
SEQ IDNO-19.pro
SEQ IDNO-20.pro
SEQ IDNO-21.pro
SEQ IDNO-22.pro
SEQ IDNO-24.pro
SEQ IDNO-23.pro
SEQ IDNO-25.pro
SEQ IDNO-27.pro
SEQ IDNO-29.pro
SEQ IDNO-31.pro
SEQ IDNO-33.pro

FIG. 14



	1	2	3	4	5	6	7	8	9	10	11	12	
1		53.7	87.5	37.4	48.3	42.2	38.8	46.9	66.7	59.2	53.7	67.3	1
2	49.8		53.5	43.1	52.3	49.0	48.4	49.0	52.9	48.0	81.7	55.0	2
3	8.3	56.9		38.9	48.6	43.8	39.6	47.9	66.0	59.7	52.8	67.4	3
4	92.5	87.5	86.9		57.4	52.1	52.4	45.6	36.4	34.5	42.6	38.4	4
5	56.9	60.3	57.8	51.8		69.8	67.9	64.2	46.1	48.0	52.3	48.3	5
6	74.2	72.8	71.6	65.8	33.7		86.9	58.6	44.2	43.2	49.7	46.4	6
7	74.2	72.8	71.6	61.6	35.7	12.3		60.1	45.5	43.9	48.4	47.7	7
8	69.9	71.5	70.9	77.5	41.8	48.9	46.1		45.5	45.9	51.0	49.0	8
9	27.9	53.2	33.2	97.3	71.0	75.4	71.9	75.4		66.2	50.6	82.8	9
10	32.7	59.3	38.4	99.2	66.3	69.0	69.0	73.6	31.2		49.3	68.9	10
11	49.3	16.0	57.8	91.4	63.5	66.5	69.7	70.0	56.3	56.6		55.0	11
12	23.9	52.8	30.7	87.9	64.9	67.4	64.2	69.4	14.0	23.9	51.8		12
	1	2	3	4	5	6	7	8	9	10	11	12	

FIG. 15

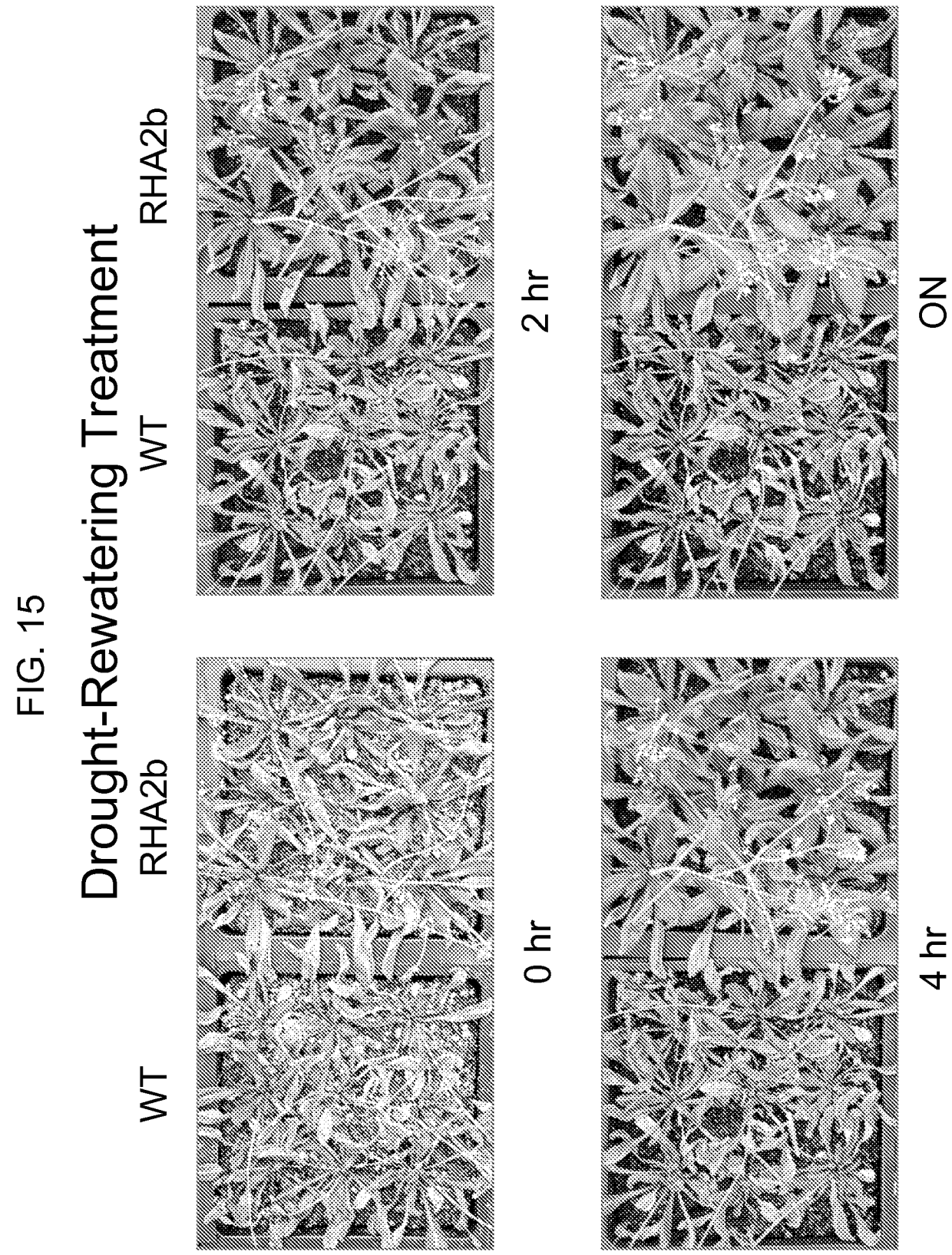
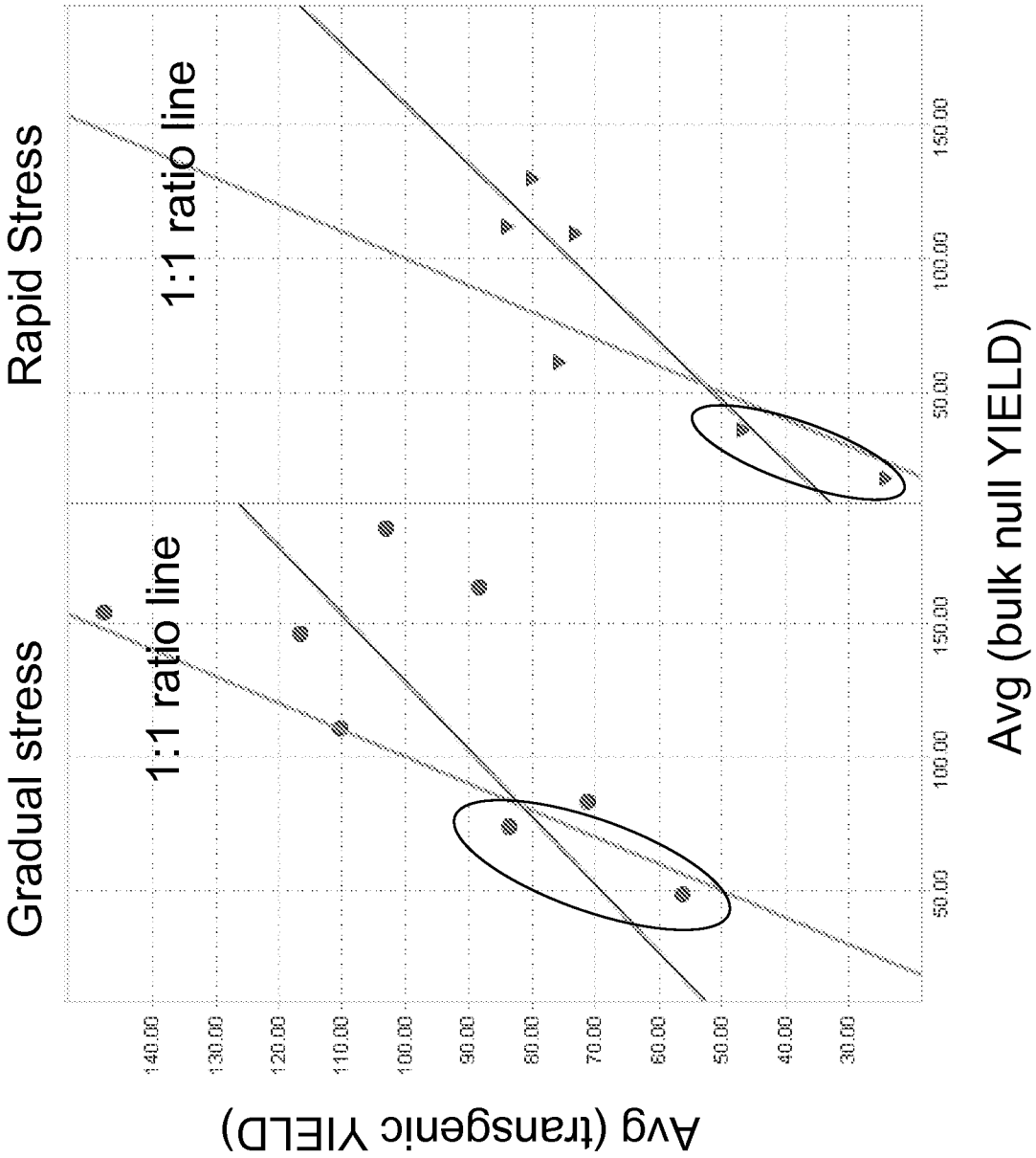

Drought-Rewatering Treatment
WT
RHA2b
RHA2b
WT
0 hr
2 hr
4 hr
ON

FIG. 16


Gradual stress
Rapid Stress
1:1 ratio line
1:1 ratio line
Avg (transgenic YIELD)
Avg (bulk null YIELD)
140.00
130.00
120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
50.00
100.00
150.00
50.00
100.00
150.00

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
BB1716PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2010/026651	09/03/2010	09/03/2009
Applicant		
E. I. du Pont de Nemours and Company		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 7 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☒ **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   ☐ the text is approved as submitted by the applicant
   ☒ the text has been established by this Authority to read as follows:

DROUGHT TOLERANT PLANTS AND METHODS INVOLVING GENES ENCODING TYPE C3HC4 RING FINGER ZINC-FINGER FAMILY POLYPEPTIDES

5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1
      ☒ as suggested by the applicant
      ☐ as selected by this Authority, because the applicant failed to suggest a figure
      ☐ as selected by this Authority, because this figure better characterizes the invention
   b. ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/026651

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/026651

A. CLASSIFICATION OF SUBJECT MATTER
INV. 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)
C12N

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, BIOSIS, Sequence Search, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/008396 A2 (UNIV MICHIGAN STATE [US]; HAN KYUNG-HWAN [US]; KO JAE-HEUNG [US] TRUST) 17 January 2008 (2008-01-17) * abstract. p. 2:1 - 8.15, 12:22 - 13:6, 43:6 - 46:19, example I-X fig. 10, SEQ ID No: 265, claims *	1-20
X	JENSEN R B ET AL: "Widespread occurrence of a highly conserved RING-H2 zinc finger motif in the model plant Arabidopsis thaliana" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL LNKD- DOI:10.1016/S0014-5793(98)01143-0, vol. 436, no. 2, 2 October 1998 (1998-10-02), pages 283-287, XP004258438 ISSN: 0014-5793 * abstract, p. 283, fig. 1-3 *	13-17

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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
19 May 2010	23/08/2010

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, Fax: (+31-70) 340-3016	Puonti-Kaerlas, J

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	XIUHONG YANG ET AL: "Molecular cloning and characterization of a gene encoding RING zinc fi nger ankyrin protein from drought-tolerant Artemisia desertorum" JOURNAL OF BIOSCIENCES, INDIAN ACADEMY OF SCIENCES, XX, vol. 33, no. 1, 1 March 2008 (2008-03-01), pages 103-112, XP007912976 ISSN: 0250-5991	1-20
A	XU ET AL: "Overexpression of a TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (Oryza sativa L.)" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 582, no. 7, 4 March 2008 (2008-03-04), pages 1037-1043, XP022550919 ISSN: 0014-5793	1-20
A	VYDEHI KANNEGANTI ET AL: "Overexpression of OsiSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice" PLANT MOLECULAR BIOLOGY, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 66, no. 5, 18 January 2008 (2008-01-18), pages 445-462, XP019579643 ISSN: 1573-5028	1-20
A	KAIMAO LIU ET AL: "Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice" PLANTA ; AN INTERNATIONAL JOURNAL OF PLANT BIOLOGY, SPRINGER, BERLIN, DE LNKD-DOI:10.1007/S00425-007-0548-5, vol. 226, no. 4, 5 June 2007 (2007-06-05), pages 1007-1016, XP019542347 ISSN: 1432-2048	1-20

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/026651

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see additional sheet(s)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention: 1; Claims: 16(completely); 1-15, 17-20(partially)

a polynucleotide encoding a Zinc-Finger (C3HC4-type RING finger) family polypeptide according to SEQ ID No: 17, and subject-matter relating thereto

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Inventions: 2-12; Claims: 1-15, 17-20(all partially)

as invention 1, but relating to SEQ ID Nos 19, 20, 21, 22, 23, 24, 25, 27, 29, 31 and 33, respectively

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/026651

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008008396 A2	17-01-2008	AU 2007272993 A1	17-01-2008
		CA 2658391 A1	17-01-2008
		EP 2046962 A2	15-04-2009
		US 2008034449 A1	07-02-2008