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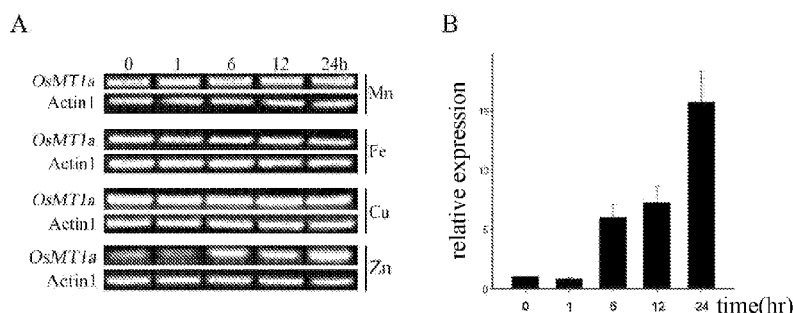


FIGURE 3

(57) Abstract: Compositions and methods for imparting improved properties to plants using the metallothionein gene *OsMT1a* and its products, including polypeptides and transgenic plants and seeds. Improved properties include improved drought tolerance and increased zinc uptake.

TITLE OF THE INVENTION

[0001] Metallothionein Gene Conferring Abiotic Stress Tolerance in Plants and Uses Thereof

TECHNICAL FIELD OF THE INVENTION

[0002] The invention relates generally to compositions and methods for conferring abiotic stress tolerance to plants, including polynucleotides, polypeptides, vectors and host cells. The present invention also relates generally to plants transformed by the aforementioned compositions and methods.

BACKGROUND OF THE INVENTION

[0003] Metallothioneins (MTs) are ubiquitous low-molecular weight, cysteine-rich proteins that can bind metals via mercaptide bonds. Since the first MT was characterized from horse kidneys as cadmium-binding proteins, numerous MT genes have been identified in both eukaryotes and prokaryotes. Based on the number and arrangement of cysteine residues, all the plant MTs belong to class II (in contrast to the vertebrate class I) and can be further subdivided into four types based on their amino acid sequences. Expression analysis of these four types of MTs revealed that type 1 MTs are expressed preferentially in roots, whereas type 2 MTs are found mainly in the leaves, type 3 MTs are expressed at high levels in the ripe fruits and *Arabidopsis* leaves, and expression of type 4 MTs, also known as the Ec type, is only found in developing seeds.

[0004] In contrast to the reports about various MTs gene structure and expression patterns, the functions of MTs are still elusive. Their striking metal-binding property suggests that MTs play a principal role in metal homeostasis and detoxification. In plants, MTs participated not only in maintaining the homeostasis of essential copper (Cu) and zinc (Zn) at micronutrient levels, but also in the detoxification of non-essential toxic metals such as cadmium (Cd) and arsenic (As). There is also evidence that MTs are possibly involved in the process of seed development and leaf senescence.

[0005] Moreover, accumulating evidence points to an alternative role for plant MTs in ameliorating oxidative damage. It is known that both abiotic and biotic stresses cause increased reactive oxygen species (ROS), and the generation of ROS is often accompanied by increased activities of many antioxidant enzymes during various stresses. The different ROS, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2(\Delta g)$), and hydroxyl radicals ($OH^{\bullet}$), may lead to non-specific oxidation of proteins and membrane lipids

or DNA damage. To minimize the affects of oxidative stress, plants have evolved a complex antioxidant system, including low molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). However, the underlying mechanisms for MTs role as antioxidants are poorly elucidated.

[0006] It is desirable that the uncertainties surrounding MTs' roles be solved, because the genetic manipulation of MTs may lead to improvements in crop production, such as crops that are more resistant to one or more biotic and abiotic stresses.

BRIEF SUMMARY OF THE INVENTION

[0007] The present invention relates to isolated OsMT1a polynucleotides, polypeptides, vectors and host cells expressing isolated *OsMT1a* polynucleotides capable of imparting a variety of properties to plants, such as improved drought tolerance.

[0008] The isolated *OsMT1a* polynucleotides provided herein include nucleic acids comprising (a) a nucleic acid comprising a nucleotide sequence of SEQ ID NO: 1; (b) a nucleic acid comprising a nucleotide sequence at least 90% identical to (a); (c) a nucleic acid comprising a nucleotide sequence that specifically hybridizes to the complement of (a) under stringent hybridization conditions and which encodes a metallothionein; (d) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence of SEQ ID NO: 2; (e) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence derived from (d) by substitution, deletion or addition of one or several amino acids in the amino acid sequence in (d) and having metallothionein activity; and (f) a nucleic acid comprising a nucleotide sequence that is the complement of any one of (a)-(e).

[0009] The isolated OsMT1a polypeptides provided herein include (a) a polypeptide sequence of SEQ ID NO: 2; and (b) a polypeptide sequence derived from (a) by substitution, deletion or addition of one or several amino acids in the amino acid sequence in (a) and having metallothionein activity.

[0010] The host cells provided herein include those comprising the isolated polynucleotides and vectors of the present invention. The host cell can be from an animal, plant, or microorganism, such as *E. coli*. Plant cells are particularly contemplated. The host cell can be isolated, excised, or cultivated. The host cell may also be part of a plant.

[0011] The present invention further relates to a plant or a part of a plant that comprises a host cell of the present invention. Plants such as rice, maize and soybean are particularly contemplated. The present invention also relates to the transgenic seeds of the plants.

[0012] The present invention further relates to a method for producing a plant comprising regenerating a transgenic plant from a host cell of the present invention, or hybridizing a transgenic plant of the present invention to another non-transgenic plant. Plants produced by these methods are also encompassed by the present invention, and plants having improved tolerance to drought and improved zinc uptake are particularly contemplated, as are crop plants, such as rice, maize and soybean.

[0013] The present invention further relates to methods of altering a trait in a plant or part of a plant using the isolated polynucleotides, polypeptides, constructs and vectors of the present invention. These traits include improved tolerance to drought and improved zinc uptake. Preferably the aforementioned traits are altered so that they are increased or otherwise improved. In one embodiment, one or more traits of a plant are altered by expressing in a plant an isolated nucleic acid such as (a) a nucleic acid comprising a nucleotide sequence of SEQ ID NO: 1; (b) a nucleic acid comprising a nucleotide sequence at least 90% identical to (a); (c) a nucleic acid comprising a nucleotide sequence that specifically hybridizes to the complement of (a) under stringent hybridization conditions and which encodes a metallothionein; (d) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence of SEQ ID NO: 2; (e) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence derived from (d) by substitution, deletion or addition of one or several amino acids in the amino acid sequence in (d) and having metallothionein activity; and (f) a nucleic acid comprising a nucleotide sequence that is the complement of any one of (a)-(e). In another embodiment, one or more traits of a plant are altered by expressing in a plant an isolated hypermorphic *OsMT1a* allele. In another embodiment, one or more traits of a plant are altered by increasing the expression of a OsMT1a nucleic acid or polypeptide in the plant. In yet another embodiment, one or more traits of a plant are altered by altering the function of a OsMT1a polypeptide in the plant.

[0014] The present invention further relates to plants, plant parts and transgenic seeds created through the aforementioned methods of altering a trait in a plant. Such contemplated plants, plant parts and transgenic seeds may be created directly from the aforementioned methods. Alternatively, the contemplated plants, plant parts and transgenic seeds may be

derived from a host cell (e.g., regenerated from a host cell) or produced by crossing a transgenic plant with one or more altered traits with a non-transgenic plant.

[0015] The present invention further relates to methods of increasing enzymatic activity in a plant comprising expressing an isolated *OsMT1a* polynucleotide in accordance with the invention in the plant, wherein the enzymatic activity is that of an enzyme selected from the group consisting of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). In certain embodiments, the activity of one or more of these antioxidative enzymes is increased by increasing the expression one or more *OsMT1a* polypeptides in a host cell, plant or plant part. In other embodiments, the activity of one or more of these antioxidative enzymes is decreased by decreasing the expression of one or more *OsMT1a* polypeptides in a host cell, plant or plant part.

[0016] The present invention further relates to methods of increasing the expression of a zinc finger transcription factor in a plant comprising expressing an isolated *OsMT1a* polynucleotide in accordance with the invention in the plant, wherein the zinc finger transcription factor is encoded by a gene selected from the group consisting of *Ossiz*, *ZF1* and *WRKY71*. In certain embodiments, the expression of one or more of these zinc finger transcription factor genes is increased by increasing the expression one or more *OsMT1a* polypeptides in a host cell, plant or plant part. In other embodiments, the activity of one or more of these zinc finger transcription factor genes is decreased by decreasing the expression of one or more *OsMT1a* polypeptides in a host cell, plant or plant part.

[0017] The present invention further relates to methods of identifying *OsMT1a* binding agents and inhibitors. In one embodiment, the method comprises (a) providing an isolated *OsMT1a* protein; (b) contacting the isolated *OsMT1a* protein with an agent under conditions sufficient for binding; (c) assaying binding of the agent to the isolated *OsMT1a* protein; and (d) selecting an agent that demonstrates specific binding to the isolated *OsMT1a* protein. In another embodiment, the method comprises (a) providing a host cell expressing a *OsMT1a* protein; (b) contacting the host cell with an agent; (c) assaying expression of *OsMT1a* protein; and (d) selecting an agent that induces altered expression of *OsMT1a* protein. In yet another embodiment, the method comprises (a) providing a plant or part of a plant expressing a *OsMT1a* protein; (b) contacting the plant or the part of the plant with an agent; (c) assaying for alteration of a trait of the plant or the part of the plant; and (d) selecting an agent that alters the trait. The traits to be assayed are those known to be affected by *OsMT1a* expression (e.g., drought tolerance, zinc uptake). Preferably agents that increase or

otherwise improve these traits are selected. However, agents that negatively impact a trait are contemplated as well.

[0018] The present invention also relates to methods of inhibiting *OsMT1a* in a plant using the binding agents and inhibitors identified by the methods herein.

BRIEF SUMMARY OF THE SEVERAL VIEWS OF THE DRAWINGS

[0019] Figure 1 shows *OsMT1a* expression in Nipponbare by Northern blotting. Lane 1 is from flower. Lane 2 is from leaf. Lane 3 is from shoots. Lane 4 is from root.

[0020] Figure 2 shows *OsMT1a* expression in metal-treated Brazil upland rice.

[0021] Figure 3 shows *OsMT1a* expression in non-biologically stressed Brazil upland rice.

[0022] Figure 4 shows the phenotypical change and dehydration rate in wild type Nipponbare (WT) and *OsMT1a*-transformed rice treated with 300 mM mannitol.

[0023] Figure 5 shows CAT, APX and POD activity assay results.

[0024] Figure 6A shows the relative expression of *Ossiz*, *ZFI* and *WRKY71* in *OsMT1a*-transformed plants. Figure 6B shows the synergistic expression of exogenous *OsMT1a* and *Ossiz* in wild type and *OsMT1a*-transformed plants. Figure 6C shows the time course of expression of *Ossiz* in zinc treated plants.

DETAILED DESCRIPTION OF THE INVENTION

[0025] *OsMT1a* Nucleic Acids and Proteins

[0026] As used herein, the terms “nucleic acid”, “polynucleotide”, “polynucleotide molecule”, “polynucleotide sequence” and plural variants are used interchangeably to refer to a wide variety of molecules, including single strand and double strand DNA and RNA molecules, cDNA sequences, genomic DNA sequences of exons and introns, chemically synthesized DNA and RNA sequences, and sense strands and corresponding antisense strands. Polynucleotides of the invention may also comprise known analogs of natural nucleotides that have similar properties as the reference natural nucleic acid.

[0027] As used herein, the terms “polypeptide”, “protein” and plural variants are used interchangeably and refer to a compound made up of a single chain of amino acids joined by peptide bonds. Polypeptides of the invention may comprise naturally occurring amino acids, synthetic amino acids, genetically encoded amino acids, non-genetically encoded amino

acids, and combinations thereof. Polypeptides may include both L-form and D-form amino acids.

[0028] Representative non-genetically encoded amino acids include but are not limited to 2-aminoadipic acid; 3-aminoadipic acid; β -aminopropionic acid; 2-aminobutyric acid; 4-aminobutyric acid (piperidinic acid); 6-aminocaproic acid; 2-aminoheptanoic acid; 2-aminoisobutyric acid; 3-aminoisobutyric acid; 2-aminopimelic acid; 2,4-diaminobutyric acid; desmosine; 2,2'-diaminopimelic acid; 2,3-diaminopropionic acid; N-ethylglycine; N-ethylasparagine; hydroxylysine; allo-hydroxylysine; 3-hydroxyproline; 4-hydroxyproline; isodesmosine; allo-isoleucine; N-methylglycine (sarcosine); N-methylisoleucine; N-methylvaline; norvaline; norleucine; and ornithine.

[0029] Representative derivatized amino acids include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine.

[0030] As used herein, the term "isolated" refers to polynucleotides and polypeptides that, but for at least one act of man, do not exist in whatever form or amount they are found. Exemplary embodiments include polynucleotides and polypeptides that are partially, substantially or wholly purified from other molecular species; polynucleotides and polypeptides that are heterologous to a particular cell, organism, or part of an organism; polynucleotides and polypeptides that are not heterologous to a particular cell, organism, or part of an organism, but are expressed at an altered level as a result of the at least one act of man; and polynucleotides and polypeptides that are expressed in the progeny or other downstream products (e.g., fruit) of a cell, organism, or part of an organism subject to the at least one act of man.

[0031] Exemplary *OsMT1a* polynucleotides of the invention are set forth as SEQ ID NO: 1 and substantially identical sequences encoding OsMT1a proteins capable of altering a trait of a plant, for example, drought tolerance.

[0032] Exemplary OsMT1a polypeptides of the invention are set forth as SEQ ID NO: 2 and substantially identical proteins capable of altering a trait of a plant, for example, drought tolerance.

[0033] Substantially identical sequences are those that have at least 60%, preferably at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence using a sequence comparison algorithm or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In an especially preferred embodiment, the sequences are substantially identical over the entire length of the coding regions. Furthermore, substantially identical nucleic acids or proteins perform substantially the same function. Substantially identical sequences may be polymorphic sequences, i.e., alternative sequences or alleles in a population. An allelic difference may be as small as one base pair. Substantially identical sequences may also comprise mutagenized sequences, including sequences comprising silent mutations. A mutation may comprise one or more residue changes, a deletion of one or more residues, or an insertion of one or more additional residues. Substantially identical nucleic acids are also identified as nucleic acids that hybridize specifically to or hybridize substantially to a reference sequence (e.g., SEQ ID NO: 1).

[0034] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0035] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see, Ausubel et al., *infra*).

[0036] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information

(<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., 1990). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA*, 89:10915 (1989)).

[0037] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see e.g., Karlin & Altschul, *Proc. Natl. Acad. Sci. USA*, 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[0038] Substantially identical sequences may be polymorphic sequences, i.e., alternative sequences or alleles in a population. An allelic difference may be as small as one base pair. Substantially identical sequences may also comprise mutagenized sequences, including sequences comprising silent mutations. A mutation may comprise one or more residue

changes, a deletion of one or more residues, or an insertion of one or more additional residues.

[0039] Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are those under which a nucleic acid probe will typically hybridize to its target sequence but to no other sequences when that sequence is present in a complex nucleic acid mixture (e.g., total cellular DNA or RNA). Stringent hybridization conditions and stringent hybridization wash conditions in the context of nucleic acid hybridization experiments such as Southern and Northern blot analyses are both sequence- and environment-dependent. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I chapter 2, Elsevier, New York (1993). Generally, highly stringent hybridization and wash conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH.

[0040] The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide with 1 mg of heparin at 42 °C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72 °C for about 15 minutes. Another example of stringent wash conditions is a 0.2X SSC wash at 65 °C for 15 minutes (*see*, Sambrook, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An exemplary medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1X SSC at 45 °C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4X – 6X SSC at 40 °C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0 M sodium ions, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30 °C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the

proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[0041] The following are examples of hybridization and wash conditions that may be used to identify nucleotide sequences that are substantially identical to reference nucleotide sequences of the present invention. A substantially identical nucleotide sequence preferably hybridizes to a reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50 °C with washing in 2X SSC, 0.1% SDS at 50 °C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50 °C with washing in 1X SSC, 0.1% SDS at 50 °C, still more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50 °C with washing in 0.5X SSC, 0.1% SDS at 50 °C, even more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50 °C with washing in 0.1X SSC, 0.1% SDS at 50 °C, and most preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50 °C with washing in 0.1X SSC, 0.1% SDS at 65 °C.

[0042] A further indication that two nucleic acid sequences or proteins are substantially identical is that the proteins encoded by the nucleic acids are substantially identical, share an overall three-dimensional structure, are biologically functional equivalents, or are immunologically cross-reactive with, or specifically bind to, each other. Nucleic acid molecules that do not hybridize to each other under stringent conditions are still substantially identical if the corresponding proteins are substantially identical. This may occur, for example, when two nucleotide sequences comprise conservatively substituted variants as permitted by the genetic code. This also includes degenerate codon substitutions wherein the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (see Batzer et al., *Nucleic Acids Res.*, 19:5081(1991); Ohtsuka et al., *J. Biol. Chem.*, 260:2605-2608 (1985); and Rossolini et al. *Mol. Cell Probes*, 8:91-98 (1994)). However, both the polynucleotides and the polypeptides of the present invention may be conservatively substituted at one or more residues. Examples of conservative amino acid substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine; the substitution of one basic residue such as lysine, arginine or histidine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

[0043] Nucleic acids of the invention also comprise nucleic acids complementary to SEQ ID NO: 1 and subsequences and elongated sequences of SEQ ID NO: 1 and complementary

sequences thereof. Complementary sequences are two nucleotide sequences that comprise antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between base pairs. Like other polynucleotides in accordance with the present invention, complementary sequences may be substantially similar to one another as described previously. A particular example of a complementary nucleic acid segment is an antisense oligonucleotide.

[0044] A subsequence is a sequence of nucleic acids that comprises a part of a longer nucleic acid sequence. An exemplary subsequence is a probe or a primer. An elongated sequence is one in which nucleotides (or other analogous molecules) are added to a nucleic acid sequence. For example, a polymerase (e.g., a DNA polymerase) may add sequences at the 3' terminus of the nucleic acid molecule. In addition, the nucleotide sequence may be combined with other DNA sequences, such as promoters, promoter regions, enhancers, polyadenylation signals, introns, additional restriction enzyme sites, multiple cloning sites, and other coding segments. Thus, the present invention also provides vectors comprising the disclosed nucleic acids, including vectors for recombinant expression, wherein a nucleic acid of the invention is operatively linked to a functional promoter. When operatively linked to a nucleic acid, a promoter is in functional combination with the nucleic acid such that the transcription of the nucleic acid is controlled and regulated by the promoter region. Vectors refer to nucleic acids capable of replication in a host cell, such as plasmids, cosmids, and viral vectors.

[0045] Polynucleotides of the present invention may be cloned, synthesized, altered, mutagenized, or combinations thereof. Standard recombinant DNA and molecular cloning techniques used to isolate nucleic acids are known in the art. Site-specific mutagenesis to create base pair changes, deletions, or small insertions is also known in the art (see e.g., Sambrook et al. (eds.) Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Silhavy et al., Experiments with Gene Fusions, 1984, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover & Hames, DNA Cloning: A Practical Approach, 2nd ed., 1995, IRL Press at Oxford University Press, Oxford/New York; Ausubel (ed.) Short Protocols in Molecular Biology, 3rd ed., 1995, Wiley, New York).

[0046] Isolated polypeptides of the invention may be purified and characterized using a variety of standard techniques that are known to the skilled artisan (see e.g., Schröder et al., The Peptides, 1965, Academic Press, New York; Bodanszky, Principles of Peptide Synthesis,

2nd rev. ed. 1993, Springer-Verlag, Berlin/ New York; Ausubel (ed.), Short Protocols in Molecular Biology, 3rd ed., 1995, Wiley, New York).

[0047] The present invention also encompasses methods for detecting a nucleic acid molecule that encodes a OsMT1a protein. Such methods may be used to detect *OsMT1a* gene variants or altered gene expression. Sequences detected by methods of the invention may be detected, subcloned, sequenced, and further evaluated by any measure well known in the art using any method usually applied to the detection of a specific DNA sequence. Thus, the nucleic acids of the present invention may be used to clone genes and genomic DNA comprising the disclosed sequences. Alternatively, the nucleic acids of the present invention may be used to clone genes and genomic DNA of related sequences. Levels of a *OsMT1a* nucleic acid molecule may be measured, for example, using an RT-PCR assay (see e.g., Chiang, *J. Chromatogr. A.*, 806:209-218 (1998) and references cited therein).

[0048] The present invention also encompasses genetic assays using *OsMT1a* nucleic acids for quantitative trait loci (QTL) analysis and to screen for genetic variants, for example by allele-specific oligonucleotide (ASO) probe analysis (Conner et al., *Proc. Natl. Acad. Sci. USA*, 80(1):278-282 (1983)), oligonucleotide ligation assays (OLAs) (Nickerson et al., *Proc. Natl. Acad. Sci. USA*, 87(22):8923-8927 (1990)), single-strand conformation polymorphism (SSCP) analysis (Orita et al., *Proc. Natl. Acad. Sci. USA*, 86(8):2766-2770 (1989)), SSCP/heteroduplex analysis, enzyme mismatch cleavage, direct sequence analysis of amplified exons (Kestila et al., *Mol. Cell*, 1(4):575-582 (1998); Yuan et al., *Hum. Mutat.*, 14(5):440-446 (1999)), allele-specific hybridization (Stoneking et al., *Am. J. Hum. Genet.*, 48(2):370-382 (1991)), and restriction analysis of amplified genomic DNA containing the specific mutation. Automated methods may also be applied to large-scale characterization of single nucleotide polymorphisms (Wang et al., *Am. J. Physiol.*, 1998, 274(4 Pt 2):H1132-1140 (1992); Brookes, *Gene*, 234(2):177-186 (1999)). Preferred detection methods are non-electrophoretic, including, for example, the TAQMANTM allelic discrimination assay, PCR-OLA, molecular beacons, padlock probes, and well fluorescence (see Landegren et al., *Genome Res.*, 8:769-776 (1998) and references cited therein).

[0049] The present invention also encompasses functional fragments of a OsMT1a polypeptide, for example, fragments that have the ability to alter a plant trait similar to that of SEQ ID NO: 2. Functional polypeptide sequences that are longer than the disclosed sequences are also encompassed. For example, one or more amino acids may be added to the N-terminus or C-terminus of an antibody polypeptide. Such additional amino acids may be

employed in a variety of applications, including but not limited to purification applications. Methods of preparing elongated proteins are known in the art.

[0050] The present invention also encompasses methods for detecting a OsMT1a polypeptide. Such methods can be used, for example, to determine levels of OsMT1a protein expression and correlate the level of expression with the presence or change in phenotype, trait, or level of expression in a different gene or gene product. In certain embodiments, the method involves an immunochemical reaction with an antibody that specifically recognizes a OsMT1a protein. Techniques for detecting such antibody-antigen conjugates or complexes are known in the art and include but are not limited to centrifugation, affinity chromatography and other immunochemical methods (see e.g., Ishikawa Ultrasensitive and Rapid Enzyme Immunoassay, 1999, Elsevier, Amsterdam/New York, United States of America; Law, Immunoassay: A Practical Guide, 1996, Taylor & Francis, London/Bristol, Pennsylvania, United States of America; Liddell et al., Antibody Technology, 1995, Bios Scientific Publishers, Oxford, United Kingdom; and references cited therein).

[0051] *OsMT1a* Expression Systems

[0052] An expression system refers to a host cell comprising a heterologous nucleic acid and the protein encoded by the heterologous nucleic acid. For example, a heterologous expression system may comprise a host cell transfected with a construct comprising a *OsMT1a* nucleic acid encoding a OsMT1a protein operatively linked to a promoter, or a cell line produced by introduction of *OsMT1a* nucleic acids into a host cell genome. The expression system may further comprise one or more additional heterologous nucleic acids relevant to *OsMT1a* function, such as targets of *OsMT1a* transcriptional activation or repression activity. These additional nucleic acids may be expressed as a single construct or multiple constructs.

[0053] A construct for expressing a OsMT1a protein may include a vector sequence and a *OsMT1a* nucleotide sequence, wherein the *OsMT1a* nucleotide sequence is operatively linked to a promoter sequence. A construct for recombinant *OsMT1a* expression may also comprise transcription termination signals and sequences required for proper translation of the nucleotide sequence. Preparation of an expression construct, including addition of translation and termination signal sequences, is known to one skilled in the art.

[0054] The promoter may be any polynucleotide sequence which shows transcriptional activity in the chosen plant cells, plant parts, or plants. The promoter may be native or analogous, or foreign or heterologous, to the plant host and/or to the DNA sequence of the

invention. Where the promoter is native or endogenous to the plant host, it is intended that the promoter is found in the native plant into which the promoter is introduced. Where the promoter is foreign or heterologous to the DNA sequence of the invention, the promoter is not the native or naturally occurring promoter for the operably linked DNA sequence of the invention. The promoter may be inducible or constitutive. It may be naturally-occurring, may be composed of portions of various naturally-occurring promoters, or may be partially or totally synthetic. Guidance for the design of promoters is provided by studies of promoter structure, such as that of Harley et al., *Nucleic Acids Res.*, 15:2343-61 (1987). Also, the location of the promoter relative to the transcription start may be optimized (see e.g., Roberts et al., *Proc. Natl. Acad. Sci. USA*, 76:760-4 (1979)). Many suitable promoters for use in plants are well known in the art.

[0055] For example, suitable constitutive promoters for use in plants include the promoters from plant viruses, such as the peanut chlorotic streak caulimovirus (PCISV) promoter (U.S. Patent No. 5,850,019); the 35S and 19S promoters from cauliflower mosaic virus (CaMV) (Odell et al., *Nature*, 313:810-812 (1985) and U.S. Patent No. 5,352,605); the promoters of *Chlorella* virus methyltransferase genes (U.S. Patent No. 5,563,328) and the full-length transcript promoter from figwort mosaic virus (FMV) (U.S. Patent No. 5,378,619); the promoters from such genes as rice actin (McElroy et al., *Plant Cell*, 2:163-171 (1990)); ubiquitin (Binet et al., *Plant Science*, 79:87-94 (1991)), maize (Christensen et al., *Plant Molec. Biol.*, 12: 619-632 (1989)), and arabidopsis (Norris et al., *Plant Molec. Biol.*, 21:895-906 (1993); and Christensen et al., *Plant Mol. Biol.*, 18:675-689 (1982)); pEMU (Last et al., *Theor. Appl. Genet.*, 81:581-588 (1991)); MAS (Velten et al., *EMBO J.*, 3:2723-2730 (1984)); maize H3 histone (Lepetit et al., *Mol. Gen. Genet.*, 1992, 231:276-285 (1992); and Atanassova et al., *Plant J.*, 2(3):291-300 (1992)); *Brassica napus* ALS3 (PCT International Publication No. WO 97/41228); and promoters of various *Agrobacterium* genes (e.g., U.S. Patent Nos. 4,771,002; 5,102,796; 5,182,200; and 5,428,147).

[0056] Suitable inducible promoters for use in plants include the promoter from the ACE1 system which responds to copper (Mett et al., *Proc. Natl. Acad. Sci. USA*, 90:4567-4571 (1993)); the promoter of the maize *In2* gene which responds to benzenesulfonamide herbicide safeners (Hershey et al., *Mol. Gen. Genetics*, 227:229-237 (1991); and Gatz et al., *Mol. Gen. Genetics*, 243:32-38 (1994)); and the promoter of the Tet repressor from Tn10 (Gatz et al., *Mol. Gen. Genet.*, 227:229-237 (1991)). Another inducible promoter for use in plants is one that responds to an inducing agent to which plants do not normally respond. An exemplary inducible promoter of this type is the inducible promoter from a steroid hormone

gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone (Skena et al., *Proc. Natl. Acad. Sci. USA*, 88:10421 (1991)) or the recent application of a chimeric transcription activator, XVE, for use in an estrogen receptor-based inducible plant expression system activated by estradiol (Zuo et al., *Plant J.*, 24:265-273 (2000)). Other inducible promoters for use in plants are described in EP 332104, PCT International Publication Nos. WO 93/21334 and WO 97/06269. Promoters composed of portions of other promoters and partially or totally synthetic promoters can also be used (see e.g., Ni et al., *Plant J.*, 7:661-676 (1995) and PCT International Publication No. WO 95/14098 describing such promoters for use in plants).

[0057] Tissue-specific or tissue-preferential promoters useful for the expression of the genes of the invention in plants. Such promoters are disclosed in WO 93/07278. Other tissue specific promoters useful in the present invention include the cotton rubisco promoter disclosed in U.S. Patent No. 6,040,504; the rice sucrose synthase promoter disclosed in U.S. Patent No. 5,604,121; and the cestrum yellow leaf curling virus promoter disclosed in PCT International Publication No. WO 01/73087. Chemically inducible promoters useful for directing the expression of the novel dense and erect panicle gene in plants are disclosed in U.S. Patent No. 5,614,395.

[0058] The promoter may include, or be modified to include, one or more enhancer elements to thereby provide for higher levels of transcription. Suitable enhancer elements for use in plants include the PCISV enhancer element (U.S. Patent No. 5,850,019), the CaMV 35S enhancer element (U.S. Patent Nos. 5,106,739 and 5,164,316) and the FMV enhancer element (Maiti et al., *Transgenic Res.*, 6:143-156 (1997)). See also PCT International Publication No. WO 96/23898.

[0059] Such constructs can contain a 'signal sequence' or 'leader sequence' to facilitate co-translational or post-translational transport of the peptide of interest to certain intracellular structures such as the chloroplast (or other plastid), endoplasmic reticulum, or Golgi apparatus, or to be secreted. For example, the construct can be engineered to contain a signal peptide to facilitate transfer of the peptide to the endoplasmic reticulum. A signal sequence is known or suspected to result in cotranslational or post-translational peptide transport across the cell membrane. In eukaryotes, this typically involves secretion into the Golgi apparatus, with some resulting glycosylation. A leader sequence refers to any sequence that, when translated, results in an amino acid sequence sufficient to trigger co-translational transport of the peptide chain to a sub-cellular organelle. Thus, this includes leader sequences targeting transport and/or glycosylation by passage into the endoplasmic reticulum, passage to

vacuoles, plastids including chloroplasts, mitochondria, and the like. Plant expression cassettes may also contain an intron, such that mRNA processing of the intron is required for expression.

[0060] Such constructs can also contain 5' and 3' untranslated regions. A 3' untranslated region is a polynucleotide located downstream of a coding sequence. Polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor are 3' untranslated regions. A 5' untranslated region is a polynucleotide located upstream of a coding sequence.

[0061] The termination region may be native with the transcriptional initiation region, may be native with the sequence of the present invention, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions (see e.g., Guerineau et al., *Mol. Gen. Genet.*, 262:141-144 (1991); Proudfoot, *Cell*, 64:671-674 (1991); Sanfacon et al., *Genes Dev.*, 5:141-149 (1991); Mogen et al., *Plant Cell*, 2:1261-1272 (1990); Munroe et al., *Gene*, 91:151-158 (1990); Ballas et al., *Nucleic Acids Res.*, 17:7891-7903 (1989); and Joshi et al., *Nucleic Acid Res.*, 15:9627-9639 (1987)).

[0062] Where appropriate, the vector and *OsMT1a* sequences may be optimized for increased expression in the transformed host cell. That is, the sequences can be synthesized using host cell-preferred codons for improving expression, or may be synthesized using codons at a host-preferred codon usage frequency. Generally, the GC content of the polynucleotide will be increased (see e.g., Campbell et al., *Plant Physiol.*, 92:1-11 (1990) for a discussion of host-preferred codon usage). Methods are known in the art for synthesizing host-preferred polynucleotides (see e.g., U.S. Patent Nos. 6,320,100; 6,075,185; 5,380,831; and 5,436,391, U.S. Application Publication Nos. 20040005600 and 20010003849, and Murray et al., *Nucleic Acids Res.*, 17:477-498 (1989)).

[0063] In certain embodiments, polynucleotides of interest are targeted to the chloroplast for expression. In this manner, where the polynucleotide of interest is not directly inserted into the chloroplast, the expression cassette may additionally contain a polynucleotide encoding a transit peptide to direct the nucleotide of interest to the chloroplasts. Such transit peptides are known in the art (see e.g., Von Heijne et al., *Plant Mol. Biol. Rep.*, 9:104-126 (1991); Clark et al., *J. Biol. Chem.*, 264:17544-17550 (1989); Della-Cioppa et al., *Plant Physiol.*, 84:965-968 (1987); Romer et al., *Biochem. Biophys. Res. Commun.*, 196:1414-1421 (1993); and Shah et al., *Science*, 233:478-481 (1986)). The polynucleotides of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for

differences in codon usage between the plant nucleus and this organelle. In this manner, the polynucleotides of interest may be synthesized using chloroplast-preferred codons (see e.g., U.S. Patent No. 5,380,831).

[0064] A plant expression cassette (i.e., a *OsMT1a* open reading frame operatively linked to a promoter) can be inserted into a plant transformation vector, which allows for the transformation of DNA into a cell. Such a molecule may consist of one or more expression cassettes, and may be organized into more than one vector DNA molecule. For example, binary vectors are plant transformation vectors that utilize two non-contiguous DNA vectors to encode all requisite cis- and trans-acting functions for transformation of plant cells (Hellens et al., *Trends in Plant Science*, 5:446-451 (2000)).

[0065] A plant transformation vector comprises one or more DNA vectors for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that comprise more than one contiguous DNA segment. These vectors are often referred to in the art as binary vectors. Binary vectors as well as vectors with helper plasmids are most often used for *Agrobacterium*-mediated transformation, where the size and complexity of DNA segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules. Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a polynucleotide of interest (i.e., a polynucleotide engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired).

[0066] For certain target species, different antibiotic or herbicide selectable markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene, which confers resistance to kanamycin and related antibiotics (Messing & Vierra, *Gene*, 19:259-268 (1982); and Bevan et al., *Nature*, 304:184-187 (1983)), the *bar* gene, which confers resistance to the herbicide phosphinothricin (White et al., *Nucl. Acids Res.*, 18: 1062 (1990), and Spencer et al., *Theor. Appl. Genet.*, 79: 625-631 (1990)), the *hph* gene, which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, *Mol. Cell. Biol.*, 4:2929-2931 (1984)), the *dhfr* gene, which confers resistance to methotrexate (Bourouis et al., *EMBO J.*, 2(7):1099-1104 (1983)), the *EPSPS* gene, which confers resistance to glyphosate (U.S. Patent Nos. 4,940,935 and 5,188,642), and the mannose-6-phosphate isomerase gene, which provides the ability to metabolize mannose (U.S. Patent Nos. 5,767,378 and 5,994,629).

[0067] Also present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker sequence and the sequence of interest are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from *Agrobacterium* to plant cells. This plasmid often contains the virulence functions (*Vir* genes) that allow infection of plant cells by *Agrobacterium*, and transfer of DNA by cleavage at border sequences and vir-mediated DNA transfer, as is understood in the art (Hellens et al., 2000). Several types of *Agrobacterium* strains (e.g., LBA4404, GV3101, EHA101, EHA105, etc.) can be used for plant transformation. The second plasmid vector is not necessary for introduction of polynucleotides into plants by other methods such as, e.g., microprojection, microinjection, electroporation, and polyethylene glycol.

[0068] In another embodiment, a nucleotide sequence of the present invention is directly transformed into a plastid genome. A major advantage of plastid transformation is that plastids are generally capable of expressing bacterial genes without substantial modification, and plastids are capable of expressing multiple open reading frames under control of a single promoter. Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817 and 5,545,818, in PCT International Application Publication WO 95/16783, and in McBride et al., *Proc. Natl. Acad. Sci. USA*, 91:7301-7305 (1994). The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, e.g., using biolistics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and *rps12* genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab et al., *Proc. Natl. Acad. Sci. USA*, 87:8526-8530 (1990); Staub et al., *Plant Cell*, 4:39-45 (1992)). This results in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allows creation of a plastid targeting vector for introduction of foreign genes (Staub et al., *EMBO J.*, 12:601-606 (1993)). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme

aminoglycoside-3'-adenyltransferase (Svab et al., *Proc. Natl. Acad. Sci. USA*, 90:913-917 (1993)). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga *Chlamydomonas reinhardtii* (Goldschmidt-Clermont, *Nucl. Acids Res.*, 19:4083-4089 (1991)). Other selectable markers useful for plastid transformation are known in the art. Typically, approximately 15-20 cell division cycles following transformation are required to reach a homoplastic state. Plastid expression, in which genes are inserted by homologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment, a nucleotide sequence of the present invention is inserted into a plastid-targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplastic for plastid genomes containing a nucleotide sequence of the present invention are obtained, and are preferentially capable of high expression of the nucleotide sequence.

[0069] Host Cells

[0070] Host cells are cells into which a heterologous nucleic acid molecule of the invention may be introduced. Representative eukaryotic host cells include yeast and plant cells, as well as prokaryotic hosts such as *E.coli* and *B. subtilis*. Preferred host cells for functional assays substantially or completely lack endogenous expression of a OsMT1a protein.

[0071] A host cell strain may be chosen which modulates the expression of the recombinant sequence, or modifies and processes the gene product in a specific manner. For example, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins). Appropriate cell lines or host cells may be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system may be used to produce a non-glycosylated core protein product, and expression in yeast will produce a glycosylated product.

[0072] The present invention further encompasses recombinant expression of a OsMT1a protein in a stable cell line. Methods for generating a stable cell line following transformation of a heterologous construct into a host cell are known in the art (see e.g., Joyner, Gene Targeting: A Practical Approach, 1993, Oxford University Press, Oxford/New York). Thus, transformed cells, tissues, and plants are understood to encompass not only the

end product of a transformation process, but also transgenic progeny or propagated forms thereof.

[0073] *OsMT1a* Knockout Plants

[0074] The present invention also provides *OsMT1a* knockout plants comprising a disruption of a *OsMT1a* locus. A disrupted gene may result in expression of an altered level of full-length *OsMT1a* protein or expression of a mutated variant *OsMT1a* protein. Plants with complete or partial functional inactivation of the *OsMT1a* gene may be generated, e.g., by expressing an amorphic (i.e., null mutation) or hypomorphic *OsMT1a* allele in the plant.

[0075] A knockout plant in accordance with the present invention may also be prepared using anti-sense, double-stranded RNA, or ribozyme *OsMT1a* constructs, driven by a universal or tissue-specific promoter to reduce levels of *OsMT1a* gene expression in somatic cells, thus achieving a “knock-down” phenotype. The present invention also provides the generation of plants with conditional or inducible inactivation of *OsMT1a*.

[0076] The present invention also encompasses transgenic plants with specific “knocked-in” modifications in the disclosed *OsMT1a* gene. In certain embodiments, a “knocked-in” transgenic plant expresses an antimorphic (i.e., dominant negative) allele. In other embodiments, a “knocked-in” transgenic plant expresses a hypermorphic (i.e., a gain of function) allele.

[0077] *OsMT1a* knockout plants may be prepared in monocot or dicot plants, such as maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugar beet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, Arabidopsis, and woody plants such as coniferous and deciduous trees. Rice, maize and soybean are particularly contemplated. As used herein, a plant refers to a whole plant, a plant organ (e.g., root, stem, leaf, flower bud, or embryo), a seed, a plant cell, a propagule, an embryo, other plant parts (e.g., protoplasts, pollen, pollen tubes, ovules, embryo sacs, zygotes) and progeny of the same. Plant cells can be differentiated or undifferentiated (e.g., callus, suspension culture cells, protoplasts, leaf cells, root cells, phloem cells, pollen).

[0078] For preparation of a *OsMT1a* knockout plant, introduction of a polynucleotide into plant cells is accomplished by one of several techniques known in the art, including but not

limited to electroporation or chemical transformation (see e.g., Ausubel, ed. (1994) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Indianapolis, Indiana). Markers conferring resistance to toxic substances are useful in identifying transformed cells (having taken up and expressed the test polynucleotide sequence) from non-transformed cells (those not containing or not expressing the test polynucleotide sequence). In one aspect of the invention, genes are useful as a marker to assess introduction of DNA into plant cells. Transgenic plants, transformed plants, or stably transformed plants, or cells, tissues or seed of any of the foregoing, refer to plants that have incorporated or integrated exogenous polynucleotides into the plant cell. Stable transformation refers to introduction of a polynucleotide construct into a plant such that it integrates into the genome of the plant and is capable of being inherited by progeny thereof.

[0079] In general, plant transformation methods involve transferring heterologous DNA into target plant cells (e.g., immature or mature embryos, suspension cultures, undifferentiated callus, protoplasts, *etc.*), followed by applying a maximum threshold level of appropriate selection (depending on the selectable marker gene) to recover the transformed plant cells from a group of untransformed cell mass. Explants are typically transferred to a fresh supply of the same medium and cultured routinely. Subsequently, the transformed cells are differentiated into shoots after placing on regeneration medium supplemented with a maximum threshold level of selecting agent (i.e., temperature and/or herbicide). The shoots are then transferred to a selective rooting medium for recovering rooted shoot or plantlet. The transgenic plantlet then grow into mature plant and produce fertile seeds (see e.g., Hiei et al., *Plant J.*, 6:271-282 (1994); and Ishida et al., *Nat. Biotechnol.*, 14:745-750 (1996)). A general description of the techniques and methods for generating transgenic plants are found in Ayres et al., *CRC Crit. Rev. Plant Sci.*, 13:219-239 (1994); and Bommineni et al., *Maydica*, 42:107-120 (1997). Since the transformed material contains many cells, both transformed and non-transformed cells are present in any piece of subjected target callus or tissue or group of cells. The ability to kill non-transformed cells and allow transformed cells to proliferate results in transformed plant cultures. Often, the ability to remove non-transformed cells is a limitation to rapid recovery of transformed plant cells and successful generation of transgenic plants. Molecular and biochemical methods can then be used for confirming the presence of the integrated nucleotide(s) of interest in the genome of transgenic plant.

[0080] Generation of transgenic plants may be performed by one of several methods, including but not limited to introduction of heterologous DNA by *Agrobacterium* into plant

cells (*Agrobacterium*-mediated transformation), bombardment of plant cells with heterologous foreign DNA adhered to particles, and various other non-particle direct-mediated methods to transfer DNA (see e.g., Hiei et al., *Plant J.*, 6:271-282 (1994); Ishida et al., *Nat. Biotechnol.*, 14:745-750 (1996); Ayres et al., *CRC Crit. Rev. Plant Sci.*, 13:219-239 (1994); and Bommineni et al., *Maydica*, 1997, 42:107-120 (1997)).

[0081] There are three common methods to transform plant cells with *Agrobacterium*. The first method is co-cultivation of *Agrobacterium* with cultured isolated protoplasts. This method requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts. The second method is transformation of cells or tissues with *Agrobacterium*. This method requires (a) that the plant cells or tissues can be transformed by *Agrobacterium* and (b) that the transformed cells or tissues can be induced to regenerate into whole plants. The third method is transformation of seeds, apices or meristems with *Agrobacterium*. This method requires micropropagation.

[0082] The efficiency of transformation by *Agrobacterium* may be enhanced by using a number of methods known in the art. For example, the inclusion of a natural wound response molecule such as acetosyringone (AS) to the *Agrobacterium* culture has been shown to enhance transformation efficiency with *Agrobacterium tumefaciens* (Shahla et al., *Plant Molec. Biol.*, 8:291-298 (1987)). Alternatively, transformation efficiency may be enhanced by wounding the target tissue to be transformed. Wounding of plant tissue may be achieved, for example, by punching, maceration, bombardment with microprojectiles (see e.g., Bidney et al., *Plant Molec. Biol.*, 18:301-313 (1992)).

[0083] In one embodiment, the plant cells are transfected with vectors via particle bombardment (i.e., with a gene gun). Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument described in U.S. Patent No. 5,584,807. This method involves coating the polynucleotide sequence of interest onto heavy metal particles, and accelerating the coated particles under the pressure of compressed gas for delivery to the target tissue.

[0084] Other particle bombardment methods are also available for the introduction of heterologous polynucleotide sequences into plant cells. Generally, these methods involve depositing the polynucleotide sequence of interest upon the surface of small, dense particles of a material such as gold, platinum, or tungsten. The coated particles are themselves then coated onto either a rigid surface, such as a metal plate, or onto a carrier sheet made of a fragile material such as mylar. The coated sheet is then accelerated toward the target biological tissue. The use of the flat sheet generates a uniform spread of accelerated particles

that maximizes the number of cells receiving particles under uniform conditions, resulting in the introduction of the polynucleotide sample into the target tissue.

[0085] Specific initiation signals may also be used to achieve more efficient translation of sequences encoding the polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide of interest, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers that are appropriate for the particular cell system that is used, such as those described in the literature (Scharf et al., *Results Probl. Cell Differ.*, 20:125 (1994)).

[0086] The cells that have been transformed may be grown into plants in accordance with conventional ways (see e.g., McCormick et al., *Plant Cell Rep.*, 5:81-84 (1986)). These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as transgenic seed) having a polynucleotide of the invention, for example, an expression cassette of the invention, stably incorporated into their genome.

[0087] Transgenic plants of the invention can be homozygous for the added polynucleotides; i.e., a transgenic plant that contains two added sequences, one sequence at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains the added sequences according to the invention, germinating some of the seed produced and analyzing the resulting plants produced for enhanced enzyme activity (i.e., herbicide resistance) and/or increased plant yield relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

[0088] It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous polynucleotides. Selfing of appropriate progeny can produce plants that are homozygous for all added exogenous polynucleotides that encode a polypeptide of the present invention. Back-crossing to a parental plant and outcrossing with a non-transgenic plant are also contemplated.

[0089] Following introduction of DNA into plant cells, the transformation or integration of the polynucleotide into the plant genome is confirmed by various methods such as analysis of polynucleotides, polypeptides and metabolites associated with the integrated sequence.

[0090] OsMT1a Inhibitors

[0091] The present invention further discloses assays to identify OsMT1a binding partners and OsMT1a inhibitors. OsMT1a antagonists/inhibitors are agents that alter the function of a OsMT1a protein e.g., by altering chemical and biological activities or properties. Methods of identifying inhibitors involve assaying a reduced level or quality of OsMT1a function in the presence of one or more agents. Exemplary OsMT1a inhibitors include small molecules as well as biological inhibitors as described herein below.

[0092] As used herein, the term “agent” refers to any substance that potentially interacts with a OsMT1a nucleic acid or protein, including any of synthetic, recombinant, or natural origin. An agent suspected to interact with a protein may be evaluated for such an interaction using the methods disclosed herein.

[0093] Exemplary agents include but are not limited to peptides, proteins, nucleic acids, small molecules (e.g., chemical compounds), antibodies or fragments thereof, nucleic acid-protein fusions, any other affinity agent, and combinations thereof. An agent to be tested may be a purified molecule, a homogenous sample, or a mixture of molecules or compounds.

[0094] A small molecule refers to a compound, for example an organic compound, with a molecular weight of less than about 1,000 daltons, more preferably less than about 750 daltons, still more preferably less than about 600 daltons, and still more preferably less than about 500 daltons. A small molecule also preferably has a computed log octanol-water partition coefficient in the range of about -4 to about +14, more preferably in the range of about -2 to about +7.5.

[0095] Exemplary nucleic acids that may be used to disrupt *OsMT1a* function include antisense RNA and small interfering RNAs (siRNAs) (see e.g., U.S. Application Publication No. 20060095987. These inhibitory molecules may be prepared based upon the *OsMT1a*

gene sequence and known features of inhibitory nucleic acids (see e.g., Van der Krol et al., *Plant Cell*, 2:291-299 (1990); Napoli et al., *Plant Cell*, 2:279-289 (1990); English et al., *Plant Cell*, 8:179-188 (1996); and Waterhouse et al., *Nature Rev. Genet.*, 2003, 4:29-38 (2003).

[0096] Agents may be obtained or prepared as a library or collection of molecules. A library may contain a few or a large number of different molecules, varying from about ten molecules to several billion molecules or more. A molecule may comprise a naturally occurring molecule, a recombinant molecule, or a synthetic molecule. A plurality of agents in a library may be assayed simultaneously. Optionally, agents derived from different libraries may be pooled for simultaneous evaluation.

[0097] Representative libraries include but are not limited to a peptide library (U.S. Patent Nos. 6,156,511, 6,107,059, 5,922,545, and 5,223,409), an oligomer library (U.S. Patent Nos. 5,650,489 and 5,858,670), an aptamer library (U.S. Patent Nos. 7,338,762; 7,329,742; 6,949,379; 6,180,348; and 5,756,291), a small molecule library (U.S. Patent Nos. 6,168,912 and 5,738,996), a library of antibodies or antibody fragments (U.S. Patent Nos. 6,174,708, 6,057,098, 5,922,254, 5,840,479, 5,780,225, 5,702,892, and 5,667,988), a library of nucleic acid-protein fusions (U.S. Patent No. 6,214,553), and a library of any other affinity agent that may potentially bind to a OsMT1a protein.

[0098] A library may comprise a random collection of molecules. Alternatively, a library may comprise a collection of molecules having a bias for a particular sequence, structure, or conformation, for example, as for inhibitory nucleic acids (see e.g., U.S. Patent Nos. 5,264,563 and 5,824,483). Methods for preparing libraries containing diverse populations of various types of molecules are known in the art, for example as described in U.S. patents cited herein above. Numerous libraries are also commercially available.

[0099] A control level or quality of *OsMT1a* activity refers to a level or quality of wild type *OsMT1a* activity, for example, when using a recombinant expression system comprising expression of SEQ ID NO: 2. When evaluating the inhibiting capacity of an agent, a control level or quality of *OsMT1a* activity comprises a level or quality of activity in the absence of the agent. A control level may also be established by a phenotype or other measureable trait.

[00100] Methods of identifying OsMT1a inhibitors also require that the inhibiting capacity of an agent be assayed. Assaying the inhibiting capacity of an agent may comprise determining a level of *OsMT1a* gene expression; determining DNA binding activity of a recombinantly expressed OsMT1a protein; determining an active conformation of a OsMT1a protein; or determining a change in a trait in response to binding of a OsMT1a inhibitor (e.g., drought tolerance, zinc uptake). In particular embodiments, a method of identifying a

OsMT1a inhibitor may comprise (a) providing a cell, plant, or plant part expressing a OsMT1a protein; (b) contacting the cell, plant, or plant part with an agent; (c) examining the cell, plant, or plant part for a change in a trait as compared to a control; and (d) selecting an agent that induces a change in the trait as compared to a control. Any of the agents so identified in the disclosed inhibitory or binding assays (see hereinafter) may be subsequently applied to a cell, plant or plant part as desired to effectuate a change in that cell, plant or plant part. For example, disruption of a *OsMT1a* gene (e.g., SEQ ID NO: 1) or inhibition of a OsMT1a polynucleotide or polypeptide (e.g., SEQ ID NO: 2) would likely alter one or more plant traits in a non-desirable fashion (e.g., decrease drought tolerance).

[00101] The present invention also encompasses a rapid and high throughput screening method that relies on the methods described herein. This screening method comprises separately contacting a OsMT1a protein with a plurality of agents. In such a screening method the plurality of agents may comprise more than about 10^4 samples, or more than about 10^5 samples, or more than about 10^6 samples.

[00102] The *in vitro* and cellular assays of the invention may comprise soluble assays, or may further comprise a solid phase substrate for immobilizing one or more components of the assay. For example, a OsMT1a protein, or a cell expressing a OsMT1a protein, may be bound directly to a solid state component via a covalent or non-covalent linkage. Optionally, the binding may include a linker molecule or tag that mediates indirect binding of a OsMT1a protein to a substrate.

[00103] OsMT1a Binding Assays

[00104] The present invention also encompasses methods of identifying of a OsMT1a inhibitor by determining specific binding of a substance (e.g., an agent described previously) to a OsMT1a protein. For example, a method of identifying a OsMT1a binding partner may comprise: (a) providing a OsMT1a protein of SEQ ID NO: 2; (b) contacting the OsMT1a protein with one or more agents under conditions sufficient for binding; (c) assaying binding of the agent to the isolated OsMT1a protein; and (d) selecting an agent that demonstrates specific binding to the OsMT1a protein. Specific binding may also encompass a quality or state of mutual action such that binding of an agent to a OsMT1a protein is inhibitory.

[00105] Specific binding refers to a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biological materials. The binding of an agent to a OsMT1a protein may be considered specific if the binding affinity is about $1 \times 10^4 \text{ M}^{-1}$ to about $1 \times 10^6 \text{ M}^{-1}$ or greater. Specific binding also refers

to saturable binding. To demonstrate saturable binding of an agent to a OsMT1a protein, Scatchard analysis may be carried out as described, for example, by Mak et al., *J. Biol. Chem.*, 264:21613-21618 (1989).

[00106] Several techniques may be used to detect interactions between a OsMT1a protein and an agent without employing a known competitive inhibitor. Representative methods include, but are not limited to, Fluorescence Correlation Spectroscopy, Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Spectroscopy, and BIACORE® technology, each technique described herein below. These methods are amenable to automated, high-throughput screening.

[00107] Fluorescence Correlation Spectroscopy (FCS) measures the average diffusion rate of a fluorescent molecule within a small sample volume. The sample size may be as low as 10^3 fluorescent molecules and the sample volume as low as the cytoplasm of a single bacterium. The diffusion rate is a function of the mass of the molecule and decreases as the mass increases. FCS may therefore be applied to protein-ligand interaction analysis by measuring the change in mass and therefore in diffusion rate of a molecule upon binding. In a typical experiment, the target to be analyzed (e.g., a OsMT1a protein) is expressed as a recombinant protein with a sequence tag, such as a poly-histidine sequence, inserted at the N-terminus or C-terminus. The expression is mediated in a host cell, such as *E.coli*, yeast, *Xenopus* oocytes, or mammalian cells. The protein is purified using chromatographic methods. For example, the poly-histidine tag may be used to bind the expressed protein to a metal chelate column such as Ni^{2+} chelated on iminodiacetic acid agarose. The protein is then labeled with a fluorescent tag such as carboxytetramethylrhodamine or BODIPY™ reagent (available from Molecular Probes of Eugene, Oregon). The protein is then exposed in solution to the potential ligand, and its diffusion rate is determined by FCS using instrumentation available from Carl Zeiss, Inc. (Thornwood of New York, New York). Ligand binding is determined by changes in the diffusion rate of the protein.

[00108] Surface-Enhanced Laser Desorption/Ionization (SELDI) was developed by Hutchens & Yip, *Rapid Commun. Mass Spectrom.*, 1993, 7:576-580. When coupled to a time-of-flight mass spectrometer (TOF), SELDI provides a technique to rapidly analyze molecules retained on a chip. It may be applied to ligand-protein interaction analysis by covalently binding the target protein, or portion thereof, on the chip and analyzing by mass spectrometry the small molecules that bind to this protein (Worrall et al., *Anal Chem.*, 1998, 70(4):750-756 (1998)). In a typical experiment, a target protein (e.g., a OsMT1a protein) is recombinantly expressed and purified. The target protein is bound to a SELDI chip either by

utilizing a poly-histidine tag or by other interaction such as ion exchange or hydrophobic interaction. A chip thus prepared is then exposed to the potential ligand via, for example, a delivery system able to pipet the ligands in a sequential manner (autosampler). The chip is then washed in solutions of increasing stringency, for example a series of washes with buffer solutions containing an increasing ionic strength. After each wash, the bound material is analyzed by submitting the chip to SELDI-TOF. Ligands that specifically bind a target protein are identified by the stringency of the wash needed to elute them.

[00109] BIACORE® relies on changes in the refractive index at the surface layer upon binding of a ligand to a target protein (e.g., a OsMT1a protein) immobilized on the layer. In this system, a collection of small ligands is injected sequentially in a 2-5 microliter cell, wherein the target protein is immobilized within the cell. Binding is detected by surface plasmon resonance (SPR) by recording laser light refracting from the surface. In general, the refractive index change for a given change of mass concentration at the surface layer is practically the same for all proteins and peptides, allowing a single method to be applicable for any protein. In a typical experiment, a target protein is recombinantly expressed, purified, and bound to a BIACORE® chip. Binding may be facilitated by utilizing a poly-histidine tag or by other interaction such as ion exchange or hydrophobic interaction. A chip thus prepared is then exposed to one or more potential ligands via the delivery system incorporated in the instruments sold by Biacore (Uppsala, Sweden) to pipet the ligands in a sequential manner (autosampler). The SPR signal on the chip is recorded and changes in the refractive index indicate an interaction between the immobilized target and the ligand. Analysis of the signal kinetics of on rate and off rate allows the discrimination between non-specific and specific interaction (see also Homola et al., *Sensors and Actuators*, 54:3-15 (1999) and references therein).

[00110] Conformational Assays

[00111] The present invention also encompasses methods of identifying OsMT1a binding partners and inhibitors that rely on a conformational change of a OsMT1a protein when bound by or otherwise interacting with a substance (e.g., an agent described previously). For example, application of circular dichroism to solutions of macromolecules reveals the conformational states of these macromolecules. The technique may distinguish random coil, alpha helix, and beta chain conformational states.

[00112] To identify inhibitors of a OsMT1a protein, circular dichroism analysis may be performed using a recombinantly expressed OsMT1a protein. A OsMT1a protein is purified,

for example by ion exchange and size exclusion chromatography, and mixed with an agent. The mixture is subjected to circular dichroism. The conformation of a OsMT1a protein in the presence of an agent is compared to a conformation of a OsMT1a protein in the absence of the agent. A change in conformational state of a OsMT1a protein in the presence of an agent identifies a OsMT1a binding partner or inhibitor. Representative methods are described in U.S. Patent Nos. 5,776,859 and 5,780,242. Antagonistic activity of the inhibitor may be assessed using functional assays, such as assaying for altered stress tolerance as described herein.

[00113] In accordance with the disclosed methods, cells expressing OsMT1a may be provided in the form of a kit useful for performing an assay of OsMT1a function. For example, a kit for detecting a OsMT1a may include cells transfected with DNA encoding a full-length OsMT1a protein and a medium for growing the cells.

[00114] Assays of OsMT1a activity that employ transiently transfected cells may include a marker that distinguishes transfected cells from non-transfected cells. A marker may be encoded by or otherwise associated with a construct for *OsMT1a* expression, such that cells are simultaneously transfected with a nucleic acid molecule encoding *OsMT1a* and the marker. Representative detectable molecules that are useful as markers include but are not limited to a heterologous nucleic acid, a protein encoded by a transfected construct (e.g., an enzyme or a fluorescent protein), a binding protein, and an antigen.

[00115] Assays employing cells expressing recombinant *OsMT1a* or plants expressing *OsMT1a* may additionally employ control cells or plants that are substantially devoid of native *OsMT1a* and, optionally, proteins substantially similar to a OsMT1a protein. When using transiently transfected cells, a control cell may comprise, for example, an untransfected host cell. When using a stable cell line expressing a OsMT1a protein, a control cell may comprise, for example, a parent cell line used to derive the *OsMT1a*-expressing cell line.

[00116] Anti-OsMT1a Antibodies

[00117] In another aspect of the invention, a method is provided for producing an antibody that specifically binds a OsMT1a protein. According to the method, a full-length recombinant OsMT1a protein is formulated so that it may be used as an effective immunogen, and used to immunize an animal so as to generate an immune response in the animal. The immune response is characterized by the production of antibodies that may be collected from the blood serum of the animal.

[00118] An antibody is an immunoglobulin protein, or antibody fragments that comprise an antigen binding site (e.g., Fab, modified Fab, Fab', F(ab')₂ or Fv fragments, or a protein having at least one immunoglobulin light chain variable region or at least one immunoglobulin heavy chain region). Antibodies of the invention include diabodies, tetrameric antibodies, single chain antibodies, tretravalent antibodies, multispecific antibodies (e.g., bispecific antibodies), and domain-specific antibodies that recognize a particular epitope. Cell lines that produce anti-OsMT1a antibodies are also encompassed by the invention.

[00119] Specific binding of an antibody to a OsMT1a protein refers to preferential binding to a OsMT1a protein in a heterogeneous sample comprising multiple different antigens. Substantially lacking binding describes binding of an antibody to a control protein or sample, i.e., a level of binding characterized as non-specific or background binding. The binding of an antibody to an antigen is specific if the binding affinity is at least about 10^{-7} M or higher, such as at least about 10^{-8} M or higher, including at least about 10^{-9} M or higher, at least about 10^{-11} M or higher, or at least about 10^{-12} M or higher.

[00120] OsMT1a antibodies prepared as disclosed herein may be used in methods known in the art relating to the expression and activity of OsMT1a proteins, e.g., for cloning of nucleic acids encoding a OsMT1a protein, immunopurification of a OsMT1a protein, and detecting a OsMT1a protein in a plant sample, and measuring levels of a OsMT1a protein in plant samples. To perform such methods, an antibody of the present invention may further comprise a detectable label, including but not limited to a radioactive label, a fluorescent label, an epitope label, and a label that may be detected in vivo. Methods for selection of a label suitable for a particular detection technique, and methods for conjugating to or otherwise associating a detectable label with an antibody are known to one skilled in the art.

[00121] Examples

[00122] The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations which are evident as a result of the teachings provided herein.

[00123]

Example 1

[00124]

Identification of the *OsMT1a* gene

[00125] *OsMT1a* was directly obtained from Brazil upland rice using suppression subtractive hybridization. The 222 bp cDNA sequence of *OsMT1a* is composed of an open reading frame of 74 amino acids (SEQ ID NO: 2). The predicted protein contains two cysteine-rich domains in the N- and C-terminal regions, which is characteristic of metallothioneins. More specifically, *OsMT1a* contains six Cys-Xaa-Cys motifs (where Xaa represents another amino acid) that are distributed equally among the N- and C-terminal domains, and an approximately 40-amino acid spacer containing aromatic amino acids, which is typical for most type 1 MTs in plants.

[00126] A BLAST search of the predicted peptide in the NCBI database demonstrated that *OsMT1a* showed high homology with many other type 1 plant MT proteins, especially with monocotyledonous *Zea mays* (73.7%) and *Hordeum vulgare* (67.6%).

[00127]

Example 2

[00128]

Expression patterns of *OsMT1a*

[00129] To examine the expression pattern of *OsMT1a* in different organs, Northern blot analysis was carried out with total RNAs extracted from different organs, including flowers, leaves, shoots and roots. As shown in Figure 1, *OsMT1a* was expressed predominantly in roots, with much lower expression in leaves and flowers, and even lower expression in shoots. These organ expression patterns are consistent with previous reports that type 1 MT genes are preferentially expressed in roots.

[00130]

Example 3

[00131]

Effect of abiotic stresses on *OsMT1a* expression

[00132] To determine the effect of several abiotic factors on *OsMT1a* expression in two rice varieties, 2-week-old seedlings of upland rice cv. Iapar 9 and lowland rice cv. Nipponbare were subjected to 150 mM NaCl, 7% PEG 6000 and 10 μ M ABA treatment for 0.5, 6 and 24 hours. Total RNA was extracted from leaves and used for Northern analysis. While there was no significant response in Nipponbare plants to all three treatments, *OsMT1a* expression in Iapar 9 significantly increased after 0.5 hours as a result of 7% PEG 6000 treatment, though *OsMT1a* expression did not change in response to 150 mM NaCl and ABA treatment (see Figure 2).

[00133] To find out whether different metal ions affected the expression level of *OsMT1a*, seedlings of Iapar 9 and Nipponbare were exposed to 10^{-5} M of Mn^{2+} , Cu^{2+} , Fe^{3+} , or Zn^{2+} for 1, 6, 12 and 24 hours and transcript levels were examined by real time PCR analysis performed with Chromo 4 (MJ Research, USA) using SYBR® Green I (10,0009, Molecular Probes, USA) according to the manufacturer's instructions. The PCR reaction was carried out with a mixture containing 10 ng of cDNA template, 5 pmol of primers and SYBR® Green I in a total volume of 20 μ l. The standard thermal profile was as follows: 95 °C for 4 min; 30 cycles of 95 °C for 30 seconds; 56 °C (*OsMT1a*, AK108249, JRC0332)/61 °C (JRC0189) for 30 seconds; and 72 °C for 30 seconds; then 72 °C for 10 minutes. The fluorescence signal was captured at the end of each cycle and melting curve analysis was performed from 65 to 95 °C with data capture every 0.3 °C during a 1 second hold. *Actin1* was used as a quantification control. Reactions with the cDNA template replaced by nuclease-free water were run with each primer pair as a blank control. Quantification consisted of at least three independent replicates.

[00134] Although the expression levels of *OsMT1a* were not altered by Mn^{2+} , Cu^{2+} or Fe^{3+} , Zn^{2+} induced expression of *OsMT1a*. In fact, *OsMT1a* levels in Iapar 9 increased dramatically (5.9-fold at 6 h and 15.7-fold at 24 h; see Figure 3). A similar pattern was observed in Nipponbare (data not shown).

[00135] Example 4

[00136] Effect of overexpression of *OsMT1a* on zinc uptake in rice and yeast

[00137] To examine whether *OsMT1a* is involved in accumulating certain heavy metals, the full-length ORF of *OsMT1a* was introduced into the binary vector under the control of the rice *Actin1* promoter, generating plasmid *pActini:OsMT1a*. The *pActini:OsMT1a* plasmid was introduced into *Agrobacterium tumefaciens* AGL1 and transformed into rice as described by Liu et al. (2007). The independent transgenic plants were further confirmed by PCR assay, and were then transferred to a greenhouse. Wild type and *OsMT1a*-overexpressing plants were further cultivated, and the aerial parts of mature plants and grains (*sans* hull) were collected and washed thoroughly in distilled water, dewatered and then oven-dried at 80 °C for 24 hours. The dry samples were milled and 200 mg aliquots were digested in a microwave oven (Ethos touch control advanced microwave lab station; Milestone, Inc, Italy) with 9 mL concentrated HNO_3 and 2 mL H_2O_2 at 180 °C for 20 minutes. After cooling, the digests were diluted to 25 mL with distilled water, and analyzed for metal content using an

Optima-2000™ DV Inductively Coupled Plasma-Optic Emission spectrometer (ICP-OES, Perkin Elmer, USA).

[00138] As shown in Table 1, the zinc content of leaves and seeds from transgenic lines L6 and L7 was increased significantly compared to wild type. In contrast, no significant difference was observed in manganese, copper or iron levels in transgenic plants and wild type controls. Furthermore, transgenic and wild type plants showed no differences in cadmium accumulation (data not shown).

[00139] Table 1

	Plant	Mn	Fe	Zn	Cu
Leaves	WT	382.67±41.80	76.69±2.05	13.00±0.40	5.39±0.12
	L6	463.20±37.99	77.37±4.17	18.90±0.80**	5.48±0.26
	L7	476.09±38.27	78.56±2.05	20.04±1.03**	5.82±0.15
Seeds	WT	16.99±0.29	6.01±0.82	9.79±0.16	2.03±0.06
	L6	20.60±1.95	6.73±0.38	14.14±0.62**	2.16±0.11
	L7	16.62±1.35	6.08±0.25	15.07±1.02**	2.28±0.11

The level of different metals (µg/g dry wt) was measured in transgenic plants harboring the *pACTIN1::OsMT1a* using inductive coupled plasma spectroscopy (ICP-OES). The data represent the average from six independent measurements with standard deviation. The asterisks (**) indicate significant differences from the control at P<0.01 by Student's t-test.

[00140] To examine whether *OsMT1a* specifically facilitates zinc accumulation, *OsMT1a* was ectopically expressed in yeast. The yeast (*Saccharomyces cerevisiae*) BY4741 wild type (*MATa his3 leu2 met15 ura3*) was employed for the heavy metal accumulation assay. The expression vector p181AINE carries the yeast alcohol dehydrogenase (ADH2) promoter and terminator and b-isopropylmalate dehydrogenase gene (LEU2) as the selective marker.

[00141] The open reading frame of *OsMT1a* was amplified by PCR with specific primers and the PCR product was inserted into the expression cassette, designated as p181AINE::*OsMT1a*. The p181AINE::*OsMT1a* and the control plasmid p181AINE vector alone were separately transformed into yeast strain BY4741 by the lithium acetate procedure described by Gietz and Schiestl (1991), and the transformants were selected by growth on leucine-deficient SD medium. The overnight cultures of yeast strains containing p181AINE and p181AINE::*OsMT1a* were diluted 300-fold in the same medium supplemented with 1

mM ZnCl₂. After 10 hours growth at 30 °C to the late exponential phase, yeast cells were collected for metal uptake analysis.

[00142] Under 1 mM Zn²⁺ treatment, yeast cells expressing *OsMT1a* accumulated 2.4-fold more zinc than controls, while no difference in manganese, copper or iron levels were observed between transgenic and wild type yeast (see Table 2).

[00143] Table 2

Metal ion	Before treatment		10 hours after 1 mM ZnCl ₂ treatment	
	p181AINE	p181AINE:: <i>OsMT1a</i>	p181AINE	p181AINE:: <i>OsMT1a</i>
Mn	79.03±1.42	79.80±5.78	50.62±1.29	64.6±5.21
Fe	63.26±8.25	66.27±4.86	49.97±1.2	59.83±7.15
Zn	220.67±22.56	263.03±48.30	777.13±74.3	1876.06±188.33**
Cu	5.81±0.33	5.00±0.21	5.28±0.28	4.50±0.85

[00144] Example 5

[00145] Effect of overexpression of *OsMT1a* on drought resistance in rice

[00146] The seedlings of wild type and *OsMT1a*-overexpressing lines L6 and L7 were treated with MS medium containing 300 mM mannitol for 10 days in order to evaluate the effect of overexpression of *OsMT1a* on drought resistance. As shown in Figure 4A, wild type plants looked almost dead, with yellow, wilting and droopy leaves after 10 days, whereas L6 and L7 displayed weaker symptoms with green and curled leaves (see Figure 3a).

[00147] A dehydration test was also performed. The seeds of *OsMT1a*-overexpressing plants were presoaked for imbibition in distilled water at 37°C for 2 days and the soaked seeds were then sown on pots and grew for another 2 months in a greenhouse. For each sample, leaf tips around 0.5 g were cut and weighed as the initial weight and then put at room temperature and weighed at consecutive time points within 1 hour. The dehydration rate was determined by weight loss, and each experiment was performed with six replicates.

[00148] As shown in Figure 4B, dehydration test revealed that transgenic lines L6 and L7 showed a slower dehydration rate in comparison to wild type, with a 0.66% lag from the beginning of drought stress, rising up until 3.75% 1 hour later.

[00149] Example 6

[00150] Effect of overexpression of *OsMT1a* on antioxidant enzyme activity in rice

[00151] Two-week seedlings were exposed to 1 mM H₂O₂ for 5 hours. One gram of frozen shoots was homogenized in 1 mL of ice-cold solution containing 100 mM Tris-HCl (pH 7.0), 20% glycerol, and 1% PVP. The homogenates were then centrifuged at 10,000xg for 30 minutes. The aliquots of supernatants were immediately used for the analysis of catalase (CAT) activity, and the rest was stored at -20 °C for subsequent analysis of peroxidase (POD) and ascorbate peroxidase (APX).

[00152] Protein content was determined as described by Bradford (1976). CAT activity was determined as described by Aebi (1983), by monitoring the disappearance of H₂O₂ by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 0.3 mL of 3% H₂O₂, 2.5 mL of 0.05 M phosphate buffer (pH 7.0) with 0.2 mL extract.

[00153] POD activity was measured as described by Abeles and Biles (1991), by the H₂O₂-dependent oxidation of benzidine at 530 nm, in a reaction mixture containing 2 mL of 0.2 M acetate buffer (pH 4.8), 0.2 mL of 3% H₂O₂, 0.2 mL of 0.04 M benzidine and 0.1 mL extracted protein.

[00154] APX activity was assayed by monitoring the ascorbic acid-dependent reduction of H₂O₂ at 265 nm in a reaction mixture containing 2 mL of 0.05 M phosphate buffer (pH 6.5), 0.2 mL of 3% H₂O₂, and 0.2 mL of 50 µM ascorbate. The reaction was started by adding 0.1 mL extracted protein as described by Arrigoni et al (1992). The unit of enzyme activity was defined as the changes in absorbance per minute per mg protein.

[00155] As shown in Figure 5, CAT, POD, and APX activities all significantly increased in L7 plants as compared to wild type plants (81, 32 and 17%, respectively). Likewise, the transgenic rice line L6 showed higher CAT (42%) and POD (23%) enzyme activity compared to wild type plants. These data suggest that *OsMT1a*-overexpressing rice might have a more efficient antioxidant system with improved antioxidative enzyme activities.

[00156] Example 7

[00157] Altered gene expression in *OsMT1a*-overexpressing rice

[00158] The expression levels of three zinc finger transcription factors, AK108249 (designated as *Ossiz*), *ZFI* (Accession No. AF332876) and *WRKY71* (Accession No. NM_001052629), which have been confirmed to play an important role in plant stress tolerance, were examined in *OsMT1a*-overexpressing rice. As shown in Figure 6A,

expression of *Ossiz*, *ZFI* and *WRKY71* were increased by 9.6-, 5.2- and 2.9-fold respectively, compared to levels in wild type plants.

[00159] *Ossiz* expression was also induced by Zn^{2+} treatment. The expression reached plateau at 6 hours and then declined gradually, and the final expression pattern was similar to *OsMT1a* (see Figure 6C). Northern analysis further confirmed the synergistic expression of *Ossiz* and *OsMT1a* in *OsMT1a*-overexpressing rice lines, suggesting *Ossiz* is very likely located downstream of *OsMT1a* (see Figure 6B).

[00160] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[00161] While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention can be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims include all such embodiments and equivalent variations.

[00162]

SEQUENCE LISTING

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 tgcagctgcg gttccagctg caggtgcaac ccttgcaact gt 222

<210> 2
 <211> 74
 <212> PRT
 <213> *Oryza glaberrima*

<400> 2

Met Ser Cys Ser Cys Gly Ser Ser Cys Ser Cys Gly Ser Asn Cys Ser
 1 5 10 15

Cys Gly Lys Lys Tyr Pro Asp Leu Glu Glu Lys Ser Ser Ser Thr Lys
 20 25 30

Ala Thr Val Val Leu Gly Val Ala Pro Glu Lys Lys Ala Gln Gln Phe
 35 40 45

Glu Ala Ala Ala Glu Ser Gly Glu Thr Ala His Gly Cys Ser Cys Gly
 50 55 60

Ser Ser Cys Arg Cys Asn Pro Cys Asn Cys
 65 70

CLAIMS

What is claimed is:

1. An isolated OsMT1a polynucleotide selected from the group consisting of:
 - (a) a nucleic acid comprising a nucleotide sequence of SEQ ID NO: 1;
 - (b) a nucleic acid comprising a nucleotide sequence at least 90% identical to (a);
 - (c) a nucleic acid comprising a nucleotide sequence that specifically hybridizes to the complement of (a) under stringent hybridization conditions and which encodes a metallothionein;
 - (d) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence of SEQ ID NO: 2;
 - (e) a nucleic acid comprising an open reading frame encoding a OsMT1a protein comprising a polypeptide sequence derived from (d) by substitution, deletion or addition of one or several amino acids in the amino acid sequence in (d) and having metallothionein activity; and
 - (f) a nucleic acid comprising a nucleotide sequence that is the complement of any one of (a)-(e).
2. A vector comprising the isolated OsMT1a polynucleotide of claim 1.
3. A host cell which expresses the vector of claim 2.
4. The host cell of claim 3, wherein the cell is selected from the group consisting of animal cell, plant cell, and microorganism cell.
5. A transgenic plant or seed comprising the host cell of claim 4.
6. The transgenic plant or seed of claim 5, wherein the plant is a monocot.
7. The transgenic plant or seed claim 5, wherein the plant is a dicot.
8. The transgenic plant or seed of claim 5, wherein the transgenic plant is selected from the group consisting of maize, wheat, barley, rye, sweet potato, bean, pea,

chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugar beet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, and Arabidopsis.

9. An isolated OsMT1a polypeptide, comprising an amino acid sequence selected from the group consisting of:

- (a) a polypeptide sequence of SEQ ID NO: 2; and
- (b) a polypeptide sequence derived from (a) by substitution, deletion or addition of one or several amino acids in the amino acid sequence in (a) and having metallothionein activity.

10. A method for producing a transgenic plant comprising regenerating a transgenic plant from the host cell according to claim 3.

11. A method for producing a transgenic plant comprising crossing a transgenic plant according to claim 5 with a non-transgenic plant.

12. A plant produced by the method according to claim 10 or 11 or a transgenic seed derived therefrom.

13. A method of improving drought tolerance in a plant comprising expressing the isolated polynucleotide of claim 1 in the plant.

14. A method of increasing zinc uptake in a plant comprising expressing the isolated polynucleotide of claim 1 in the plant.

15. A method of increasing enzymatic activity in a plant comprising expressing the isolated polynucleotide of claim 1 in the plant, wherein the enzymatic activity is that of an enzyme selected from the group consisting of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX).

16. A method of increasing the expression of a zinc finger transcription factor in a plant comprising expressing the isolated polynucleotide of claim 1 in the plant, wherein the zinc finger transcription factor is encoded by a gene selected from the group consisting of *Ossiz*, *ZF1* and *WRKY71*.

17. A plant produced by the method according to any one of claims 13 to 16 or a transgenic seed derived therefrom.

18. A method for producing a transgenic plant comprising crossing the plant according to claim 17 with a non-transgenic plant.

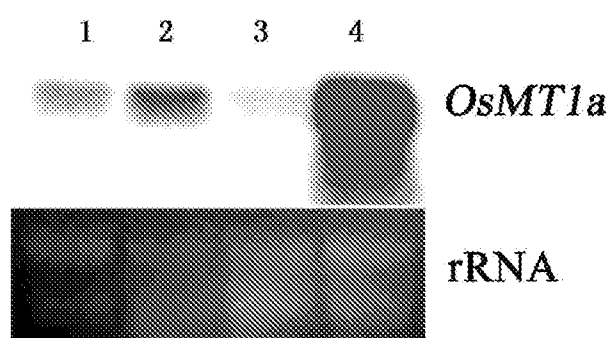


FIGURE 1

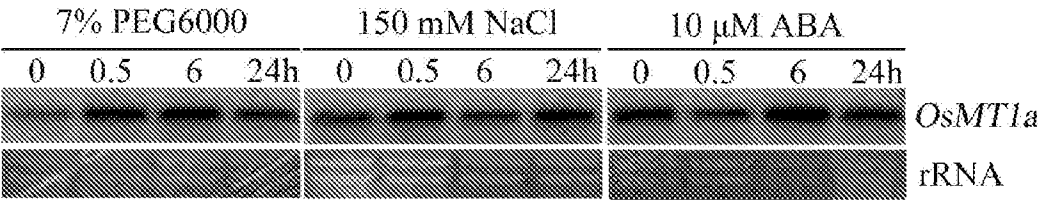


FIGURE 2

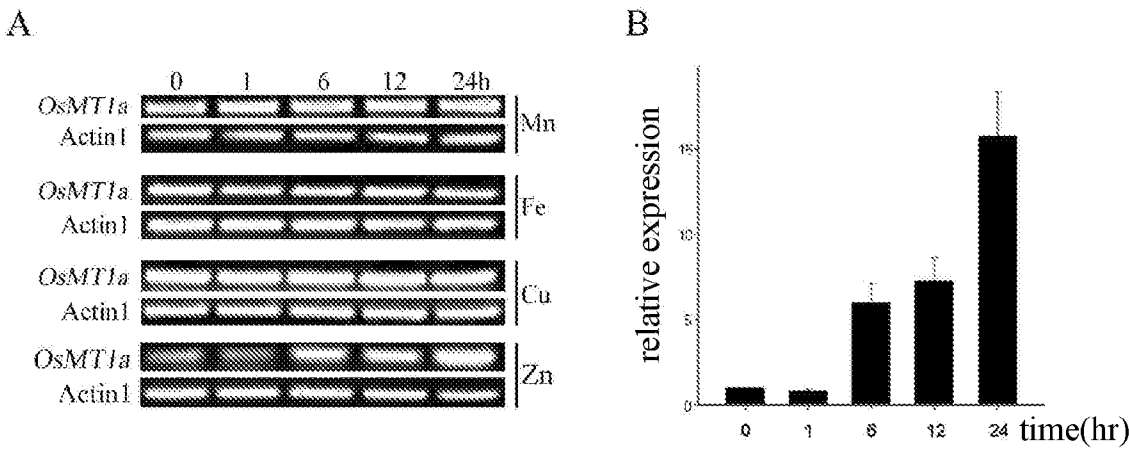


FIGURE 3

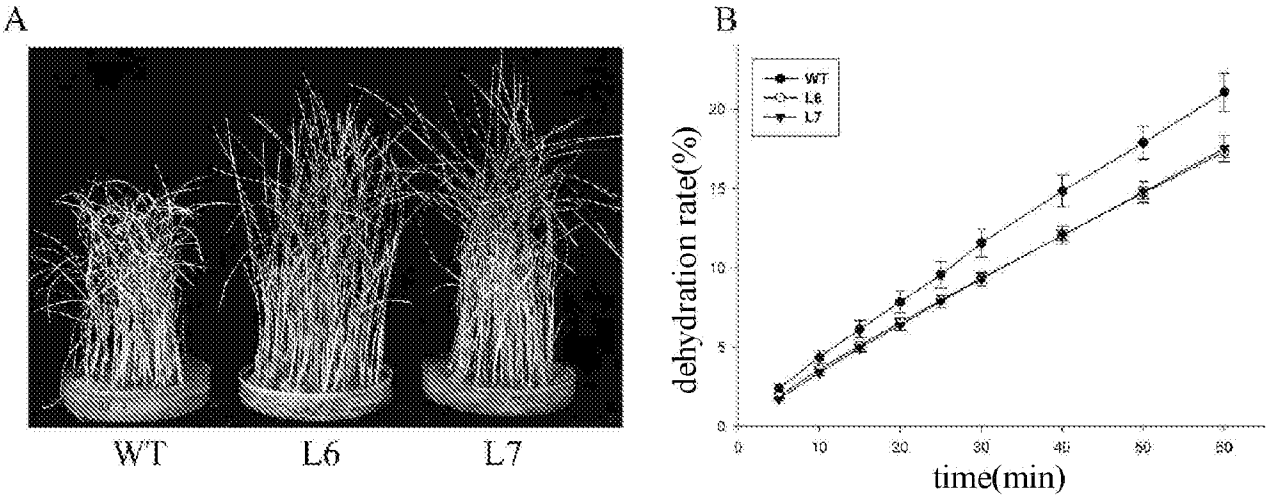


FIGURE 4

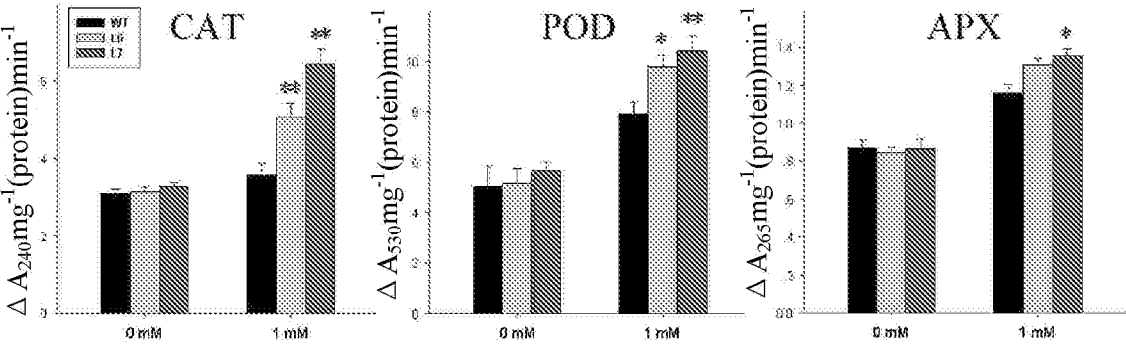


FIGURE 5

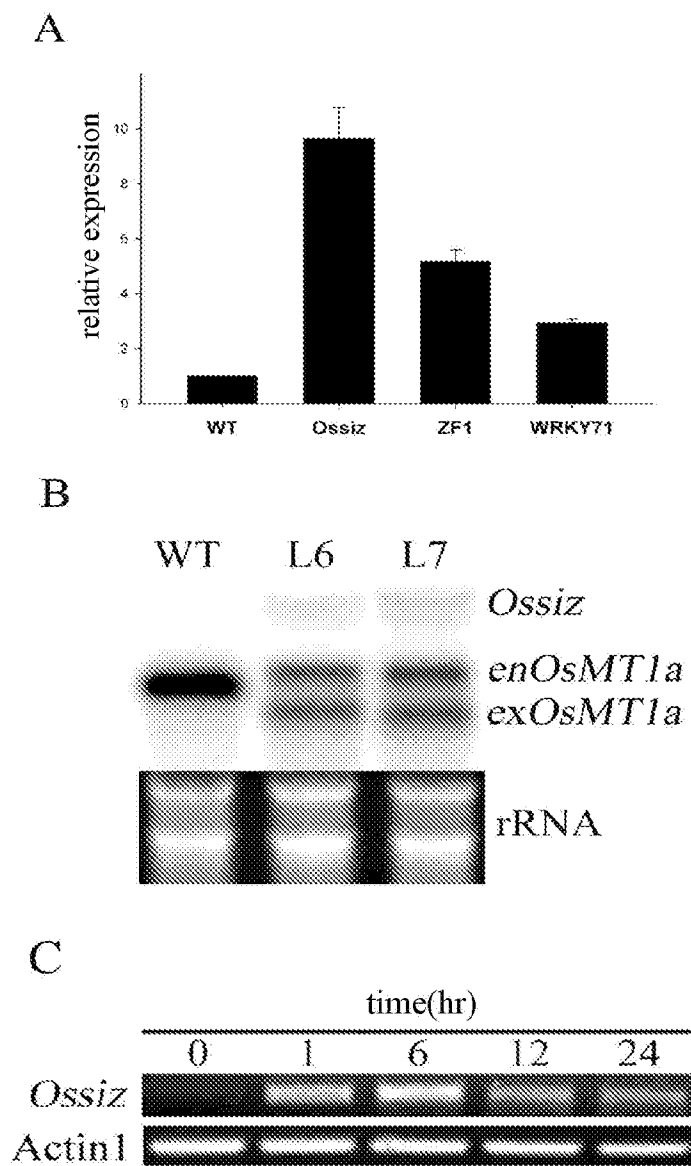


FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2010/050755

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/825 C12N15/82

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K C12N

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, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO YANG ET AL: "OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice" PLANT MOLECULAR BIOLOGY, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 70, no. 1-2, 20 February 2009 (2009-02-20), pages 219-229, XP019686543 ISSN: 1573-5028 the whole document	1-18
X	US 2007/016974 A1 (BYRUM JOSEPH R [US] ET AL) 18 January 2007 (2007-01-18) claims 1-9 page 3, column 2 - page 4, column 1 -/--	1-8, 10, 11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :-

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

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"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

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"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

22 June 2010

Date of mailing of the international search report

02/07/2010

Name and mailing address of the ISA/

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Authorized officer

Behrens, Joyce

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2010/050755

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	& DATABASE Geneseq [Online] 12 June 2008 (2008-06-12), "Rice Expressed Sequence Tag, SEQ ID 39666." XP002588149 retrieved from EBI accession no. GSN:AQF07337 Database accession no. AQF07337 the whole document ----- HSIEH H M ET AL: "NOVEL STRESS-INDUCIBLE METALLOTHIONEIN-LIKE GENE FROM RICE" EXPERIMENTAL HEMATOLOGY, ELSEVIER INC, US, vol. 28, 1 January 1995 (1995-01-01), pages 381-389, XP000921148 ISSN: 0301-472X * abstract figure 1 & DATABASE UniProt [Online] 24 July 2007 (2007-07-24), "RecName: Full=Metallothionein-like protein 1A; AltName: Full=Class I metallothionein-like protein 1A; AltName: Full=OsMT-I-1a; Short=OsMT1a;" XP002588150 retrieved from EBI accession no. UNIPROT:POC5B3 Database accession no. POC5B3 the whole document -----	9
A	WU Y ET AL: "Isolation and expression analysis of salt up-regulated ESTs in upland rice using PCR-based subtractive suppression hybridization method" PLANT SCIENCE, ELSEVIER IRELAND LTD, IE LNKD- DOI:10.1016/J.PLANTSCI.2004.10.020, vol. 168, no. 3, 1 March 2005 (2005-03-01) , pages 847-853, XP025296240 ISSN: 0168-9452 [retrieved on 2005-03-01] * abstract table 1 page 851, column 1 -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2010/050755

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007016974 A1	18-01-2007	US 2008168582 A1	10-07-2008