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(54) MANIPULATION OF AMMONIUM TRANSPORTERS (AMTS) TO IMPROVE NITROGEN USE EFFICIENCY IN HIGHER PLANTS

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(57) ABSTRACT

The present invention provides polynucleotides and related polypeptides of the protein AMT. The invention provides genomic sequence for the AMT gene. AMT is responsible for controlling nitrogen utilization efficiency in plants.

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AtAMT2

<u>acttggtggaataatgacagggttgtttercacacctgatototggtttttggtacttcccctcccagcggacca</u>
g<u>tacttcctcccaggggacaatggogggaataatgacaggttttttgcacocctgatcctcgcgttttg</u>
gtacttcctcccaggggacaatggoggaatattacggtggcaatgttttgcacoccgatcctcgcgttttg

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MANIPULATION OF AMMONUM TRANSPORTERS (AMTS) TO IMPROVE NITROGEN USE EFFICIENCY IN HIGHER PLANTS

CROSS REFERENCE

[0001] This utility application claims the benefit U.S. Provisional Application No. 60/893,901, filed Mar. 9, 2007, which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates generally to the field of molecular biology.

BACKGROUND OF THE INVENTION

0003) Nitrogen (N) is the most abundant inorganic nutrient taken up from the soil by plants for growth and development. Maize roots absorb most of the N from the soil in the form of nitrate, the majority of which is transported to the leaf for reduction and assimilation. Nitrate is reduced to nitrite by nitrate reductase (NR) in the cytosol and then nitrite is transported into chloroplast where it is reduced by nitrite reductase
(NiR) to ammonium. Ammonium is assimilated into glutamine by the glutamine synthase-glutamate synthase system (Crawford and Glass, (1998) Trends in Plant Science 3:389-395.). Also, it has long been known that significant amounts of N are lost from the plant aerial parts by volatil ization (Glyan'ko, et al., (1980) "Effect of autumn frost and forms of nitrogen on translocation of nitrogen compounds to spring wheat grain", Agrokhimiya 8:19-26; Hooker, et al., (1980) "Gaseous N losses from winter wheat", Agronomy Journal 72(5):789-792; Silva, et al., (1981) "Nitrogen vola-
tilization from rice leaves. II. Effects of source of applied nitrogen in nutrient culture solution", Crop Science $21 (6)$: 913–916: Stutte, et al., (1981) "Nitrogen volatilization from rice leaves. I. Effects of genotype and air temperature", Crop
Science 21(4):596-600; Foster, et al., (1986) "Glutamine synthetase activity and foliar nitrogen volatilization in response to temperature and inhibitor chemicals" Annals of Botany 57(3):305-307; Parton, et al., (1988) "Ammonia volatiliza tion from spring wheat plants" Agronomy Journal 80(3):419-425; Kamiji, et al., (1989) "Measurement of ammonium ening period." Japanese Journal of Crop Science 58(1): 140-142; Morgan, et al., (1989) "Characteristics of ammonia vola tilization from spring wheat", Crop Science 29(3):726-731;
O'Deen, (1989) "Wheat volatilized ammonia and resulting nitrogen isotopic fractionation." Agronomy Journal 81(6): 980-985; Guindo, et al., (1994) "Nitrogen loss from rice plants during grain fill and oven drying", Arkansas Farm Research 43(1):12-13; Heckathorn, et al., (1995) "Ammonia volatilization during drought in perennial C4 grasses of tallgrass prairie." Oecologia 101(3):361-365; Cabezas, et al., (1997). "NH3-N volatilization in a maize crop: I Effect of irrigation and partial substitution of urea by ammonium sulphate", Revista Brasileira de Ciencia do Solo 21(3):481-487). Experimental evidence supports the loss of N through ammonium and not through N oxides (Hooker, et al., 1980). Treatment with chemicals that inhibit glutamine or glutamate synthase activities led to increased loss of ammonium through volatilization (Foster, et al., 1986). Loss of N is not only limited to C-3 species as C-4 plants have also been reported to lose N through volatilization (Heckathorn, et al., 1995).

[0004] Manipulation of AMTs can be utilized to improve NUE by causing increased dry matter, thereby contributing to an increase in plant yield. Two of the ways to improved dry matter accumulation are: 1) reduce N loss through volatiliza tion and 2) reduce N content of the plant so that more dry matter can be accumulated in the form of low-energy con stituents, e.g., starch or cellulose.

[0005] For ammonium to be lost from the leaf, it must first pass through a facilitated channel since it is highly hydrophilic. Ammonium transporters (AMTs) were originally discovered as ammonium transporters but some recent studies
have shown that at least in some cases AMTs can act as gas channels (Soupene, et al., (2002) Proc Natl Acad Sci USA 99:3926-3931; Kustu and Inwood, (2006) Transfus Clin Biol 13:103-110). An amtB knock-out mutant of Salmonella grows better on poor N source, apparently because it can sequester more N by keeping it from leaking back out (Soupene, et al., 2002). This application details an invention which is used to manipulate AMTs in higher plants to improve NUE. The inventors identified chloroplast-specific and/or leaf-preferred AMT(s) and knocked them out/down to minimize the loss of ammonium, which resulting in better N assimilation/NUE. In addition, work was not limited only to the chloroplast-localized AMTs but will also down-regula tion of the AMTs that are localized to other organelles/mem branes.

SUMMARY OF THE INVENTION

[0006] The present invention provides polynucleotides, related polypeptides and all conservatively modified variants of the present AMT sequences. The invention provides sequences for the AMT genes. Six Arabidopsis, 7 maize, 17 rice, and 11 soybean AMT genes were identified. Table 1 lists these genes and their seq id numbers.

TABLE 1

SEQUENCE ID NUMBER	IDENTITY
SEQ ID NOS: 1	AtAMT 1 polynucleotide
SEQ ID NOS: 2	AtAMT 1 polypeptide
SEQ ID NO: 3	AtAMT 1;2 polynucleotide
SEQ ID NO: 4	AtAMT 1;2 polypeptide
SEQ ID NO: 5	AtAMT 1;3 polynucleotide
SEQ ID NO: 6	AtAMT 1;3 polypeptide
SEQ ID NO: 7	AtAMT 2 polynucleotide
SEQ ID NO: 8	AtAMT 2 polypeptide
SEO ID NO: 9	AtAMT 3 polynucleotide
SEQ ID NO: 10	AtAMT 3 polypeptide
SEO ID NO: 11	AtAMT 4 polynucleotide
SEQ ID NO: 12	AtAMT 4 polypeptide
SEQ ID NO: 13	ZmAMT 1 polynucleotide
SEO ID NO: 14	ZmAMT 1 polypeptide
SEQ ID NO: 15	ZmAMT 2 polynucleotide
SEO ID NO: 16	ZmAMT 2 polypeptide
SEO ID NO: 17	ZmAMT 3 polynucleotide
SEO ID NO: 18	ZmAMT 3 polypeptide
SEQ ID NO: 19	ZmAMT 4 polynucleotide
SEO ID NO: 20	ZmAMT 4 polypeptide
SEO ID NO: 21	ZmAMT 5 polynucleotide
SEQ ID NO: 22	ZmAMT 5 polypeptide
SEQ ID NO: 23	ZmAMT 6 polynucleotide
SEQ ID NO: 24	ZmAMT 6 polypeptide
SEQ ID NO: 25	ZmAMT 7 polynucleotide
SEQ ID NO: 26	ZmAMT 7 polypeptide
SEQ ID NO: 27	OsAMT 1 polynucleotide

TABLE 1-continued

SEQUENCE ID NUMBER	IDENTITY
SEO ID NO: 28	OsAMT 1 polypeptide
SEQ ID NO: 29	OsAMT 2 polynucleotide
SEQ ID NO: 30	OsAMT 2 polypeptide
SEQ ID NO: 31	OsAMT 3 polynucleotide
SEQ ID NO: 32	OsAMT 3 polypeptide
SEQ ID NO: 33	OsAMT 4 polynucleotide
SEQ ID NO: 34	OsAMT 4 polypeptide
SEQ ID NO: 35	OsAMT 5 polynucleotide
SEQ ID NO: 36	OsAMT 5 polypeptide
SEO ID NO: 37	OsAMT 6 polynucleotide
SEQ ID NO: 38	OsAMT 6 polypeptide
SEQ ID NO: 39	OsAMT 7 polynucleotide
SEQ ID NO: 40	OsAMT 7 polypeptide
SEQ ID NO: 41	OsAMT 8 polynucleotide
SEQ ID NO: 42	OsAMT 8 polypeptide
SEQ ID NO: 43	OsAMT 9 polynucleotide
SEQ ID NO: 44	OsAMT 9 polypeptide
SEQ ID NO: 45	OsAMT 10 polynucleotide
SEQ ID NO: 46	OsAMT 10 polypeptide
SEQ ID NO: 47	OsAMT 11 polynucleotide
SEQ ID NO: 48	OsAMT 11 polypeptide
SEO ID NO: 49	OsAMT 12 polynucleotide
SEQ ID NO: 50	OsAMT 12 polypeptide
SEQ ID NO: 51	OsAMT 13 polynucleotide
SEQ ID NO: 52	OsAMT 13 polypeptide
SEQ ID NO: 53	OsAMT 14 polynucleotide
SEQ ID NO: 54	OsAMT 14 polypeptide
SEQ ID NO: 55	OsAMT 15 polynucleotide
SEQ ID NO: 56	OsAMT 15 polypeptide
SEQ ID NO: 57	OsAMT 16 polynucleotide
SEQ ID NO: 58	OsAMT 16 polypeptide
SEQ ID NO: 59	OsAMT 17 polynucleotide
SEO ID NO: 60	OsAMT 17 polynucleotide
SEQ ID NO: 61	GmAMT 1 polynucleotide
SEO ID NO: 62	GmAMT 1 polypeptide
SEQ ID NO: 63	GmAMT 2 polynucleotide
SEQ ID NO: 64	GmAMT 2 polypeptide
SEQ ID NO: 65	GmAMT 3 polynucleotide
SEO ID NO: 66	GmAMT 3 polypeptide
SEQ ID NO: 67	GmAMT 4 polynucleotide
SEQ ID NO: 68	GmAMT 4 polypeptide
SEO ID NO: 69	GmAMT 5 polynucleotide
SEQ ID NO: 70	GmAMT 5 polypeptide
SEQ ID NO: 71	GmAMT 6 polynucleotide
SEQ ID NO: 72	GmAMT 6 polypeptide
SEQ ID NO: 73	GmAMT 7 polynucleotide
SEQ ID NO: 74	GmAMT 7 polypeptide
SEQ ID NO: 75	GmAMT 8 polynucleotide
SEQ ID NO: 76	GmAMT 8 polypeptide
SEQ ID NO: 77	GmAMT 9 polynucleotide
SEQ ID NO: 78	GmAMT 9 polypeptide
SEQ ID NO: 79	GmAMT 10 polynucleotide
SEQ ID NO: 80	GmAMT 10 polypeptide
SEQ ID NO: 81	GmAMT 11 polynucleotide
SEQ ID NO: 82	GmAMT 11 polypeptide

[0007] Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising an isolated polynucleotide sequence encoding an AMT protein. One embodiment of the invention is an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence comprising SEQID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81; (b) the nucleotide sequence encoding an amino acid sequence comprising SEQ ID NO: 2. 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32,34, 36,38, 40, 42, 44, 46,48, 50, 52,54, 56,58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78. 80 or 82; and (c) the nucleotide sequence com prising at least 70% sequence identity to SEQ ID NO: 1, 3, 5,

7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33,35, 37, 39, 41, 43,45, 47,49,51,53,55, 57, 59, 61, 63, 65, 67, 69, 71,73, 75, 77, 79 or 81, wherein said polynucleotide encodes a polypeptide having AMT transporter activity.

[0008] Compositions of the invention include an isolated polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence comprising SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36,38, 40, 42, 44, 46,48, 50, 52, 54,56, 58, 60, 62, 64, 66, 68, 70, 72, 74,76, 78, 80 or 82; and (b) the amino acid sequence comprising at least 70% sequence iden tity to SEQID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32,34, 36,38, 40,42, 44, 46,48, 50, 52, 54, 56,58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80 or 82, wherein said polypeptide has AMT transporter activity.

[0009] In another aspect, the present invention relates to a recombinant expression cassette comprising a nucleic acid as described. Additionally, the present invention relates to a vector containing the recombinant expression cassette. Fur ther, the vector containing the recombinant expression cas sette can facilitate the transcription and translation of the nucleic acid in a host cell. The present invention also relates to the host cells able to express the polynucleotide of the present invention. A number of host cells could be used, such as but not limited to, microbial, mammalian, plant, or insect.

[0010] In yet another embodiment, the present invention is directed to a transgenic plant or plant cells, containing the nucleic acids of the present invention. Preferred plants con taining the polynucleotides of the present invention include but are not limited to maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, tomato, switchgrass, myscanthus, triticale and millet. In another embodiment, the transgenic plant is a maize plant or plant cells. Another embodiment is the transgenic seeds from the trans genic plant. Another embodiment of the invention includes plants comprising an amt polypeptide of the invention oper ably linked to a promoter that drives expression in the plant. The plants of the invention can have altered AMT as com pared to a control plant. In some plants, the AMT is altered in a vegetative tissue, a reproductive tissue, or a vegetative tissue and a reproductive tissue. Plants of the invention can have at least one of the following phenotypes including but not lim ited to: increased leaf size, increased ear size, increased seed size, increased endosperm size, alterations in the relative size of embryos and endosperms leading to changes in the relative levels of protein, oil, and/or starch in the seeds, absence of tassels, absence of functional pollen bearing tassels, or increased plant size.

[0011] Another embodiment of the invention would be plants that have been genetically modified at a genomic locus, wherein the genomic locus encodes an amt polypeptide of the invention.

[0012] Methods for increasing the activity of an amt polypeptide in a plant are provided. The method can comprise introducing into the plant an amt polynucleotide of the invention. Providing the polypeptide can decrease the number of cells in plant tissue, modulating the tissue growth and size.

[0013] Methods for reducing or eliminating the level of an amt polypeptide in the plant are provided. The level or activity of the polypeptide could also be reduced or eliminated in specific tissues, causing increased AMT in said tissues. Reducing the level and/or activity of the AMT polypeptide increases the number of cells produced in the associated tissue.

[0014] Compositions further include plants and seed having a DNA construct comprising a nucleotide sequence of interest operably linked to a promoter of the current invention. In specific embodiments, the DNA construct is stably integrated into the genome of the plant. The method comprises introducing into a plant a nucleotide sequence of inter est operably linked to a promoter of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0015] FIG. 1: Phylogentic tree of AMTs from Arabidopsis, rice, soybean and maize

[0016] Phylogenetic analyses of all the AMTs from $Arabi$ dopsis, rice, maize and soybean are shown in FIG. 1. The length of the line at the base of the figure represents an equivalent of 10 amino acid differences and could be used to approximate the amino acid differences between different ammonium transporter proteins from the individual branch lengths.

[0017] FIG. 2: Expression analysis of ZM-AMTs

[0018] In order to identify leaf specific/preferred/expressed AMT(s) in maize, Lynx MPSS expression analyses in -300 libraries reveal that ZmAMT1 (SEQ ID NO: 14), 2, 7 are expressed both in roots and leaves whereas ZmAMT4 (SEQ ID NO: 20) is a root preferred AMT. ZmAMT6 (SEQ ID NO: 24) expresses at very low level in comparison to other Zm AMTs. In case of ZmAMT5 there was no specific Lynx tag available.

[0019] FIG. 3: Characterization of atamt1:2 T-DNA knock-Out mutant

[0020] In cTP prediction analyses, AtAMT1;2 (SEQ ID NO: 4) posses a putative cTP. For functional analyses of AtAMT1:2 (SEQ ID NO: 4) and to determine it's role in N-assimilation, analyses identified a T-DNA mutant line (SM_3.15680) from the Arabidopsis T-DNA mutant data base. In this mutant line T-DNA was inserted in c-terminal of AtAMT1;2 (SEQ ID NO: 4) gene (FIG. 4A). Genomic PCRs using AtAMT1;2 (SEQ ID NO: 4) gene and T-DNA specific primers show that T-DNA is indeed inserted in the AtAMT1;2
(SEQ ID NO: 4) (FIG. 4B). AtAMT1;2 (SEQ ID NO: 4) gene specific primers flanking the T-DNA insert couldn't amplify any DNA region in mutant plants where as an expected PCR product was detected in wild type plant (FIG. 4B, upper panel). Similarly, genomic PCR with AtAMT1:2 (SEQ ID NO: 4) specific forward primer and T-DNA specific reverse primers amplify an expected product in mutant lines and nothing in wild type plants as expected (FIG. 4B, lower panel). Saturated RT-PCRs (35 cycles) analyses couldn't detect a full length atamt1:2 mRNA in mutant (FIG. 4C, upper panel) suggesting that AtAMT1;2 (SEQ ID NO: 4) is completely knocked out in this T-DNA mutant. Actin control RT-PCR worked fine in both mutant and wild type plants (FIG. 4C, lower panel).

[0021] FIG. 4: Knock-out of multiple AMTs in Arabidopsis by single RNAi vector

[0022] Six AMT genes are present in $Arabidopsis$ genome. Hence, it is very likely that due to functional redundancy one might need to manipulate the expression of multiple AMTs simultaneously. Analyses of the DNA sequence of all these AMTs was performed which identified the high homology regions among them. There is a stretch of \sim 200 bp among AtAMT1:2 (SEQ ID NO: 4), AtAMT1 (SEQ ID NO: 2), AMT1;3 (SEQ ID NO: 6), At3g24290 (SEQ ID NO: 10) and At4g28700 (SEQ ID NO: 12) where as AMT2 (SEQ ID NO: 8) stood independent. Amplification of these regions was

accomplished (bold and underlined in FIG. 4) by PCR from AtAMT1;2 (SEQ ID NO: 4) and AtAMT2 (SEQ ID NO: 8) and a multi-way ligation was performed to make an inverted repeat using ADH-intron as a spacer. The RNAi cassette of these hybrid inverted repeats is driven by constitutive or root specific or leaf specific promoter.

[0023] FIG. 5: Knock-out/down of multiple AMTs in Maize by single RNAi vector
[0024] Detailed analyses of all 7 maize AMTs were per-

formed to identify the DNA regions showing high homology among different ZmAMTs. This analysis reveals that ZmAMT1 (SEQ ID NO: 14) and ZmAMT5 (SEQ ID NO: 22), ZmAMT3 (SEQ ID NO: 18) and ZmAMT4 (SEQ ID NO: 20) and ZmAMT2 (SEQ ID NO: 16), ZmAMT6 (SEQ ID NO: 24) and ZmAMT7 (SEQ ID NO: 26) form three separate groups and there is a very high homology in stretches of DNA sequences with in each group. Three DNA fragments (bold and underlined in FIG. 5) from ZmAMT 1, 4 and 7 (SEQ ID NOS: 14, 20 and 26) representing each of the dif ferent groups were amplified by PCR. Multi-way ligations were performed to make inverted repeats with hybrid of these 3 fragments and ADH intron as a spacer to facilitate the formation of stem-loop structure. This RNAi cassette of 'ZmAMT1 (SEQ ID NO: 14):ZmAMT4 (SEQ ID NO: 20): ZmAMT7 (SEQ ID NO: 26)' inverted repeats was driven by a constitutive (Zm-UBI promoter) or leaf-specific promoter. MOPAT driven by Zm-UBI promoter was used as herbicide resistance marker for selected. In addition to that RFP driven by a pericarp specific promoter LTP2 was also used to sort out the transgenic seeds (red) from there segregating non-trans genic seeds.

DETAILED DESCRIPTION OF THE INVENTION

[0025] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the tech niques employed or contemplated herein are standard meth odologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting. The following is presented by way of illustration and is not intended to limit the scope of the invention.

[0026] The present inventions now will be described more fully hereinafter with reference to the accompanying draw ings, in which some, but not all embodiments of the invention are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodi ments are provided so that this disclosure will satisfy appli cable legal requirements. Like numbers refer to like elements throughout.

0027 Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

[0028] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of botany, microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Langenheim and Thimann, BOTANY: PLANT BIOLOGY AND ITS RELA TION TO HUMAN AFFAIRS, John Wiley (1982); CELL CULTURE AND SOMATIC CELL GENETICS OF PLANTS, vol. 1, Vasil, ed. (1984); Stanier, et al., THE MICROBIAL WORLD, $5th$ ed., Prentice-Hall (1986); Dhringra and Sinclair, BASIC PLANT PATHOLOGY METH ODS, CRC Press (1985); Maniatis, et al., MOLECULAR CLONING: A LABORATORY MANUAL (1982); DNA CLONING, vols. I and II, Glover, ed. (1985): OLIGO NUCLEOTIDE SYNTHESIS, Gait, ed. (1984); NUCLEIC ACID HYBRIDIZATION, Hames and Higgins, eds. (1984): and the series METHODS IN ENZYMOLOGY, Colowick and Kaplan, eds, Academic Press, Inc., San Diego, Calif.

[0029] Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orien tation, respectively. Numeric ranges are inclusive of the num bers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The terms defined below are more fully defined by reference to the specification as a whole.

[0030] In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

[0031] By "microbe" is meant any microorganism (including both eukaryotic and prokaryotic microorganisms), such as fungi, yeast, bacteria, actinomycetes, algae and protozoa, as well as other unicellular structures.

0032. By "amplified' is meant the construction of multiple copies of a nucleic acid sequence or multiple copies comple mentary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., DIAGNOSTIC MOLECULAR MICROBIOL-OGY: PRINCIPLES AND APPLICATIONS, Persing, et al., eds., American Society for Microbiology, Washington, D.C. (1993). The product of amplification is termed an amplicon. [0033] The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids that encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methion ine; one exception is Micrococcus rubens, for which GTG is the methionine codon (Ishizuka, et al., (1993) J. Gen. Microbiol. 139:425-32) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a invention, is implicit in each described polypeptide sequence and incorporated herein by reference.

[0034] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percent age of amino acids in the encoded sequence is a "conserva tively modified variant' when the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1,2,3,4, 5, 7 or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is gen erally at least 30%, 40%, 50%, 60%, 70%, 80% or 90%, preferably 60-90% of the native protein for it's native sub strate. Conservative substitution tables providing function ally similar amino acids are well known in the art.

[0035] The following six groups each contain amino acids that are conservative substitutions for one another:

[0036] 1) Alanine (A), Serine (S), Threonine (T);
[0037] 2) Aspartic acid (D), Glutamic acid (E);

[0037] 2) Aspartic acid (D), Glutamic acid (E);
[0038] 3) Asparagine (N), Glutamine (O);

[0038] 3) Asparagine (N), Glutamine (Q);
[0039] 4) Arginine (R), Lysine (K);

4) Arginine (R), Lysine (K);

[0040] 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

0041 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton, PROTEINS, W.H. Freeman and Co. (1984).

 (1984) .
 (0042) As used herein, "consisting essentially of" means the inclusion of additional sequences to an object polynucle-
otide where the additional sequences do not selectively hybridize, under stringent hybridization conditions, to the same cDNA as the polynucleotide and where the hybridiza tion conditions include a wash step in $0.1 \times SSC$ and 0.1% sodium dodecyl sulfate at 65° C.

 $[0043]$ By "encoding" or "encoded," with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack Such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal' genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium Mycoplasma capricolum (Yamao, et al., (1985) Proc. Natl. Acad. Sci. USA 82:2306-9), or the ciliate Macro nucleus, may be used when the nucleic acid is expressed using these organisms.
[0044] When the nucleic acid is prepared or altered syn-

thetically, advantage can be taken of known codon prefer-

ences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of donous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledonous plants or dicotyledonous plants as these preferences have been shown to differ (Murray, et al., (1989) Nucleic Acids Res. 17:477-98 and herein incorporated by reference). Thus, the maize pre ferred codon for a particular amino acid might be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray, et al., Supra.

[0045] As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived
or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

[0046] By "host cell" is meant a cell, which comprises a heterologous nucleic acid sequence of the invention, which contains a vector and Supports the replication and/or expres sion of the expression vector. Host cells may be prokaryotic cells such as E. coli, or eukaryotic cells such as yeast, insect, plant, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells, includ ing but not limited to maize, sorghum, sunflower, soybean, wheat, alfalfa, rice, cotton, canola, barley, millet, switchgrass, myscanthus, triticale, and tomato. A particularly preferred monocotyledonous host cell is a maize host cell.

[0047] The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two singlestranded nucleic acid sequences selectively hybridized with each other.

[0048] The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection' or "transforma tion" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochon drial DNA), converted into an autonomous replicon, or tran siently expressed (e.g., transfected mRNA).

[0049] The terms "isolated" refers to material, such as a nucleic acid or a protein, which is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment. Nucleic acids, which are "isolated", as defined herein, are also referred to as "heterologous" nucleic acids. Unless otherwise stated, the term "AMT nucleic acid" means a nucleic acid comprising a polynucleotide (AMT polynucleotide') encoding a full length or partial length AMT polypeptide.

[0050] As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise lim ited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single

stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

[0051] By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules, which comprise and sub stantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, GUIDE TOMOLECULAR CLONING TECHNIQUES, from the series METHODS IN ENZYMOL OGY. vol. 152, Academic Press, Inc., San Diego, Calif. (1987); Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2^{nd} ed., vols. 1-3 (1989); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, et al., eds. Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994 Supplement).

[0052] As used herein "operably linked" includes reference to a functional linkage between a first sequence, such as a promoter, and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

[0053] As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds, suspension cul tures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants, which can be used in the methods of the invention, is generally as broad as the class of higher plants amenable to transformation techniques, includ ing both monocotyledonous and dicotyledonous plants including species from the genera: Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Dau cus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Cap sicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciahorium, Helian thus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocal lis, Nemesis, Pelargonium, Pamieum, Pennisetum, Ranuncu lus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Allium, and Triticum. A particularly preferred plant is Zea mays.

[0054] As used herein, "yield" may include reference to bushels per acre of a grain crop at harvest, as adjusted for grain moisture (15% typically for maize, for example). Grain moisture is measured in the grain at harvest. The adjusted test weight of grain is determined to be the weight in pounds per bushel, adjusted for grain moisture level at harvest.

[0055] As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural ribonucle otide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide (s). A polynucleotide can be full-length or a Subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAS or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAS or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucle otides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including inter alia, simple and complex cells.

[0056] The terms "polypeptide," "peptide," and "protein" are used interchangeably hereinto refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

[0057] As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells such *Agrobac*terium or Rhizobium. Examples are promoters that preferen tially initiate transcription in certain tissues, such as leaves, roots, seeds, fibres, xylem vessels, tracheids, or sclerenchyma. Such promoters are referred to as "tissue preferred." A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible' or "regulatable' pro moter is a promoter, which is under environmental control. Examples of environmental conditions that may effect tran scription by inducible promoters include anaerobic conditions or the presence of light. Another type of promoter is a developmentally regulated promoter, for example, a promoter that drives expression during pollen development. Tissue pre ferred, cell type specific, developmentally regulated, and inducible promoters constitute the class of "non-constitutive' promoters. A "constitutive' promoter is a promoter, which is active under most environmental conditions.

[0058] The term "AMT polypeptide" refers to one or more amino acid sequences. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof. A "AMT protein" comprises an amt polypeptide. Unless otherwise stated, the term "AMT nucleic acid" means a nucleic acid comprising a polynucleotide ("AMT polynucleotide") encoding an amt polypeptide. [0059] As used herein "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention; or may have reduced or eliminated expression of a native gene. The term "recombinant' as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation,

natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

[0060] As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or Syn thetically, with a series of specified nucleic acid elements, which permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

[0061] The term "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein'). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

[0062] The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization condi tions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 40% sequence identity, prefer ably 60-90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

[0063] The terms "stringent conditions" or "stringent" hybridization conditions' include reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-
dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which can be up to 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Optimally, the probe is approximately 500 nucleotides in length, but can vary greatly in length from less than 500 nucleotides to equal to the entire length of the target sequence. [0064] Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucle otides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide or Den hardt's. Exemplary low stringency conditions include hybrid ization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1x to 2xSSC (20xSSC=3.0M NaC1/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency con ditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37 \degree C., and a wash in 0.5 \times to 1 \times SSC at 55 to 60° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in $0.1 \times$ SSC at 60 to 65 \degree C. Specificity is typically the function of post-hybridization washes, the criti

cal factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, (1984) Anal. Biochem., 138:267-84: T_m =81.5° C.+16.6 (log M)+0.41 (% GC)-0.61 (% form)-500/L: where M is the molarity of monovalent cations, 96 GC is the percentage of guanosine and cytosine nucleotides in the DNA,% form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1° C. for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/ or wash at 1, 2, 3 or 4°C. lower than the thermal melting point (T_m) ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9 or 10° C. lower than the thermal melting point (T_m) ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15 or 20° C. lower than the thermal melting point (T_m) . Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the strin gency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45° C. (aqueous solution) or 32° C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, LABO RATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY-HYBRIDIZATION WITH NUCLEIC ACID PROBES, part 1, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays." Elsevier, N.Y. (1993); and CURRENT PRO TOCOLS IN MOLECULAR BIOLOGY, chapter 2, Ausubel, et al., eds, Greene Publishing and Wiley-Interscience, New tion high stringency is defined as hybridization in $4 \times SSC$, 5xDenhardt's (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin in 500 ml of water), 0.1 mg/ml boiled salmon sperm DNA, and 25 mM Na phosphate at 65° C., and a wash in $0.1 \times$ SSC, 0.1% SDS at 65° C.

[0065] As used herein, "transgenic plant" includes reference to a plant, which comprises within its genome a heter ologous polynucleotide. Generally, the heterologous poly nucleotide is stably integrated within the genome Such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic' is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertili zation, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

[0066] As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

[0067] The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides or polypeptides: (a) "reference sequence." (b) "comparison window," (c) "sequence identity," (d) "percentage of sequence identity," and (e) "substantial identity." [0068] As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A refer ence sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

[0069] As used herein, "comparison window" means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100 or longer. Those of skill in the art under stand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is Subtracted from the number of matches.

[0070] Methods of alignment of nucleotide and amino acid sequences for comparison are well known in the art. The local homology algorithm (BESTFIT) of Smith and Waterman, (1981) Adv. Appl. Math 2:482, may conduct optimal align ment of sequences for comparison; by the homology align ment algorithm (GAP) of Needleman and Wunsch, (1970) J. Mol. Biol. 48:443-53; by the search for similarity method (Tfasta and Fasta) of Pearson and Lipman, (1988) Proc. Natl. Acad. Sci. USA 85:2444; by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif., GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG® programs (Accelrys, Inc., San Diego, Calif.).). The CLUSTAL program is well described by Higgins and Sharp, (1988) Gene 73:237-44: Higgins and Sharp, (1989) CABIOS 5:151-3: Corpet, et al., (1988) Nucleic Acids Res. 16:10881-90; Huang, et al., (1992) Computer Applications in the Biosciences 8:155-65, and Pearson, et al., (1994) Meth. Mol. Biol. 24:307-31. The preferred program to use for optimal global alignment of multiple sequences is PileUp (Feng and Doolittle, (1987) J. Mol. *Evol.*, 25:351-60 which is similar to the method described by Higgins and Sharp, (1989) CABIOS 5:151-53 and hereby incorporated by reference). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide

database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, CUR RENT PROTOCOLS IN MOLECULAR BIOLOGY, Chap ter 19, Ausubel, et al., eds. Greene Publishing and Wiley Interscience, New York (1995).

[0071] GAP uses the algorithm of Needleman and Wunsch, supra, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap posi tions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than Zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package are 8 and 2. respectively. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 100. Thus, for example, the gap creation and gap extension penalties can be 0, 1,2,3,4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or greater.

[0072] GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the num ber of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Pack age is BLOSUM62 (see, Henikoff and Henikoff, (1989) Proc. Natl. Acad. Sci. USA 89:10915).

[0073] Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters (Alts chul, et al., (1997) Nucleic Acids Res. 25:3389-402).

[0074] As those of ordinary skill in the art will understand, BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences, which may be homopoly meric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-com plexity filter programs can be employed to reduce such lowcomplexity alignments. For example, the SEG (Wooten and Federhen, (1993) Comput. Chem. 17:149-63) and XNU (Cla verie and States, (1993) Comput. Chem. 17:191-201) low complexity filters can be employed alone or in combination. [0075] As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences, which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recog nized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino

acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydropho bicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative sub stitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substi tution. Sequences, which differ by such conservative substi tutions, are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of Zero, a conservative substitution is given a score between zero and 1.
The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, (1988) Computer Applic. Biol. Sci. 4:11-17, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif., USA).

[0076] As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not com prise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0077] The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has between 50-100% sequence identity, preferably at least 50% sequence identity, preferably at least 60% sequence identity, preferably at least 70%, more preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters.
One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame posi tioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence iden tity of between 55-100%, preferably at least 55%, preferably at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

[0078] Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. The degeneracy of the genetic code allows for many amino acids substitutions that lead to variety in the nucleotide sequence that code for the same amino acid, hence it is possible that the DNA sequence could code for the same polypeptide but not hybridize to each other under stringent conditions. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide, which the first nucleic acid encodes, is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

[0079] The terms "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with between 55-100% sequence identity to a reference sequence preferably at least $55%$ sequence identity, preferably 60% preferably 70%, more preferably 80%, most preferably at least 90% or 95% sequence identity to the reference sequence
over a specified comparison window. Preferably, optimal a lignment is conducted using the homology alignment algorithm of Needleman and Wunsch, supra. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. In addi tion, a peptide can be substantially identical to a second peptide when they differ by a non-conservative change if the epitope that the antibody recognizes is substantially identical. Peptides, which are "substantially similar" share sequences as, noted above except that residue positions, which are not identical, may differ by conservative amino acid changes.

[0080] The invention discloses AMT polynucleotides and polypeptides. The novel nucleotides and proteins of the invention have an expression pattern which indicates that they regulate ammonium transport and thus play an important role in plant development. The polynucleotides are expressed in various plant tissues. The polynucleotides and polypeptides thus provide an opportunity to manipulate plant development to alter seed and vegetative tissue development, timing or composition. This may be used to create a plant with altered N composition in source and sink.

Nucleic Acids

[0081] The present invention provides, inter alia, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising an amt polynucleotide.
[0082] The present invention also includes polynucleotides

optimized for expression in different organisms. For example, for expression of the polynucleotide in a maize plant, the sequence can be altered to account for specific codon preferences and to alter GC content as according to Murray, et al., Supra. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray et al., supra.

I0083) The AMT nucleic acids of the present invention comprise isolated AMT polynucleotides which are inclusive of:

- [0084] (a) a polynucleotide encoding an AMT polypeptide and conservatively modified and polymorphic variants thereof;
- I0085 (b) a polynucleotide having at least 70% sequence identity with polynucleotides of (a) or (b):
- [0086] (c) complementary sequences of polynucleotides of (a) or (b) .

Construction of Nucleic Acids

[0087] The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified, or otherwise constructed from a fungus or bacteria.

[0088] The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a con Venient means to purify the proteins of the present invention. The nucleic acid of the present invention-excluding the polynucleotide sequence—is optionally a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression sequences to optimize their func tion in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning Vectors, expression vectors, adapters, and linkers is well known in the art. Exemplary nucleic acids include such vec tors as: M13, lambda ZAP Express, lambda ZAP II, lambda gt10, lambda gt11, pBK-CMV, pBK-RSV, pBluescript II, pWE15, SuperCos 1, SurfZap, Uni-ZAP, pBC, pBS+/-, pSG5, pBK, pCR-Script, pET, pSPUTK, p3'SS, pGEM, pSK+/-, pGEX, pSPORTI and II, pOPRSVI CAT, pOPI3
CAT, pXT1, pSG5, pPbac, pMbac, pMC1neo, pOG44, pOG45, pFRTßGAL, pNEOßGAL, pRS403, pRS404, pRS405, prS406, pRS413, pRS414, pRS415, pRS416, lambda MOSSlox, and lambda MOSElox. Optional vectors for the present invention, include but are not limited to, lambda ZAP II, and pGEX. For a description of various nucleic acids see, e.g., Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, Calif); and, Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, Ill.).

Synthetic Methods for Constructing Nucleic Acids

[0089] The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang, et al., (1979) Meth. Enzymol. 68:90-9; the phosphodiester method of Brown, et al., (1979) Meth. Enzymol. 68:109-51; the dieth ylphosphoramidite method of Beaucage, et al., (1981) Tetra. Letts. 22(20):1859-62; the solid phase phosphoramidite triester method described by Beaucage, et al., supra, e.g., using an automated synthesizer, e.g., as described in Needham VanDevanter, et al., (1984) *Nucleic Acids Res.* 12:6159-68; and, the solid support method of U.S. Pat. No. 4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

UTRs and Codon Preference

[0090] In general, translational efficiency has been found to be regulated by specific sequence elements in the 5' non coding or untranslated region (5' UTR) of the RNA. Positive sequence motifs include translational initiation consensus sequences (Kozak, (1987) Nucleic Acids Res. 15:8125) and the 5<G> 7 methyl GpppG RNA cap structure (Drummond, et al., (1985) Nucleic Acids Res. 13:7375). Negative elements include stable intramolecular 5' UTR stem-loop structures (Muesing, et al., (1987) Cell 48:691) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5' UTR (Kozak, supra, Rao, et al., (1988) Mol. and Cell. Biol. 8:284). Accordingly, the present invention provides 5' and/or 3' UTR regions for modulation of translation of heter ologous coding sequences.

[0091] Further, the polypeptide-encoding segments of the polynucleotides of the present invention can be modified to alter codon usage. Altered codon usage can be employed to alter translational efficiency and/or to optimize the coding sequence for expression in a desired host or to optimize the codon usage in a heterologous sequence for expression in maize. Codon usage in the coding regions of the polynucle-
otides of the present invention can be analyzed statistically using commercially available software packages such as "Codon Preference' available from the University of Wiscon sin Genetics Computer Group. See, Devereaux, et al., (1984) Nucleic Acids Res. 12:387-395; or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.). Thus, the present invention provides a codon usage frequency characteristic of the coding region of at least one of the polynucleotides of the present invention. The number of polynucleotides (3 nucleotides per amino acid) that can be used to determine a codon usage frequency can be any integer from 3 to the number of polynucleotides of the present invention as provided herein. Optionally, the polynucleotides will be full-length sequences. An exemplary number of sequences for statistical analysis can be at least 1, 5, 10, 20, 50 or 100.

Sequence Shuffling

[0092] The present invention provides methods for sequence shuffling using polynucleotides of the present invention, and compositions resulting therefrom. Sequence shuffling is described in PCT publication No. 96/19256. See also, Zhang, et al., (1997) Proc. Natl. Acad. Sci. USA 94:4504-9; and Zhao, et al., (1998) Nature Biotech 16:258 61. Generally, sequence shuffling provides a means for gen erating libraries of polynucleotides having a desired charac teristic, which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides, which comprise sequence regions, which have substantial sequence identity and can be homologously recombined in vitro or in vivo. The population of sequence-recombined polynucleotides com prises a Subpopulation of polynucleotides which possess desired or advantageous characteristics and which can be selected by a suitable selection or screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be an altered K_m and/or K_{cat} over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled wild-type polynucle otide. In yet other embodiments, a protein or polynucleotide optimum as compared to the non-shuffled wild-type polynucleotide. The increase in Such properties can be at least 110%, 120%, 130%, 140% or greater than 150% of the wildtype value.

Recombinant Expression Cassettes

[0093] The present invention further provides recombinant expression cassettes comprising a nucleic acid of the present invention. A nucleic acid sequence coding for the desired polynucleotide of the present invention, for example a cDNA or a genomic sequence encoding a polypeptide long enough to code for an active protein of the present invention, can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the tran scription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

[0094] For example, plant expression vectors may include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring
inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

[0095] A plant promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive' promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the 1'- or 2'-promoter derived from T-DNA of Agrobacterium tumefaciens, the Smas promoter, the cin namyl alcohol dehydrogenase promoter (U.S. Pat. No. 5,683, 439), the Nos promoter, the rubisco promoter, the GRP1-8 promoter, the 35S promoter from cauliflower mosaic virus (CaMV), as described in Odell, et al., (1985) Nature 313:810 2: rice actin (McElroy, et al., (1990) Plant Cell 163-171): ubiquitin (Christensen, et al., (1992) Plant Mol. Biol. 12:619 632 and Christensen, et al., (1992) Plant Mol. Biol. 18:675 89); PEMU (Last, et al., (1991) Theor. Appl. Genet. 81:581 8); MAS (Velten, et al., (1984) *EMBO J.* 3:2723-30); and maize H3 histone (Lepetit, et al., (1992) Mol. Gen. Genet. 23 1:276-85; and Atanassvoa, et al., (1992) Plant Journal 2(3):291-300); ALS promoter, as described in PCT Applica tion Number WO 96/30530; and other transcription initiation regions from various plant genes known to those of skill. For the present invention ubiquitin is the preferred promoter for expression in monocot plants.

[0096] Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here effect transcription by inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. Examples of inducible promoters are the Adh1 promoter, which is inducible by hypoxia or cold stress, the Hsp70 pro moter, which is inducible by heat stress, and the PPDK pro moter, which is inducible by light.

[0097] Examples of promoters under developmental control include promoters that initiate transcription only, or pref erentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

[0098] If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from a variety of plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene. Examples of such regulatory elements include, but are not limited to, 3' termination and/or polyadenylation regions such as those of the Agrobacterium tumefaciens nopaline synthase (nos) gene (Bevan, et al., (1983) Nucleic Acids Res. 12:369-85); the potato proteinase inhibitor II (PINII) gene (Keil, et al., (1986) Nucleic Acids Res. 14:5641-50; and An, et al., (1989) Plant Cell 1:115-22); and the CaMV 19S gene (Mogen, et al., (1990) Plant Cell 2:1261-72).

[0099] An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold (Buchman and Berg, (1988) Mol. Cell Biol. 8:4395-4405; Callis, et al., (1987) Genes Dev. 1:1 183-200). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1 intron are known in the art. See generally, THE MAIZE HANDBOOK, Chapter 116, Freeling and Walbot, eds., Springer, N.Y. (1994).

[0100] Plant signal sequences, including, but not limited to, signal-peptide encoding DNA/RNA sequences which target proteins to the extracellular matrix of the plant cell (Dratewka-Kos, et al., (1989) J. Biol. Chem. 264:4896-900), such as the Nicotiana plumbaginifolia extension gene (De-Loose, et al., (1991) Gene 99.95-100); signal peptides which target proteins to the vacuole. Such as the Sweet potato sporamin gene (Matsuka, et al., (1991) Proc. Natl. Acad. Sci. USA 88:834) and the barley lectin gene (Wilkins, et al., (1990) Plant Cell, 2:301-13); signal peptides which cause proteins to be secreted, such as that of PRIb (Lind, et al., (1992) *Plant Mol. Biol.* 18:47-53) or the barley alpha amylase (BAA) (Rahmatullah, et al., (1989) *Plant Mol. Biol.* 12:119, and hereby incorporated by reference), or signal peptides which target proteins to the plastids such as that of rapeseed enoyl-Acp reductase (Verwaert, et al., (1994) Plant Mol. Biol. 26:189-202) are useful in the invention. The barley alpha amylase signal sequence fused to the AMT polynucleotide is the preferred construct for expression in maize for the present invention.

[0101] The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene, which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibi

otic resistance, with Suitable genes including genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotrans ferase (NPTII) gene encoding kanamycin or geneticin resis tance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resis tance to herbicides which act to inhibit the action of aceto lactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene con taining mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The bar gene encodes resistance to the herbicide basta, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

[0102] Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacte*rium tumefaciens described by Rogers, et al., (1987) Meth. Enzymol. 153:253-77. These vectors are plant integrating vectors in that on transformation, the vectors integrate a por tion of vector DNA into the genome of the host plant. Exem plary A. tumefaciens vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl, et al., (1987) Gene 61:1- 11, and Berger, et al., (1989) Proc. Natl. Acad. Sci. USA, 86:8402-6. Another useful vector herein is plasmid pBI101.2 that is available from CLONTECH Laboratories, Inc. (Palo Alto, Calif.).

Expression of Proteins in Host Cells

[0103] Using the nucleic acids of the present invention, one may express a protein of the present invention in a recombi nantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

[0104] It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various meth ods known for the expression of proteins in prokaryotes or eukaryotes will be made.

[0105] In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typi cally be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or induc ible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expres sion of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desir able to construct expression vectors which contain, at the minimum, a strong promoter, Such as ubiquitin, to direct transcription, a ribosome binding site for translational initia tion, and a transcription/translation terminator. Constitutive promoters are classified as providing for a range of constitutive expression. Thus, some are weak constitutive promoters, and others are strong constitutive promoters. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended at levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Con versely, a "strong promoter' drives expression of a coding sequence at a "high level," or about $1/10$ transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

[0106] One of skill would recognize that modifications could be made to a protein of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifi cations are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Expression in Prokaryotes

[0107] Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription ini tiation, optionally with an operator, along with ribosome binding site sequences, include Such commonly used promot ers as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al., (1977) Nature 198:1056), the tryptophan (trp) promoter system (Goeddel, et al., (1980) Nucleic Acids Res. 8:4057) and the lambda derived PL pro moter and N-gene ribosome binding site (Shimatake, et al., (1981) *Nature* 292:128). The inclusion of selection markers in DNA vectors transfected in E . *coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

[0108] The vector is selected to allow introduction of the gene of interest into the appropriate host cell. Bacterial vec tors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. Ifa plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using Bacillus sp. and Salmonella (Palva, et al., (1983) Gene 22:229-35; Mosbach, et al., (1983) Nature 302:543-5). The pGEX-4T-1 plasmid vector from Pharmacia is the preferred E. coli expression vector for the present invention.

Expression in Eukaryotes

0109) A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, the present invention can be expressed in these eukaryotic sys cells, as discussed infra, are employed as expression systems for production of the proteins of the instant invention.

[0110] Synthesis of heterologous proteins in yeast is well known. Sherman, et al., METHODS INYEAST GENETICS, Cold Spring Harbor Laboratory (1982) is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are Saccharomyces cerevisiae and Pichia pastoris. Vectors, strains, and protocols for expression in Saccharomyces and Pichia are known in the art and available from commercial Suppliers (e.g., Invitrogen). Suitable vec tors usually have expression control sequences, such as pro moters, including 3-phosphoglycerate kinase or alcohol oxi dase, and an origin of replication, termination sequences and the like as desired.

[0111] A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates or the pellets. The monitoring of the purification process can be accomplished by using Western blot techniques or radioim munoassay of other standard immunoassay techniques.

[0112] The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been devel oped in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV th promoter or pgk (phosphoglycerate kinase) promoter), an enhancer (Queen, et al., (1986) *Immunol. Rev.* 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of pro teins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas ($7th$ ed., 1992).

[0113] Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth, and *Drosophila* cell lines such as a Schneider cell line (see, e.g., Schneider, (1987) *J*. Embryol. Exp. Morphol. 27:353-65).

 $[0114]$ As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, et al., (1983) J. Virol. 45:773-81). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors (Saveria-Campo, "Bovine Pap illoma Virus DNA a Eukaryotic Cloning Vector," in DNA CLONING: A PRACTICAL APPROACH, vol. II, Glover, ed., IRL Press, Arlington, Va., pp. 213-38 (1985)).
[0115] In addition, the gene for AMT placed in the appro-

priate plant expression vector can be used to transform plant cells. The polypeptide can then be isolated from plant callus or the transformed cells can be used to regenerate transgenic plants. Such transgenic plants can be harvested, and the appropriate tissues (seed or leaves, for example) can be subjected to large scale protein extraction and purification tech niques.

Plant Transformation Methods

[0116] Numerous methods for introducing foreign genes into plants are known and can be used to insert an amt poly nucleotide into a plant host, including biological and physical plant transformation protocols. See, e.g., Miki, et al., "Proce dure for Introducing Foreign DNA into Plants." in METH ODS IN PLANT MOLECULAR BIOLOGY AND BIO TECHNOLOGY. Glick and Thompson, eds., CRC Press, with the host plant, and include chemical transfection methods such as calcium phosphate, microorganism-mediated gene transfer such as Agrobacterium (Horsch, et al., (1985) Science 227:1229-31), electroporation, micro-injection, and biolistic bombardment.

[0117] Expression cassettes and vectors and in vitro culture methods for plant cell or tissue transformation and regenera tion of plants are known and available. See, e.g., Gruber, et al., "Vectors for Plant Transformation," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOL OGY, supra, pp. 89-119.

[0118] The isolated polynucleotides or polypeptides may be introduced into the plant by one or more techniques typi cally used for direct delivery into cells. Such protocols may vary depending on the type of organism, cell, plant or plant cell, i.e. monocot or dicot, targeted for gene modification. Suitable methods of transforming plant cells include micro injection (Crossway, et al., (1986) Biotechniques 4:320-334: and U.S. Pat. No. 6,300,543), electroporation (Riggs, et al., (1986) Proc. Natl. Acad. Sci. USA 83:5602-5606, direct gene transfer (Paszkowski, et al., (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford, et al., U.S. Pat. No. 4,945,050: WO91/10725; and McCabe, et al., (1988) Biotechnology 6:923-926). Also see, Tomes, et al., Direct DNA Transfer into Intact Plant Cells Via Microprojec tile Bombardment. pp. 197-213 in Plant Cell, Tissue and Organ Culture, Fundamental Methods. eds. O. L. Gamborg & G. C. Phillips. Springer-Verlag Berlin Heidelberg N.Y., 1995: U.S. Pat. No. 5,736,369 (meristem); Weissinger, et al., (1988) Ann. Rev. Genet. 22:421-477; Sanford, et al., (1987) Particu late Science and Technology 5:27-37 (onion); Christou, et al., (1988) Plant Physiol. 87:671-674 (soybean); Datta, et al., (1990) Biotechnology 8:736-740 (rice); Klein, et al., (1988) Proc. Natl. Acad. Sci. USA 85:4305-4309 (maize): Klein, et al., (1988) Biotechnology 6:559-563 (maize): WO 91/10725 (maize): Klein, et al., (1988) Plant Physiol. 91:440-444 (maize); Fromm, et al., (1990) Biotechnology 8:833-839; and
Gordon-Kamm, et al., (1990) Plant Cell 2:603-618 (maize): Hooydaas-Van Slogteren and Hooykaas (1984) Nature (London) 311:763-764: Bytebier, et al., (1987) Proc. Natl. Acad. Sci. USA 84:5345-5349 (Liliaceae); De Wet, et al., (1985) In The Experimental Manipulation of Ovule Tissues, ed. G. P. Chapman, et al., pp. 197-209 Longman, N.Y. (pollen); Kaep pler, et al., (1990) Plant Cell Reports 9:415-418; and Kaep pler, et al., (1992) Theor: Appl. Genet. 84:560-566 (whisker mediated transformation): U.S. Pat. No. 5,693,512 (sonication); D'Halluin, et al., (1992) Plant Cell 4:1495-1505 (electroporation); Li, et al., (1993) Plant Cell Reports 12:250 255; and Christou and Ford (1995) Annals of Botany 75:407 413 (rice); Osjoda, et al., (1996) Nature Biotech. 14:745-750: Agrobacterium mediated maize transformation (U.S. Pat. No. 5,981,840); silicon carbide whisker methods (Frame, et al., (1994) Plant J. 6:941-948); laser methods (Guo, et al., (1995) Physiologia Plantarum 93:19-24); sonication methods (Bao, et al., (1997) Ultrasound in Medicine & Biology 23:953-959; Finer and Finer (2000) Lett Appl Microbiol. 30:406-10; Amoah, et al., (2001) *J Exp Bot* 52:1135-42); polyethylene glycol methods (Krens, et al., (1982) Nature 296:72-77); protoplasts of monocot and dicot cells can be transformed using electroporation (Fromm, et al., (1985) Proc. Natl. Acad. Sci. USA 82:5824-5828) and microinjection (Crossway, et al., (1986) Mol. Gen. Genet. 202:179-185); all of which are herein incorporated by reference.

Agrobacterium-Mediated Transformation

[0119] The most widely utilized method for introducing an expression vector into plants is based on the natural transfor mation system of Agrobacterium. A. tumefaciens and A. rhizogenes are plant pathogenic soil bacteria, which geneti cally transform plant cells. The Ti and Ri plasmids of A. tumefaciens and A. rhizogenes, respectively, carry genes responsible for genetic transformation of plants. See, e.g., Kado, (1991) Crit. Rev. Plant Sci. 10:1. Descriptions of the Agrobacterium vector systems and methods for Agrobacte rium-mediated gene transfer are provided in Gruber, et al., supra; Miki, et al., supra; and Moloney, et al., (1989) Plant Cell Reports 8:238.

I0120 Similarly, the gene can be inserted into the T-DNA region of a Ti or Ri plasmid derived from A. tumefaciens or A. rhizogenes, respectively. Thus, expression cassettes can be constructed as above, using these plasmids. Many control sequences are known which when coupled to a heterologous coding sequence and transformed into a host organism show
fidelity in gene expression with respect to tissue/organ specificity of the original coding sequence. See, e.g., Benfey and Chua, (1989) Science 244:174-81. Particularly suitable con trol sequences for use in these plasmids are promoters for constitutive leaf-specific expression of the gene in the various target plants. Other useful control sequences include a pro moter and terminator from the nopaline synthase gene (NOS). The NOS promoter and terminator are present in the plasmid pARC2, available from the American Type Culture Collec tion and designated ATCC 67238. If such a system is used, the virulence (vir) gene from either the Ti or Riplasmid must also be present, either along with the T-DNA portion, or via a binary system where the vir gene is present on a separate vector. Such systems, vectors for use therein, and methods of transforming plant cells are described in U.S. Pat. No. 4,658, 082: US Patent Application Number 913,914, filed Oct. 1, 1986, as referenced in U.S. Pat. No. 5,262,306, issued Nov. 16, 1993; and Simpson, et al., (1986) Plant Mol. Biol. 6:403 15 (also referenced in the 306 patent); all incorporated by reference in their entirety.

[0121] Once constructed, these plasmids can be placed into A. rhizogenes or A. tumefaciens and these vectors used to transform cells of plant species, which are ordinarily susceptible to Fusarium or Alternaria infection. Several other transgenic plants are also contemplated by the present invention including but not limited to soybean, corn, sorghum, alfalfa, rice, clover, cabbage, banana, coffee, celery, tobacco, cow pea, cotton, melon, switchgrass, myscanthus, triticale and pepper. The selection of either A. tumefaciens or A. rhizo genes will depend on the plant being transformed thereby. In general A. tumefaciens is the preferred organism for transfor mation. Most dicotyledonous plants, some gymnosperms, and a few monocotyledonous plants (e.g., certain members of the Liliales and Arales) are susceptible to infection with A. tumefaciens. A. rhizogenes also has a wide host range, embracing most dicots and some gymnosperms, which includes members of the Leguminosae, Compositae, and Chenopodiaceae. Monocot plants can now be transformed with some success. EP Patent Application Number 604 662

A1 discloses a method for transforming monocots using Agrobacterium. EP Application Number 672 752 A1 dis closes a method for transforming monocots with Agrobacte rium using the scutellum of immature embryos. Ishida, et al., discuss a method for transforming maize by exposing imma ture embryos to A. tumefaciens (Nature Biotechnology 14:745-50 (1996)).

[0122] Once transformed, these cells can be used to regenerate transgenic plants. For example, whole plants can be infected with these vectors by wounding the plant and then introducing the vector into the wound site. Any part of the plant can be wounded, including leaves, stems and roots. Alternatively, plant tissue, in the form of an explant, such as cotyledonary tissue or leaf disks, can be inoculated with these vectors, and cultured under conditions, which promote plant regeneration. Roots or shoots transformed by inoculation of plant tissue with A. rhizogenes or A. tumefaciens, containing the gene coding for the fumonisin degradation enzyme, can be used as a source of plant tissue to regenerate fumonisin resistant transgenic plants, either via somatic embryogenesis or organogenesis. Examples of such methods for regenerating plant tissue are disclosed in Shahin, (1985) Theor: Appl. Genet. 69:235-40; U.S. Pat. No. 4,658,082; Simpson, et al., supra; and US Patent Application Numbers 913.913 and 913, 914, both filed Oct. 1, 1986, as referenced in U.S. Pat. No. 5,262,306, issued Nov. 16, 1993, the entire disclosures therein incorporated herein by reference.

Direct Gene Transfer

[0123] Despite the fact that the host range for *Agrobacte*rium-mediated transformation is broad, some major cereal crop species and gymnosperms have generally been recalci trant to this mode of gene transfer, even though some success has recently been achieved in rice (Hiei, et al., (1994) The Plant Journal 6:271-82). Several methods of plant transfor mation, collectively referred to as direct gene transfer, have been developed as an alternative to Agrobacterium-mediated transformation.

[0124] A generally applicable method of plant transformation is microprojectile-mediated transformation, where DNA is carried on the Surface of microprojectiles measuring about 1 to 4 um. The expression vector is introduced into plant tissues with a biolistic device that accelerates the micro projectiles to speeds of 300 to 600 m/s which is sufficient to penetrate the plant cell walls and membranes (Sanford, et al., (1987) Part. Sci. Technol. 5:27: Sanford, (1988) Trends Bio tech 6:299; Sanford, (1990) Physiol. Plant 79:206; and Klein, et al., (1992) Biotechnology 10:268).

[0125] Another method for physical delivery of DNA to plants is sonication of target cells as described in Zang, et al., (1991) BioTechnology 9:996. Alternatively, liposome or spheroplast fusions have been used to introduce expression vectors into plants. See, e.g., Deshayes, et al., (1985) EMBO J. 4:2731; and Christou, et al., (1987) Proc. Natl. Acad. Sci. USA 84:3962. Direct uptake of DNA into protoplasts using CaCl_, precipitation, polyvinyl alcohol, or poly-L-ornithine has also been reported. See, e.g., Hain, et al., (1985) *Mol.* Gen. Genet. 199:161; and Draper, et al., (1982) Plant Cell Physiol. 23:451.

[0126] Electroporation of protoplasts and whole cells and tissues has also been described. See, e.g., Donn, et al., (1990) in Abstracts of the VIIth Int'l. Congress on Plant Cell and

Tissue Culture IAPTC, A2-38, p. 53; D'Halluin, et al., (1992) Plant Cell 4:1495-505; and Spencer, et al., (1994) Plant Mol. Biol. 24:51-61.

Increasing the Activity and/or Level of an amt Polypeptide I0127. Methods are provided to increase the activity and/or level of the AMT polypeptide of the invention. An increase in the level and/or activity of the AMT polypeptide of the invention can be achieved by providing to the plant an amt polypeptide. The AMT polypeptide can be provided by introducing the amino acid sequence encoding the AMT polypeptide into the plant, introducing into the plant a nucleotide sequence encoding an amt polypeptide or alternatively by modifying a genomic locus encoding the AMT polypeptide of the inven tion.

[0128] As discussed elsewhere herein, many methods are known the art for providing a polypeptide to a plant including, but not limited to, direct introduction of the polypeptide into the plant, introducing into the plant (transiently or stably) a polynucleotide construct encoding a polypeptide having AMT transporter activity. It is also recognized that the meth ods of the invention may employ a polynucleotide that is not capable of directing, in the transformed plant, the expression of a protein or an RNA. Thus, the level and/or activity of an amt polypeptide may be increased by altering the gene encoding the AMT polypeptide or its promoter. See, e.g., Kmiec, U.S. Pat. No. 5,565,350; Zarling, et al., PCT/US93/03868. Therefore mutagenized plants that carry mutations in AMT genes, where the mutations increase expression of the AMT gene or increase the AMT transporter activity of the encoded AMT polypeptide are provided.

Reducing the Activity and/or Level of an amt Polypeptide I0129 Methods are provided to reduce or eliminate the activity of an amt polypeptide of the invention by transform ing a plant cell with an expression cassette that expresses a polynucleotide that inhibits the expression of the AMT polypeptide. The polynucleotide may inhibit the expression of the AMT polypeptide directly, by preventing transcription
or translation of the AMT messenger RNA, or indirectly, by encoding a polypeptide that inhibits the transcription or translation of an amt gene encoding an amt polypeptide. Methods for inhibiting or eliminating the expression of a gene inaplant are well known in the art, and any such method may be used in the present invention to inhibit the expression of an amt polypeptide.

[0130] In accordance with the present invention, the expression of an amt polypeptide is inhibited if the protein level of the AMT polypeptide is less than 70% of the protein level of the same AMT polypeptide in a plant that has not been genetically modified or mutagenized to inhibit the expression of that AMT polypeptide. In particular embodiments of the invention, the protein level of the AMT polypeptide in a modified plant according to the invention is less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 5% or less than 2% of the protein level of the same AMT polypeptide in a plant that is not a mutant or that has not been genetically modified to inhibit the expression of that AMT polypeptide. The expression level of the AMT polypeptide may be measured directly, for example, by assaying for the level of AMT polypeptide expressed in the plant cell or plant, or indirectly, for example, by measuring the AMT transporter activity of the AMT polypeptide in the plant cell or plant, or by measuring the AMT in the plant. Methods for performing such assays are described elsewhere herein.

0131. In other embodiments of the invention, the activity of the AMT polypeptides is reduced or eliminated by trans forming a plant cell with an expression cassette comprising a polynucleotide encoding a polypeptide that inhibits the activ ity of an amt polypeptide. The AMT transporter activity of an amt polypeptide is inhibited according to the present inven tion if the AMT transporter activity of the AMT polypeptide is less than 70% of the AMT transporter activity of the same AMT polypeptide in a plant that has not been modified to inhibit the AMT transporter activity of that AMT polypeptide. In particular embodiments of the invention, the AMT trans porter activity of the AMT polypeptide in a modified plant according to the invention is less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10% or less than 5% of the AMT transporter activity of the same AMT polypeptide in a plant that that has not been modified to inhibit the expression of that AMT polypeptide. The AMT transporter activity of an amt polypeptide is "eliminated according to the invention when it is not detectable by the assay methods described elsewhere herein. Methods of deter mining the AMT transporter activity of an amt polypeptide are described elsewhere herein.

[0132] In other embodiments, the activity of an amt polypeptide may be reduced or eliminated by disrupting the gene encoding the AMT polypeptide. The invention encom passes mutagenized plants that carry mutations in AMT genes, where the mutations reduce expression of the AMT gene or inhibit the AMT transporter activity of the encoded AMT polypeptide.

[0133] Thus, many methods may be used to reduce or eliminate the activity of anamt polypeptide. In addition, more than one method may be used to reduce the activity of a single AMT polypeptide. Non-limiting examples of methods of reducing or eliminating the expression of AMT polypeptides are given below.

0134) 1. Polynucleotide-Based Methods:

0135) In some embodiments of the present invention, a plant is transformed with an expression cassette that is capable of expressing a polynucleotide that inhibits the expression of an amt polypeptide of the invention. The term "expression" as used herein refers to the biosynthesis of a gene product, including the transcription and/or translation of said gene product. For example, for the purposes of the present invention, an expression cassette capable of expressing a polynucleotide that inhibits the expression of at least one AMT polypeptide is an expression cassette capable of pro ducing an RNA molecule that inhibits the transcription and/or translation of at least one AMT polypeptide of the invention. The "expression" or "production" of a protein or polypeptide from a DNA molecule refers to the transcription and translation of the coding sequence to produce the protein or polypeptide, while the "expression" or "production" of a protein or polypeptide from an RNA molecule refers to the translation of the RNA coding sequence to produce the protein or polypeptide.

[0136] Examples of polynucleotides that inhibit the expression of an amt polypeptide are given below.

[0137] i. Sense Suppression/Cosuppression

[0138] In some embodiments of the invention, inhibition of the expression of an amt polypeptide may be obtained by sense suppression or cosuppression. For cosuppression, an expression cassette is designed to express an RNA molecule corresponding to all or part of a messenger RNA encoding an amt polypeptide in the "sense' orientation. Over expression of the RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the cosuppression expression cassette are screened to identify those that show the greatest inhibition of AMT polypeptide expression.

[0139] The polynucleotide used for cosuppression may correspond to all or part of the sequence encoding the AMT polypeptide, all or part of the 5' and/or 3' untranslated region of an amt polypeptide transcript, or all or part of both the coding sequence and the untranslated regions of a transcript encoding an amt polypeptide. In some embodiments where the polynucleotide comprises all or part of the coding region for the AMT polypeptide, the expression cassette is designed to eliminate the start codon of the polynucleotide so that no protein product will be translated.
[0140] Cosuppression may be used to inhibit the expression

of plant genes to produce plants having undetectable protein levels for the proteins encoded by these genes. See, for example, Broin, et al., (2002) Plant Cell 14:1417-1432. CoSuppression may also be used to inhibit the expression of multiple proteins in the same plant. See, for example, U.S. Pat. No. 5,942,657. Methods for using cosuppression to inhibit the expression of endogenous genes in plants are described in Flavell, et al., (1994) Proc. Natl. Acad. Sci. USA 91:3490-3496; Jorgensen, et al., (1996) Plant Mol. Biol. 31:957-973: Johansen and Carrington (2001) Plant Physiol. 126:930-938: Broin, et al., (2002) Plant Cell 14:1417-1432: Stoutjesdijk, et al., (2002) Plant Physiol. 129:1723-1731;Yu, et al., (2003) Phytochemistry 63:753-763; and U.S. Pat. Nos. 5,034,323, 5,283,184, and 5,942,657; each of which is herein incorporated by reference. The efficiency of cosuppression may be increased by including a poly-dT region in the expres sion cassette at a position 3' to the sense sequence and 5' of the polyadenylation signal. See, US Patent Application Publica tion Number 20020048814, herein incorporated by reference. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, optimally greater than about 65% sequence identity, more optimally greater than about 85% sequence identity, most optimally greater than about 95% sequence identity. See, U.S. Pat. Nos. 5.283,184 and 5,034, 323; herein incorporated by reference.

0141 ii. Antisense Suppression

[0142] In some embodiments of the invention, inhibition of the expression of the AMT polypeptide may be obtained by antisense suppression. For antisense suppression, the expression cassette is designed to express an RNA molecule complementary to all or part of a messenger RNA encoding the AMT polypeptide. Over expression of the antisense RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the antisense suppression expression cassette are screened to identify those that show the greatest inhibition of AMT polypeptide expression.

[0143] The polynucleotide for use in antisense suppression may correspond to all or part of the complement of the sequence encoding the AMT polypeptide, all or part of the complement of the 5' and/or 3' untranslated region of the AMT transcript, or all or part of the complement of both the coding sequence and the untranslated regions of a transcript encoding the AMT polypeptide. In addition, the antisense polynucleotide may be fully complementary (i.e., 100% iden tical to the complement of the target sequence) or partially complementary (i.e., less than 100% identical to the comple

ment of the target sequence) to the target sequence. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, U.S. Pat. No. 5.942,657. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucle otides, 200 nucleotides, 300, 400, 450, 500, 550 or greater may be used. Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu, et al., (2002) Plant Physiol. 129: 1732-1743 and U.S. Pat. Nos. 5,759,829 and 5,942,657, of antisense suppression may be increased by including a poly-dT region in the expression cassette at a position 3' to the antisense sequence and 5' of the polyadenylation signal. See, US Patent Application Publication Number 20020048814, herein incorporated by reference.

[0144] iii. Double-Stranded RNA Interference

[0145] In some embodiments of the invention, inhibition of the expression of an amt polypeptide may be obtained by double-stranded RNA (dsRNA) interference. For dsRNA interference, a sense RNA molecule like that described above for cosuppression and an antisense RNA molecule that is fully or partially complementary to the sense RNA molecule are expressed in the same cell, resulting in inhibition of the expression of the corresponding endogenous messenger RNA.

[0146] Expression of the sense and antisense molecules can be accomplished by designing the expression cassette to com prise both a sense sequence and an antisense sequence. Alter natively, separate expression cassettes may be used for the sense and antisense sequences. Multiple plant lines trans formed with the dsRNA interference expression cassette or expression cassettes are then screened to identify plant lines that show the greatest inhibition of AMT polypeptide expres sion. Methods for using dsRNA interference to inhibit the expression of endogenous plant genes are described in Water house, et al., (1998) Proc. Natl. Acad. Sci. USA 95:13959-13964, Liu, et al., (2002) Plant Physiol. 129:1732-1743, and WO 99/49029, WO 99/53050, WO 99/61631, and WO 00/49035; each of which is herein incorporated by reference. 0147 iv. Hairpin RNA Interference and Intron-Containing Hairpin RNA Interference

[0148] In some embodiments of the invention, inhibition of the expression of an amt polypeptide may be obtained by hairpin RNA (hpRNA) interference or intron-containing hair-
pin RNA (ihpRNA) interference. These methods are highly efficient at inhibiting the expression of endogenous genes. See, Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29 38 and the references cited therein.

[0149] For hpRNA interference, the expression cassette is designed to express an RNA molecule that hybridizes with itself to form a hairpin structure that comprises a single stranded loop region and a base-paired stem. The base-paired stem region comprises a sense sequence corresponding to all or part of the endogenous messenger RNA encoding the gene whose expression is to be inhibited, and an antisense sequence that is fully or partially complementary to the sense sequence. Alternatively, the base-paired stem region may cor respond to a portion of a promoter sequence controlling expression of the gene to be inhibited. Thus, the base-paired stem region of the molecule generally determines the specificity of the RNA interference. hpRNA molecules are highly efficient at inhibiting the expression of endogenous genes,

and the RNA interference they induce is inherited by subse quent generations of plants. See, for example, Chuang and Meyerowitz (2000) Proc. Natl. Acad. Sci. USA 97:4985 4990; Stoutjesdijk, et al., (2002) Plant Physiol. 129:1723-1731; and Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29-38. Methods for using hpRNA interference to inhibit or silence the expression of genes are described, for example, in Chuang and Meyerowitz (2000) Proc. Natl. Acad. Sci. USA 97:4985-4990; Stoutjesdijk, et al., (2002) Plant Physiol. 129: 1723-1731: Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29-38; Pandolfini, et al., BMC Biotechnology 3:7, and US Patent Application Publication Number 20030175965; each of which is herein incorporated by refer ence. A transient assay for the efficiency of hpRNA constructs to silence gene expression in vivo has been described by Panstruga, et al., (2003) Mol. Biol. Rep. 30:135-140, herein incorporated by reference.

[0150] For ihpRNA, the interfering molecules have the same general structure as for hpRNA, but the RNA molecule additionally comprises an intron that is capable of being spliced in the cell in which the ihpRNA is expressed. The use of an intron minimizes the size of the loop in the hairpin RNA molecule following splicing, and this increases the efficiency of interference. See, for example, Smith, et al., (2000) Nature 407:319-320. In fact, Smith, et al., show 100% suppression of endogenous gene expression using ihpRNA-mediated inter ference. Methods for using ihpRNA interference to inhibit the expression of endogenous plant genes are described, for example, in Smith, et al., (2000) Nature 407:319-320; Wes ley, et al., (2001) Plant J. 27:581-590; Wang and Waterhouse (2001) Curr. Opin. Plant Biol. 5:146-150; Waterhouse and Helliwell (2003) Nat. Rev. Genet. 4:29-38: Helliwell and Waterhouse (2003) Methods 30:289-295, and US Patent Application Publication Number 20030180945, each of which is herein incorporated by reference.

0151. The expression cassette for hpRNA interference may also be designed such that the sense sequence and the antisense sequence do not correspond to an endogenous RNA. In this embodiment, the sense and antisense sequence flank a loop sequence that comprises a nucleotide sequence corresponding to all or part of the endogenous messenger RNA of the target gene. Thus, it is the loop region that deter mines the specificity of the RNA interference. See, for example, WO 02/00904, Mette, et al., (2000) EMBO J. 19:5194-5201; Matzke, et al., (2001) Curr. Opin. Genet. Devel. 11:221-227; Scheid, et al., (2002) Proc. Natl. Acad. Sci., USA 99:13659-13662; Aufsaftz, et al., (2002) Proc. Nat'l. Acad. Sci. 99(4):16499-16506; Sijen, et al., Curr. Biol. (2001) 11:436-440), herein incorporated by reference.

0152 v. Amplicon-Mediated Interference

[0153] Amplicon expression cassettes comprise a plant virus-derived sequence that contains all or part of the target gene but generally not all of the genes of the native virus. The viral sequences present in the transcription product of the expression cassette allow the transcription product to direct its own replication. The transcripts produced by the amplicon i.e., the messenger RNA for the AMT polypeptide). Methods of using amplicons to inhibit the expression of endogenous plant genes are described, for example, in Angell and Baul combe (1997) EMBO J. 16:3675-3684, Angell and Baulcombe (1999) Plant J. 20:357-362, and U.S. Pat. No. 6,646, 805, each of which is herein incorporated by reference.

[0154] vi. Ribozymes

0155. In some embodiments, the polynucleotide expressed by the expression cassette of the invention is cata lytic RNA or has ribozyme activity specific for the messenger RNA of the AMT polypeptide. Thus, the polynucleotide causes the degradation of the endogenous messenger RNA, resulting in reduced expression of the AMT polypeptide. This method is described, for example, in U.S. Pat. No. 4,987,071, herein incorporated by reference.

[0156] vii. Small Interfering RNA or Micro RNA

[0157] In some embodiments of the invention, inhibition of the expression of an amt polypeptide may be obtained by RNA interference by expression of a gene encoding a micro RNA (miRNA). miRNAs are regulatory agents consisting of about 22 ribonucleotides. miRNA are highly efficient at inhibiting the expression of endogenous genes. See, for example, Javier, et al., (2003) Nature 425:257-263, herein incorporated by reference.

[0158] For miRNA interference, the expression cassette is designed to express an RNA molecule that is modeled on an endogenous miRNA gene. The miRNA gene encodes an RNA that forms a hairpin structure containing a 22-nucle otide sequence that is complementary to another endogenous gene (target sequence). For Suppression of AMT expression, the 22-nucleotide sequence is selected from an amt transcript sequence and contains 22 nucleotides of said AMT sequence
in sense orientation and 21 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence. miRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interfer ence they induce is inherited by Subsequent generations of plants.

[0159] 2. Polypeptide-Based Inhibition of Gene Expression

[0160] In one embodiment, the polynucleotide encodes a zinc finger protein that binds to a gene encoding an amt polypeptide, resulting in reduced expression of the gene. In particular embodiments, the zinc finger protein binds to a regulatory region of an amt gene. In other embodiments, the zinc finger protein binds to a messenger RNA encoding an amt polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described, for example, in U.S. Pat. No. 6,453,242, and meth ods for using Zinc finger proteins to inhibit the expression of genes in plants are described, for example, in US Patent Application Publication Number 20030037355; each of which is herein incorporated by reference.

[0161] 3. Polypeptide-Based Inhibition of Protein Activity [0162] In some embodiments of the invention, the polynucleotide encodes an antibody that binds to at least one AMT polypeptide, and reduces the AMT transporter activity of the AMT polypeptide. In another embodiment, the binding of the antibody results in increased turnover of the antibody-AMT complex by cellular quality control mechanisms. The expres sion of antibodies in plant cells and the inhibition of molecu lar pathways by expression and binding of antibodies to pro teins in plant cells are well known in the art. See, for example, Conrad and Sonnewald (2003) Nature Biotech. 21:35-36, incorporated herein by reference.

[0163] 4. Gene Disruption

[0164] In some embodiments of the present invention, the activity of an amt polypeptide is reduced or eliminated by disrupting the gene encoding the AMT polypeptide. The gene encoding the AMT polypeptide may be disrupted by any method known in the art. For example, in one embodiment, the gene is disrupted by transposon tagging. In another embodiment, the gene is disrupted by mutagenizing plants using random or targeted mutagenesis, and selecting for plants that have reduced AMT transporter activity.

(0165 i. Transposon Tagging

[0166] In one embodiment of the invention, transposon tagging is used to reduce or eliminate the AMT activity of one or more AMT polypeptide. Transposon tagging comprises inserting a transposon within an endogenous AMT gene to reduce or eliminate expression of the AMT polypeptide. AMT gene' is intended to mean the gene that encodes anamt polypeptide according to the invention.

[0167] In this embodiment, the expression of one or more AMT polypeptide is reduced or eliminated by inserting a transposon within a regulatory region or coding region of the gene encoding the AMT polypeptide. A transposon that is within an exon, intron, 5' or 3' untranslated sequence, a pro moter, or any other regulatory sequence of an amt gene may be used to reduce or eliminate the expression and/or activity of the encoded AMT polypeptide.

[0168] Methods for the transposon tagging of specific genes in plants are well known in the art. See, for example, Maes, et al., (1999) Trends Plant Sci. 4:90-96; Dharmapuri and Sonti (1999) FEMS Microbiol. Lett. 179:53-59; Meiss ner, et al., (2000) Plant J. 22:265-274; Phogat, et al., (2000) J. Biosci. 25:57-63; Walbot (2000) Curr. Opin. Plant Biol. 2:103-107: Gai, et al., (2000) Nucleic Acids Res. 28:94-96: Fitzmaurice, et al., (1999) Genetics 153:1919-1928). In addi tion, the TUSC process for selecting Mu insertions in selected genes has been described in Bensen, et al., (1995) Plant Cell 7:75-84; Mena, et al., (1996) Science 274: 1537-1540; and U.S. Pat. No. 5,962,764; each of which is herein incorporated by reference.

[0169] ii. Mutant Plants with Reduced Activity

0170 Additional methods for decreasing or eliminating the expression of endogenous genes in plants are also known in the art and can be similarly applied to the instant invention. These methods include other forms of mutagenesis, such as ethyl methanesulfonate-induced mutagenesis, deletion mutagenesis, and fast neutron deletion mutagenesis used in a reverse genetics sense (with PCR) to identify plant lines in which the endogenous gene has been deleted. For examples of these methods see, Ohshima, et al., (1998) Virology 243: 472-481: Okubara, et al., (1994) Genetics 137:867-874; and Quesada, et al., (2000) Genetics 154:421-436; each of which is herein incorporated by reference. In addition, a fast and automatable method for screening for chemically induced mutations, TILLING (Targeting Induced Local Lesions In Genomes), using denaturing HPLC or selective endonuclease digestion of selected PCR products is also applicable to the instant invention. See, McCallum, et al., (2000) Nat. Biotechnol. 18:455-457, herein incorporated by reference.

[0171] Mutations that impact gene expression or that interfere with the function (AMT transporter activity) of the encoded protein are well known in the art. Insertional muta tions in gene exons usually result in null-mutants. Mutations in conserved residues are particularly effective in inhibiting the AMT transporter activity of the encoded protein. Conserved residues of plant AMT polypeptides suitable for mutagenesis with the goal to eliminate AMT transporter activity have been described. Such mutants can be isolated according to well-known procedures, and mutations in dif ferent AMT loci can be stacked by genetic crossing. See, for example, Gruis, et al., (2002) Plant Cell 14:2863–2882.

[0172] In another embodiment of this invention, dominant mutants can be used to trigger RNA silencing due to gene inversion and recombination of a duplicated gene locus. See, for example, Kusaba, et al., (2003) Plant Cell 15:1455-1467. [0173] The invention encompasses additional methods for reducing or eliminating the activity of one or more AMT polypeptide. Examples of other methods for altering or mutating a genomic nucleotide sequence in a plant are known in the art and include, but are not limited to, the use of RNA:DNA vectors, RNA:DNA mutational vectors, RNA: DNA repair vectors, mixed-duplex oligonucleotides, self complementary RNA:DNA oligonucleotides, and recombi nogenic oligonucleobases. Such vectors and methods of use are known in the art. See, for example, U.S. Pat. Nos. 5,565, 350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871, 984; each of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham, et al., (1999) Proc. Natl. Acad. Sci. USA 96:8774 8778; each of which is herein incorporated by reference.

[0174] iii. Modulating AMT Transporter Activity

[0175] In specific methods, the level and/or activity of an amt regulator in a plant is decreased by increasing the level or activity of the AMT polypeptide in the plant. Methods for increasing the level and/or activity of AMT polypeptides in a plant are discussed elsewhere herein. Briefly, such methods comprise providing an amt polypeptide of the invention to a plant and thereby increasing the level and/or activity of the AMT polypeptide. In other embodiments, an amt nucleotide sequence encoding an amt polypeptide can be provided by introducing into the planta polynucleotide comprising anamt nucleotide sequence of the invention, expressing the AMT sequence, increasing the activity of the AMT polypeptide, and thereby decreasing the ammonium uptake or transport in the plant or plant part. In other embodiments, the AMT nucle otide construct introduced into the plant is stably incorpo rated into the genome of the plant.

0176). As discussed above, one of skill will recognize the appropriate promoter to use to modulate the level/activity of an amt transporter in the plant. Exemplary promoters for this embodiment have been disclosed elsewhere herein.

0177 Accordingly, the present invention further provides plants having a modified number of cells when compared to the number of cells of a control plant tissue. In one embodi ment, the plant of the invention has an increased level/activity of the AMT polypeptide of the invention and thus has an increased Ammonium transport in the plant tissue. In other embodiments, the plant of the invention has a reduced or eliminated level of the AMT polypeptide of the invention and thus has an increased NUE in the plant tissue. In other embodiments. Such plants have stably incorporated into their genome a nucleic acid molecule comprising an amt nucle otide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

[0178] iv. Modulating Root Development

[0179] Methods for modulating root development in a plant are provided. By "modulating root development' is intended any alteration in the development of the plant root when compared to a control plant. Such alterations in root devel opment include, but are not limited to, alterations in the growth rate of the primary root, the fresh root weight, the extent of lateral and adventitious root formation, the vascu lature system, meristem development, or radial expansion.

[0180] Methods for modulating root development in a plant are provided. The methods comprise modulating the level and/or activity of the AMT polypeptide in the plant. In one method, an amt sequence of the invention is provided to the plant. In another method, the AMT nucleotide sequence is provided by introducing into the plant a polynucleotide com ing the AMT sequence, and thereby modifying root development. In still other methods, the AMT nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.
 [0181] In other methods, root development is modulated by

altering the level or activity of the AMT polypeptide in the plant. A decrease in AMT activity can result in at least one or more of the following alterations to root development, includ ing, but not limited to, larger root meristems, increased in root growth, enhanced radial expansion, an enhanced vasculature system, increased root branching, more adventitious roots, and/or an increase in fresh root weight when compared to a control plant.

[0182] As used herein, "root growth" encompasses all aspects of growth of the different parts that make up the root system at different stages of its development in both mono cotyledonous and dicotyledonous plants. It is to be under stood that enhanced root growth can result from enhanced growth of one or more of its parts including the primary root, lateral roots, adventitious roots, etc.

[0183] Methods of measuring such developmental alterations in the root system are known in the art. See, for example, US Patent Application Publication Number 2003/ 0074698 and Werner, et al., (2001) PNAS 18:10487-10492, both of which are herein incorporated by reference.

[0184] As discussed above, one of skill will recognize the appropriate promoter to use to modulate root development in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters. Exem plary root-preferred promoters have been disclosed else where herein.

[0185] Stimulating root growth and increasing root mass by decreasing the activity and/or level of the AMT polypeptide also finds use in improving the standability of a plant. The term "resistance to lodging" or "standability" refers to the ability of a plant to fix itself to the soil. Forplants with an erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse (environmen tal) conditions. This trait relates to the size, depth and mor phology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the level and/ or activity of the AMT polypeptide also finds use in promot ing in vitro propagation of explants.

[0186] Furthermore, higher root biomass production due to an decreased level and/or activity of AMT activity has a direct effect on the yield and an indirect effect of production of compounds produced by root cells or transgenic root cells or cell cultures of said transgenic root cells. One example of an interesting compound produced in root cultures is shikonin, the yield of which can be advantageously enhanced by said methods.

0187. Accordingly, the present invention further provides plants having modulated root development when compared to the root development of a control plant. In some embodi ments, the plant of the invention has an increased level/activ ity of the AMT polypeptide of the invention and has enhanced root growth and/or root biomass. In other embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising an amt nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

[0188] v. Modulating Shoot and Leaf Development

[0189] Methods are also provided for modulating shoot and leaf development in a plant. By "modulating shoot and/or leaf development" is intended any alteration in the development of the plant shoot and/or leaf. Such alterations in shoot and/or leaf development include, but are not limited to, alterations in shoot meristem development, in leaf number, leaf size, leaf and stem vasculature, internode length, and leaf senescence. As used herein, "leaf development" and "shoot development encompasses all aspects of growth of the different parts that make up the leaf system and the shoot system, respectively, at different stages of their development, both in monocotyle donous and dicotyledonous plants. Methods for measuring such developmental alterations in the shoot and leaf system are known in the art. See, for example, Werner, et al., (2001) PNAS 98:10487-10492 and US Patent Application Publication Number 2003/0074698, each of which is herein incor porated by reference.

[0190] The method for modulating shoot and/or leaf development in a plant comprises modulating the activity and/or level of an AMT polypeptide of the invention. In one embodi ment, an amt sequence of the invention is provided. In other embodiments, the AMT nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising an amt nucleotide sequence of the invention, expressing the AMT sequence, and thereby modifying shoot and/or leaf development. In other embodiments, the AMT nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

[0191] In specific embodiments, shoot or leaf development is modulated by increasing the level and/or activity of the AMT polypeptide in the plant. An increase in AMT activity can result in at least one or more of the following alterations in shoot and/or leaf development, including, but not limited to, reduced leaf number, reduced leaf surface, reduced vas cular, shorterinternodes and stunted growth, and retarded leaf senescence, when compared to a control plant.

[0192] As discussed above, one of skill will recognize the appropriate promoter to use to modulate shoot and leaf devel opment of the plant. Exemplary promoters for this embodi ment include constitutive promoters, shoot-preferred pro moters, shoot meristem-preferred promoters, and leaf preferred promoters. Exemplary promoters have been disclosed elsewhere herein.

[0193] Increasing AMT activity and/or level in a plant results in shorter internodes and stunted growth. Thus, the methods of the invention find use in producing dwarf plants. In addition, as discussed above, modulation AMT activity in the plant modulates both root and shoot growth. Thus, the present invention further provides methods for altering the root/shoot ratio. Shoot or leaf development can further be modulated by decreasing the level and/or activity of the AMT polypeptide in the plant.

[0194] Accordingly, the present invention further provides plants having modulated shoot and/or leaf development when compared to a control plant. In some embodiments, the plant of the invention has an increased level/activity of the AMT polypeptide of the invention. In other embodiments, the plant of the invention has a decreased level/activity of the AMT polypeptide of the invention.

[0195] vi Modulating Reproductive Tissue Development [0196] Methods for modulating reproductive tissue development are provided. In one embodiment, methods are provided to modulate floral development in a plant. By "modulating floral development' is intended any alteration in a structure of a plant's reproductive tissue as compared to a control plant in which the activity or level of the AMT polypeptide has not been modulated. "Modulating floral development" further includes any alteration in the timing of the development of a plant's reproductive tissue (i.e., a delayed or a accelerated timing of floral development) when compared to a control plant in which the activity or level of
the AMT polypeptide has not been modulated. Macroscopic alterations may include changes in size, shape, number, or location of reproductive organs, the developmental time period that these structures form, or the ability to maintain or proceed through the flowering process in times of environ mental stress. Microscopic alterations may include changes to the types or shapes of cells that make up the reproductive Organs.

0197) The method for modulating floral development in a plant comprises modulating AMT activity in a plant. In one method, an AMT sequence of the invention is provided. AN AMT nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising an amt nucleotide sequence of the invention, expressing the AMT sequence, and thereby modifying floral development. In other embodi ments, the AMT nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

[0198] In specific methods, floral development is modulated by increasing the level or activity of the AMT polypep tide in the plant. An increase in AMT activity can result in at least one or more of the following alterations in floral devel opment, including, but not limited to, retarded flowering, reduced number of flowers, partial malesterility, and reduced seed set, when compared to a control plant. Inducing delayed flowering or inhibiting flowering can be used to enhance yield in forage crops such as alfalfa. Methods for measuring such developmental alterations infloral development are known in the art. See, for example, Mouradov, et al., (2002) The Plant Cell S11-S130, herein incorporated by reference.

[0199] As discussed above, one of skill will recognize the appropriate promoter to use to modulate floral development of the plant. Exemplary promoters for this embodiment include constitutive promoters, inducible promoters, shoot preferred promoters, and inflorescence-preferred promoters. [0200] In other methods, floral development is modulated by decreasing the level and/or activity of the AMT sequence of the invention. Such methods can comprise introducing an amt nucleotide sequence into the plant and decreasing the activity of the AMT polypeptide. In other methods, the AMT nucleotide construct introduced into the plant is stably incor porated into the genome of the plant. Decreasing expression of the AMT sequence of the invention can modulate floral development during periods of stress. Such methods are described elsewhere herein. Accordingly, the present inven tion further provides plants having modulated floral develop ment when compared to the floral development of a control plant. Compositions include plants having a decreased level/ activity of the AMT polypeptide of the invention and having an altered floral development. Compositions also include plants having a decreased level/activity of the AMT polypep through the flowering process in times of stress.

[0201] Methods are also provided for the use of the AMT sequences of the invention to increase nitrogen use efficiency. The method comprises decreasing or increasing the activity of the AMT sequences in a plant or plant part, such as the roots, shoot, epidermal cells, etc.

[0202] As discussed above, one of skill will recognize the appropriate promoter to use to manipulate the expression of AMTs. Exemplary promoters of this embodiment include constitutive promoters, inducible promoters, and root or shoot or leaf preferred promoters.

[0203] vii. Method of Use for AMT Promoter Polynucleotides

[0204] The polynucleotides comprising the AMT promoters disclosed in the present invention, as well as variants and fragments thereof, are useful in the genetic manipulation of any host cell, preferably plant cell, when assembled with a linked to a nucleotide sequence comprising a polynucleotide of interest. In this manner, the AMT promoter polynucleotides of the invention are provided in expression cassettes along with a polynucleotide sequence of interest for expres sion in the host cell of interest. As discussed in Example XX below, the AMT promoter sequences of the invention are expressed in a variety of tissues and thus the promoter sequences can find use in regulating the temporal and/or the spatial expression of polynucleotides of interest.

[0205] Synthetic hybrid promoter regions are known in the art. Such regions comprise upstream promoter elements of one polynucleotide operably linked to the promoter element of another polynucleotide. In an embodiment of the inven tion, heterologous sequence expression is controlled by a synthetic hybrid promoter comprising the AMT promoter sequences of the invention, or a variant or fragment thereof, operably linked to upstream promoter element(s) from a het erologous promoter. Upstream promoter elements that are involved in the plant defense system have been identified and may be used to generate a synthetic promoter. See, for example, Rushton, et al., (1998) Curr. Opin. Plant Biol. 1:311-315. Alternatively, a synthetic AMT promoter sequence may comprise duplications of the upstream pro moter elements found within the AMT promoter sequences.

[0206] It is recognized that the promoter sequence of the invention may be used with its native AMT coding sequences. A DNA construct comprising the AMT promoter operably linked with its native AMT gene may be used to transformany plant of interest to bring about a desired phenotypic change, such as, modulating root, shoot, leaf, floral, and embryo development, stress tolerance, and any other phenotype described elsewhere herein.

[0207] The promoter nucleotide sequences and methods disclosed herein are useful in regulating expression of any heterologous nucleotide sequence in a host plant in order to vary the phenotype of a plant. Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterolo gous products or increased expression of endogenous prod providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

[0208] Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as devel oping nations open up world markets, new crops and tech nologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and het erosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agro nomics, insect resistance, disease resistance, herbicide resis tance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like.

[0209] In certain embodiments the nucleic acid sequences of the present invention can be used in combination ("stacked') with other polynucleotide sequences of interest in order to create plants with a desired phenotype. The combi nations generated can include multiple copies of any one or more of the polynucleotides of interest. The polynucleotides of the present invention may be stacked with any gene or combination of genes to produce plants with a variety of desired trait combinations, including but not limited to traits desirable for animal feed Such as high oil genes (e.g., U.S. Pat. No. 6.232.529); balanced amino acids (e.g., hordothionins (U.S. Pat. Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703, 409); barley high lysine (Williamson, et al., (1987) Eur: J. Biochem. 165:99-106; and WO 98/20122); and high methion ine proteins (Pedersen, et al., (1986) J. Biol. Chem. 261:6279; Kirihara, et al., (1988) Gene 71:359; and Musumura, et al., (1989) Plant Mol. Biol. 12: 123)); increased digestibility (e.g., modified storage proteins (U.S. patent application Ser. No. 10/053,410, filed Nov. 7, 2001); and thioredoxins (U.S. patent application Ser. No. 10/005,429, filed Dec. 3, 2001)), the disclosures of which are herein incorporated by reference. The polynucleotides of the present invention can also be stacked with traits desirable for insect, disease or herbicide resistance (e.g., Bacillus thuringiensis toxic proteins (U.S. Pat. Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593, 881; Geiser, et al., (1986) Gene 48:109); lectins (Van Damme, et al., (1994) Plant Mol. Biol. 24:825); fumonisin detoxifica tion genes (U.S. Pat. No. 5,792.931); avirulence and disease resistance genes (Jones, et al., (1994) Science 266:789; Mar tin, et al., (1993) Science 262:1432: Mindrinos, et al., (1994) Cell 78: 1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)); and traits desirable for processing or process products such as high oil (e.g., U.S. Pat. No. 6.232.529); modified oils (e.g., fatty acid desaturase genes (U.S. Pat. No. 5,952,544: phorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., U.S. Pat. No. 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert, et al., (1988) J. Bacteriol. 170:5837-5847) facilitate expression of polyhydroxyal kanoates (PHAs)), the disclosures of which are herein incor porated by reference. One could also combine the

polynucleotides of the present invention with polynucle otides affecting agronomic traits such as male sterility (e.g., see, U.S. Pat. No. 5,583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regula tion or gene targeting (e.g., WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated by reference.

0210. In one embodiment, sequences of interest improve plant growth and/or crop yields. For example, sequences of interest include agronomically important genes that result in improved primary or lateral root systems. Such genes include, but are not limited to, nutrient/water transporters and growth induces. Examples of Such genes, include but are not limited to, maize plasma membrane H"-ATPase (MHA2) (Frias, et al., (1996) Plant Cell 8:1533-44); AKT1, a component of the potassium uptake apparatus in Arabidopsis, (Spalding, et al., (1999) J Gen Physiol 113:909-18); RML genes which activate cell division cycle in the root apical cells (Cheng, et al., (1995) Plant Physiol 108:881); maize glutamine synthetase genes (Sukanya, et al., (1994) Plant Mol Biol 26:1935-46) and hemoglobin (Duff, et al., (1997) J. Biol. Chem. 27:16749-16752, Arredondo-Peter, et al., (1997) Plant Physiol. 115: 1259-1266; Arredondo-Peter, et al., (1997) Plant Physiol 114:493-500 and references sited therein). The sequence of interest may also be useful in expressing antisense nucleotide sequences of genes that that negatively affects root develop ment.

[0211] Additional, agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, Saturated and unsat urated oils, increasing levels of lysine and Sulfur, providing essential amino acids, and also modification of starch. Hor dothionin protein modifications are described in U.S. Pat. Nos. 5,703,049, 5,885,801, 5,885,802, and 5,990,389, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Pat. No. 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson, et al., (1987) Eur: J. Biochem. 165:99-106, the disclosures of which are herein incorporated by reference.

0212 Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. patent application Ser. No. 08/740,682, filed Nov. 1, 1996, and WO 98/20133, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant pro teins such as from sunflower seed (Lilley, et al., (1989) Proceedings of the World Congress on Vegetable Protein Utiliza-
tion in Human Foods and Animal Feedstuffs, ed. Applewhite (American Oil Chemists Society, Champaign, Ill.), pp. 497-502; herein incorporated by reference); corn (Pedersen, et al., (1986).J. Biol. Chem. 261:6279; Kirihara, et al., (1988) Gene 71:359; both of which are herein incorporated by reference); and rice (Musumura, et al., (1989) Plant Mol. Biol. 12:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

[0213] Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, Bacillus thuringiensis toxic protein genes (U.S. Pat.

Nos. 5,366,892; 5,747,450; 5,736,514;5,723,756; 5,593.881: and Geiser, et al., (1986) Gene 48:109); and the like.

[0214] Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Pat. No. 5,792.931); avirulence (avr) and disease resistance (R) genes (Jones, et al., (1994) Science 266:789; Martin, et al., (1993) Science 262:1432; and Mindrinos, et al., (1994) Cell 78: 1089); and the like.

[0215] Herbicide resistance traits may include genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS) , in particular the sulfonylureatype herbicides $(e.g., the acetolactate synthase (ALS) gene$ containing mutations leading to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The bar gene encodes resistance to the herbicide basta, the nptII gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.

[0216] Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissuepreferred genes and genes with male sterility phenotypes such as QM, described in U.S. Pat. No. 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

[0217] The quality of grain is reflected in traits such as levels and types of oils, Saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. In corn, modified hordothionin proteins are described in U.S. Pat. Nos. 5,703,049, 5,885,801, 5,885,802 and 5,990,389.

[0218] Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another impor tant commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Pat. No. $5{,}602{,}321$. Genes such as β -Ketothiolase, PHBase (polyhydroxyburyrate synthase), and acetoacetyl-CoA reductase (see, Schubert, et al., (1988) J. Bacteriol. 170:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs).

0219 Exogenous products include plant enzymes and products as well as those from other sources including pro caryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

[0220] This invention can be better understood by reference to the following non-limiting examples. It will be appreciated by those skilled in the art that other embodiments of the invention may be practiced without departing from the spirit and the scope of the invention as herein disclosed and claimed.

EXAMPLES

Example 1

Isolation of AMT Sequences

[0221] A routine for identifying all members of a given species' ammonium transporter (AMT) gene family was employed. First, a diverse set of all the known available members of the gene family as protein sequences was pre pared from public and proprietary sources. This data could include orthologous sequences from other species besides these four. Then, as in the example of maize, these protein query sequences were BLAST algorithm searched against a combination of proprietary and public maize, genomic or transcript, nucleotide sequence datasets, and a non-redundant set of candidate AMTs or 'hits' was identified. These sequences were combined with any existing maize gene fam ily sequences, and then curated and edited to arrive at a new working set of unique maize AMT gene or transcript sequences and their translations. This search for gene family members was repeated. If there were recovered new sequences whose nucleotide sequences were unique (not same-gene matches), the process repeated until completion, that is until no new and distinct nucleotide sequences were found. In this way it was determined that the maize AMT family of genes consisted of at least seven members. Eleven distinct soybean sequences were found. Without the complete genome sequences of maize or soybean available, researchers were less certain of the exact gene family size, than they were for Arabidopsis (6 members) and rice (17 members). The availability of complete genome sequences for Arabidopsis and rice simplified the search, aided also by availability of fairly mature gene models and annotations for these species.

Example 2

Transformation and Regeneration of Transgenic Plants

[0222] Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the AMT sequence operably linked to the drought-inducible promoter RAB17 promoter (Villardell, et al., (1990) Plant Mol Biol 14:423-432) and the selectable marker gene PAT, which con fers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes fol low below.

[0223] Preparation of Target Tissue:

[0224] The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target Zone in preparation for bombardment.

[0225] Preparation of DNA:

[0226] A plasmid vector comprising the AMT sequence operably linked to an ubiquitin promoter is made. This plas mid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto $1.1 \mu m$ (average diameter) tungsten pellets using a CaCl₂ precipitation procedure as follows:

[0227] 100 µl prepared tungsten particles in water

[0228] 10 μ l (1 μ g) DNA in Tris EDTA buffer (1 μ g total DNA)
[0229]

[0229] 100 µ1 2.5 M CaC1₂
[0230] 10 µ1 0.1 M spermid

 $10 \mu l$ 0.1 M spermidine

[0231] Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105μ l 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bom bardment.

[0232] Particle Gun Treatment:

0233. The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

[0234] Subsequent Treatment:

[0235] Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and trans ferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are moni tored and scored for increased drought tolerance. Assays to measure improved drought tolerance are routine in the art and include, for example, increased kernel-earring capacity yields under drought conditions when compared to control maize plants under identical environmental conditions. Alter natively, the transformed plants can be monitored for a modu lation in meristem development (i.e., a decrease in spikelet formation on the ear). See, for example, Bruce, et al., (2002) Journal of Experimental Botany 53:1-13.

[0236] Bombardment and Culture Media:

102371 Bombardment medium (560Y) comprises 4.0 α /1 N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000xSIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/1 Sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-1 $H₂O$ following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I $H₂O$); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000 \times SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with $D-H₂O$ following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_2O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos (both added after sterilizing the medium and cooling to room temperature).

[0238] Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I $H₂O$) (Murashige and Skoog (1962) Physiol. Plant. 15:473), 100 mg/l myo-inositol, 0.5 mg/1 zeatin, 60 α /l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished $D-I H₂O$ after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I $H₂O$; and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60° C.). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_2O), 0.1 g/1 myo-inositol, and 40.0 g/l sucrose (brought to volume with polished $D-IH₂O$ after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_2O , sterilized and cooled to 60 \degree C.

Example 3

Agrobacterium-Mediated Transformation

[0239] For *Agrobacterium*-mediated transformation of maize with an antisense sequence of the AMT sequence of the present invention, preferably the method of Zhao is employed (U.S. Pat. No. 5,981,840, and PCT patent publication WO98/ 32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of $Agro$ bacterium, where the bacteria are capable of transferring the antisense AMT sequences to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an Agrobacterium suspension for the initiation of inoculation. The embryos are co-cultured for a time with the Agrobacterium (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "rest ing" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of Agrobacterium and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing trans formed callus is recovered (step 4: the selection step). Pref erably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants. Plants are monitored and scored for a modulation in tissue development.

Example 4

Soybean Embryo Transformation

[0240] Soybean embryos are bombarded with a plasmid containing an antisense AMT sequences operably linked to an ubiquitin promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar $A2872$, are cultured in the light or dark at 26° C. on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below. [0241] Soybean embryogenic suspension cultures can be maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C. with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inocu lating approximately 35 mg of tissue into 35 ml of liquid medium.

[0242] Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombard ment (Klein, et al., (1987) Nature (London) 327:70-73, U.S. Pat. No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations. [0243] A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell, et al., (1985) Nature 313:810-812), the hygromycin phosphotrans ferase gene from plasmid pJR225 (from *E. coli*; Gritz, et al., (1983) *Gene* 25:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Tiplasmid of Agrobacterium tumefaciens. The expression cassette comprising an antisense AMT sequence operably linked to the ubiquitin promoter can be isolated as a restriction fragment. This frag ment can then be inserted into a unique restriction site of the vector carrying the marker gene.

[0244] To 50 μ l of a 60 mg/ml 1 μ m gold particle suspension is added (in order): 5μ l DNA (1 μ g/ μ l), 20 μ l spermidine (0.1 M), and 50 μ l CaCl₂ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 ul 70% ethanol and resuspended in 40 ul of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

[0245] Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approxi mately 3.5 inches away from the retaining screen and bom barded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as

described above.
[0246] Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untrans formed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Example 5

Sunflower Meristem Tissue Transformation

[0247] Sunflower meristem tissues are transformed with an expression cassette containing an antisense AMT sequences operably linked to a ubiquitin promoter as follows (see also, European Patent Number EP 0486233, herein incorporated by reference, and Malone-Schoneberg, et al., (1994) Plant Science 103:199-207). Mature sunflower seed (Helianthus annuus L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled water.

[0248] Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer, et al. (Schrammeijer, et al., (1990) Plant Cell Rep. 9:55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral ele ments (Murashige, et al., (1962) Physiol. Plant., 15:473 497), Shepard's vitamin additions (Shepard (1980) in Emer gent Techniques for the Genetic Improvement of Crops (University of Minnesota Press, St. Paul, Minn.), 40 mg/l adenine sulfate, 30 g/l sucrose, 0.5 mg/l 6-benzyl-aminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1 mg/l gibberellic acid (GA₃), pH 5.6, and 8 g/l Phytagar.

[0249] The explants are subjected to microprojectile bombardment prior to Agrobacterium treatment (Bidney, et al., (1992) Plant Mol. Biol. 18:301-313). Thirty to forty explants are placed in a circle at the center of a 60×20 mm plate for this treatment. Approximately 4.7 mg of 1.8 mm tungsten micro projectiles are resuspended in 25 ml of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 1.5 ml aliquots are used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000® particle acceleration device.

[0250] Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the AMT gene operably linked to the ubiquitin promoter is introduced into *Agrobacterium* strain EHA105 via freezethawing as described by Holsters, et al., (1978) Mol. Gen. Genet. 163:181-187. This plasmid further comprises a kana-
mycin selectable marker gene (i.e., nptII). Bacteria for plant transformation experiments are grown overnight (28° C. and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD_{600} of about 0.4 to 0.8. The Agrobacterium cells are pelleted and resuspended at a final OD_{oo} of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l NH₄Cl, and 0.3 gm/l MgSO₄.

[0251] Freshly bombarded explants are placed in an $Agro$ bacterium suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26° C. and 18-hour days. After three days of co-cultivation, the explants are trans ferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on selection and then trans ferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differ entiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day

phytohormone treatment. Leaf samples from green, kanamy cin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assay ing for a modulation in meristem development (i.e., an alter ation of size and appearance of shoot and floral meristems). [0252] NPTII-positive shoots are grafted to Pioneer® hybrid 6440 in vitro-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the trans with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of in vitro culture. Grafts in soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T_0 plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by AMT activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T_0 plants are identified by AMT activity analysis of small portions of dry seed coty ledon.

0253) An alternative sunflower transformation protocol allows the recovery of transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surfacesterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, then rinsed three times with distilled water. Steril ized seeds are imbibed in the dark at 26°C. for 20 hours on filter paper moistened with water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-BAP, 0.25 mg/l IAA, 0.1 mg/l GA, and 0.8% Phytagar at pH 5.6) for 24 hours under the dark. The primary leaves are removed to expose the apical meristem, around 40 explants are placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

[0254] Approximately 18.8 mg of 1.8 μ m tungsten particles are resuspended in 150 ul absolute ethanol. After sonication, 8 ul of it is dropped on the center of the surface of macrocar rier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

[0255] The plasmid of interest is introduced into $A\alpha\beta\alpha\beta\alpha\beta$ terium tumefaciens strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C. in a liquid YEP medium (10 g/l yeast extract, 10 g/1 Bactopeptone, and 5 g/l NaCl, pH 7.0) in the presence of 50 ug/l kanamycin is resuspended in an inoculation medium (12.5 mM 2-mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH₄Cl and 0.3 g/l MgSO₄ at pH 5.7) to reach a final concentration of 4.0 at OD 600. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no BAP, IAA, GA3 and supplemented with $250 \mu g/ml$ cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C. incubation conditions.

[0256] Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for a modulation in

meristem development (i.e., an alteration of size and appear ance of shoot and floral meristems). After positive (i.e., a decrease in AMT expression) explants are identified, those shoots that fail to exhibit a decrease in AMT activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an additional four weeks. Devel oping buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the initial assay prior to nodal culture.

[0257] Recovered shoots positive for a decreased AMT expression are grafted to Pioneer hybrid 6440 in vitro-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-steril ized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and are rinsed three times with distilled water. The sterilized seeds are germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26° C. under the dark for three days, then incubated at 16-hour-day culture conditions. The upper por tion of selected seedling is removed, a vertical slice is made in each hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

Example 6

Identification, Phylogenetic Analysis and Chloro plast Targeting Peptide (cTP) Predictions of AMTs in Arabidopsis, Rice, Soybean and Maize

[0258] Taking a 'genomic' approach AMTs were identified in several higher plants. In Arabidopsis 6 AMTs have been identified, and phylogenetic analyses reveals that AtAMT1 (SEQ ID NO: 2) AtAMT1:2 (SEQ ID NO: 4), AtAMT1:3 (SEQ ID NO: 6) and At3g24290 (SEQ ID NO: 10) cluster in one group where as AtAMT2 (SEQ ID NO: 8) and At4g28700 (SEQ ID NO: 12) are independent. Chloroplast targeting peptide (cTP) prediction by ChloroP program reveals that AtAMT1:2 (SEQ ID NO: 4) have a putative cTP (with 55% predicted cTP In rice, soybean and maize, 17, 11, 7 AMTs have been identified, respectively. cTP prediction in AMTs proteins from maize and soybean didn't identify any AMT candidate with a putative cTP, however in rice one AMT has putative cTP with more than 50% probability. Phylogenetic analyses of all the AMTs from Arabidopsis, rice, maize and soybean are shown in FIG. 1.

Example 7

Expression Analysis of AMTs in Maize

[0259] In order to identify leaf specific/preferred/expressed AMT(s) in maize, Lynx MPSS expression analyses in -300 libraries reveal that ZmAMT1 (SEQ ID NO: 14), 2, 7 are expressed both in roots and leaves (FIG. 2) whereas ZmaMT4 (SEQ ID NO: 20) is a root preferred AMT. ZmaMT6 (SEQ ID NO: 24) expresses at very low level in comparison to other ZmAMTs. In case of ZmAMT5 there was no specific Lynx tag available. Researchers also per formed RT-PCR on leaf and roots of B73 maize and the results confirm Lynx analysis results that there is no leaf specific AMT in maize, although ZmAMT1, 2, 7 (SEQ ID NOS: 14, 16 and 26) are expressed in leaves and roots.

Example 8

CTP Predictions in Chloroplast Outer Envelope Pro teins

[0260] Initial cTP prediction couldn't detect a putative cTP in most of the higher plant AMTs analyzed. The chloroplast localized AMT (if any) has to be in the outer envelope of the chloroplast. In order to determine whether proteins localized in outer envelop of the chloroplast have any predicted cTP. researchers searched the NCBI database using 'chloroplast outer envelop/membrane' as keyword and identified the 14, 14, and 5 proteins from *Arabidopsis*, rice and maize, respectively that are suppose to be localized in outer envelop of chloroplast. Some of these are well characterized proteins and ChloroP program was used to identify putative cTP in these 33 candidate proteins and interestingly none of these proteins show any putative cTP with high probability. These observa tions suggest that either a cTP is not required or not identified/ characterized for these proteins so far. This also suggests that although most of the AMTs don't have a predicted cTP but some of them might be localized in the chloroplast outer membrane.

Example 9

Isolation and Characterization of AtAMT1:2 (SEQ ID NO: 4) T-DNA Mutant

[0261] In cTP prediction analyses, AtAMT1;2 (SEQ ID NO: 4) posses a putative cTP. For functional analyses of AtAMT1:2 (SEQ ID NO: 4) and to determine it's role in N-assimilation, researchers identified a T-DNA mutant line (SM_3.15680) from the Arabidopsis T-DNA mutant data base. The T-DNA mutant line was ordered from ABRC and the homozygous plants were Subjected to molecular analyses. In this mutant line T-DNA was inserted in c-terminal of AtAMT1;2 (SEQ ID NO: 4) gene (FIG. 3A). Genomic PCRs using AtAMT1;2 (SEQ ID NO: 4) gene and T-DNA specific primers show that T-DNA is indeed inserted in the AtAMT1:2
(SEQ ID NO: 4) (FIG. 3B). AtAMT1:2 (SEQ ID NO: 4) gene specific primers flanking the T-DNA insert couldn't amplify any DNA region in mutant plants where as an expected PCR product was detected in wild type plant (FIG. 4B, upper panel). Similarly, genomic PCR with AtAMT1:2 (SEQ ID NO: 4) specific forward primer and T-DNA specific reverse primers amplify an expected product in mutant lines and nothing in wild type plants as expected (FIG. 4B, lower panel). Saturated RT-PCRs (35 cycles) analyses couldn't detect a full length atamt1:2 mRNA in mutant (FIG. 4C, upper panel) suggesting that AtAMT1:2 (SEQ ID NO: 4) is completely knocked out in this T-DNA mutant. Actin control RT-PCR worked fine in both mutant and wild type plants (FIG. 3C, lower panel).

Example 10

Generation and Molecular Characterization of AtAMT1;2 (SEQ ID NO: 4) RNAi Lines

[0262] In addition to T-DNA mutant, another parallel approach was also undertaken for functional analysis of AtAMT1;2 (SEQ ID NO: 4). A RNAi vector containing ZM-UBI promoter driven RNAi cassette consisting of inverted repeats of AtAMT1;2 (SEQ ID NO: 4) specific DNA regions and ADH intron as a spacer was constructed. Wild type Ara bidopsis (Columbia-0) was transformed with this RNAi vec tor by *Agrobacterium* mediated 'floral-dip' method. Several transgenic lines were identified by selecting the T0 seeds for herbicide resistance in soil. Molecular characterization of these transgenic lines were performed by RT-PCR for Actin, AtAMT1:2 (SEQ ID NO: 4) RNAi cassette, endogenous AtAMT1;2 (SEQ ID NO: 4) and presence of gDNA in RNA preparations. Several lines with a significant reduced levels of AtAMT1;2 (SEQ ID NO: 4) were identified after molecular analysis.

Example 11

Sub-Cellular Localization and Regulation of Expres sion of AtAMT1:2 (SEQ ID NO: 4)

[0263] cTP prediction analyses indicate that AtAMT1:2 (SEQ ID NO: 4) contains a putative predicted cTP (but with only 55% probability). The objectives of the experiments described in this example are to determine sub-cellular local ization and regulation of expression the endogenous AtAMT1:2 (SEQ ID NO: 4). The coding sequence of AtAMT1;2 (SEQ ID NO: 4) was tagged with green fluorescent protein (GFP) as an in-frame C-terminal fusion under the control of AtAMT1;2 (SEQ ID NO: 4) native promoter and a strong constitutive (ZM-UBI) promoter. Arabidopsis trans genic lines were generated and analyzed for GFP expression by confocal microscopy. Analyses show that AtAMT1:2:GFP is localized in the plasma membrane of endodermis and the cortex in roots.

Example 12

Knock-Out/Knock-Down of Zm-AMTs in Maize

[0264] ESTs corresponding to all seven maize AMTs were identified and annotated and full length cDNA clones were obtained. Experiments to knock-out/knock-down of all these individual ZmAMTs by RNAi are in progress. TUSC screen ing experiments were used to identify knock-out mutants for three leaf expressed ZmAMT1 (SEQ ID NO: 14), ZmAMT2 (SEQ ID NO: 16) and ZmAMT7 (SEQ ID NO: 26).

Example 13

Knock-Out/Knock-Down of Multiple AtAMTs with Single RNAi Vector in Arabidopsis

[0265] Six AMT genes are present in Arabidopsis genome. Hence, it is very likely that due to functional redundancy one might need to manipulate the expression of multiple AMTs simultaneously. The DNA sequence of all these AMTs was analyzed and identified the high homology regions among them. For example there is such a stretch of \sim 200 bp among AtAMT1:2 (SEQ ID NO: 4), AtAMT1 (SEQ ID NO: 2), AMT1;3 (SEQ ID NO: 6), At3g24290 (SEQ ID NO: 10) and At4g28700 (SEQ ID NO: 12) where as AMT2 (SEQ ID NO: 8) stood independent (FIG. 4). These regions were amplified (bold and underlined in FIG. 4) by PCR from AtAMT1;2 (SEQ ID NO: 4) and AtAMT2 (SEQ ID NO: 8) and performed a multi-way ligation to make an inverted repeat using ADH-intron as a spacer. The RNAi cassette of these hybrid inverted repeats is driven by a constitutive or root-specific or leaf-specific promoter. Several transgenic Arabidopsis lines were generated for these three constructs. Molecular analyses of these lines were performed by genomic and RT-PCR. Sev eral lines were identified that expressed significantly reduced levels of multiple AtAMTs. These transgenic lines show a methyl ammonium (ammonium analog toxic to plants) toler ant/better growth phenotype as compared to wild type control when grown on MS media supplemented with 10-30 mM of methyl ammonium. These results indicate multiple AMTs were knocked-down in these lines, resulting in reduced uptake of methyl ammonium.

Example 14

Knock-Out/Knock-Down of Multiple ZmAMTs in Maize by Single RNAi Vector

[0266] In maize at least 7 AMT like genes were identified and at least 3 of them are expressed both in leaf and root (see, Example 2). For improving NUE by reducing loss of ammonia by volatilization, one might have to knock-out/knock down multiple AMTs. Detailed analyses of all 7 maize AMTs were performed to identify the DNA regions showing high homology among different ZmAMTs. This analysis reveals that ZmAMT1 (SEQ ID NO: 14) and ZmAMT5 (SEQ ID NO: 22), ZmAMT3 (SEQ ID NO: 18) and ZmAMT4 (SEQ ID NO: 20) and ZmAMT2 (SEQ ID NO: 16), ZmAMT6 (SEQ ID NO: 24) and ZmAMT7 (SEQ ID NO: 26) form three separate groups and there is a very high homology in stretches of DNA sequences with in each group (FIG. 5). Three DNA fragments (bold and underlined in FIG. 5) from ZmAMT 1, 4 and 7 (SEQ ID NOS: 14, 20 and 26) representing each of the different groups were amplified by PCR. Multi-way ligations were performed to make inverted repeats with hybrid of these 3 fragments and ADH intron as a spacer to facilitate the formation of stem-loop structure. This hybrid RNAi cassette of ZmAMT1 (SEQ ID NO: 14):ZmAMT4 (SEQ ID NO: 20):ZmAMT7 (SEQ ID NO: 26)' inverted repeats was driven by Zm-UBI promoter and a leaf-specific promoter. MOPAT driven by Zm-UBI promoter was used as herbicide resistance marker for selected. In addition to that RFP driven by a pericarp specific promoter LTP2 was also used to sort out the transgenic seeds (red) from there segregating non-transgenic seeds. Transgenic lines for the constructs were generated, with molecular analyses of the T0 events performed by genomic and RT-PCR. Several lines with significantly reduced expression of individual/multiple ZmAMTs have been identified and characterized.

Example 15

Variants of AMT Sequences

[0267] A. Variant Nucleotide Sequences of AMT that do not Alter the Encoded Amino Acid Sequence
[0268] The AMT nucleotide sequences are used to generate

variant nucleotide sequences having the nucleotide sequence

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

[0269] B. Variant Amino Acid Sequences of AMT Polypeptides

[0270] Variant amino acid sequences of the AMT polypeptides are generated. In this example, one amino acid is altered. Specifically, the open reading frames are reviewed to deter mine the appropriate amino acid alteration. The selection of the amino acid to change is made by consulting the protein alignment (with the other orthologs and other gene family members from various species). An amino acid is selected that is deemed not to be under high selection pressure (not highly conserved) and which is rather easily substituted by an amino acid with similar chemical characteristics (i.e., similar functional side-chain). Using the protein alignment set forth in FIG.2, an appropriate amino acid can be changed. Once the targeted amino acid is identified, the procedure outlined in the following section C is followed. Variants having about 70%, 75%, 80%, 85%.90% and 95% nucleic acid sequence identity are generated using this method.

[0271] C. Additional Variant Amino Acid Sequences of AMT Polypeptides

[0272] In this example, artificial protein sequences are created having 80%, 85%, 90% and 95% identity relative to the reference protein sequence. This latter effort requires identi fying conserved and variable regions from the alignment set forth in FIG. 2 and then the judicious application of an amino acid substitutions table. These parts will be discussed in more detail below.

[0273] Largely, the determination of which amino acid sequences are altered is made based on the conserved regions among AMT protein or among the other AMT polypeptides. Based on the sequence alignment, the various regions of the AMT polypeptide that can likely be altered are represented in lower case letters, while the conserved regions are represented by capital letters. It is recognized that conservative substitutions can be made in the conserved regions below without altering function. In addition, one of skill will under stand that functional variants of the AMT sequence of the invention can have minor non-conserved amino acid alter ations in the conserved domain.

[0274] Artificial protein sequences are then created that are different from the original in the intervals of 80-85%, 85-90%, 90-95% and 95-100% identity. Midpoints of these intervals are targeted, with liberal latitude of plus or minus 1%, for example. The amino acids substitutions will be effected by a custom Perl script. The substitution table is provided below in Table 2.

TABLE 2

	Substitution Table				
Amino Acid	Strongly Similar and Optimal Substitution	Rank of Order ŤΩ Change	Comment		
	L, V I. V	\mathcal{P}	50:50 substitution 50:50 substitution		

TABLE 2-continued

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

0275 First, any conserved amino acids in the protein that should not be changed is identified and "marked off" for insulation from the substitution. The start methionine will of course be added to this list automatically. Next, the changes are made. H. C. and P are not changed in any circumstance. The changes will occur with isoleucine first, sweeping N-ter minal to C-terminal. Then leucine, and so on down the list until the desired target it reached. Interim number substitu tions can be made so as not to cause reversal of changes. The list is ordered 1-17, so start with as many isoleucine changes as needed before leucine, and so on down to methionine. Clearly many amino acids will in this manner not need to be changed. L, I and V will involve a 50:50 substitution of the two alternate optimal substitutions.

[0276] The variant amino acid sequences are written as output. Perl script is used to calculate the percent identities. Using this procedure, variants of the AMT polypeptides are generating having about 80%, 85%, 90%, and 95% amino acid identity to the starting unaltered ORF nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31,33,35, 37, 39, 41,43, 45, 47,49, 51,53,55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81.

Example 16

Over-Expression of AMTs in Plants to Improve NUE

[0277] The over-expression of AMTs has been demonstrated with strong constitutively or organ-specific (e.g. in roots) expression which improves ammonium uptake (especially in low ammonium soils in anaerobic conditions typical of rice field conditions) leading to improved nitrogen use efficiency. In other plants, such as maize, typically most of the N is absorbed by roots in the form of nitrate, the available source in most soil, however there is still a considerable proportion of Navailable as ammonium. Over-expression of AMTs in these conditions leads to improved nitrogen utiliza tion. Since nitrate needs to be reduced to ammonium by an energy expensive reaction before it is assimilated, ammonium is a preferable source of N when available to the plant.

[0278] All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the cation was specifically and individually indicated by reference.

[0279] The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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- Continued

-continued

31

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65 70 75 75 80 Lys Asn Thr Met Asn Ile Met Leu. Thr Asn. Val Leu. Asp Ala Ala Ala 35 Gly Ala Ile Ser Tyr Tyr Leu Phe Gly Phe Ala Phe Ala Phe Gly Thr 100 105 Pro Ser Asn Gly Phe Ile Gly Arg His His Ser Phe Phe Ala Leu Ser
115 20 20 20 Ser Tyr Pro Glu Arg Pro Gly Ser Asp Phe Ser Phe Phe Leu Tyr Gln 130 30 Trp Ala Phe Ala Ile Ala Ala Ala Gly Ile Thr Ser Gly Ser Ile Ala
145 150 160 Glu Arg Thr Gln Phe Val Ala Tyr Leu Ile Tyr Ser Thr Phe Leu Thr 165 170 175 Gly Phe Val Tyr Pro Thr Val Ser His Trp Phe Trp Ser Ser Asp Gly 180 190 rp Ala Ser Ala Ser Arg Ser Asp Asn. Asn Lieu. Lieu. Phe Gly Ser Gly 95 2 OO 2O5 Ala Ile Asp Phe Ala Gly Ser Gly Val Val His Met Val Gly Gly Ile 210 215 22O Ala Gly Leu Cys Gly Ala Leu Val Glu Gly Pro Arg Ile Gly Arg Phe 225 230 230

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165			170			175	Thr Val Gly Ala Tyr Ser Ile Trp Gly Gly Gly Phe Leu Tyr Gln Trp		
180			185			190	Gly Val Ile Asp Tyr Ser Gly Gly Tyr Val Ile His Leu Ser Ser Gly		
195			200			205	Val Ala Gly Phe Val Ala Ala Tyr Trp Val Gly Pro Arg Pro Lys Ala		
210			215			220	Asp Arg Glu Arg Phe Pro Pro Asn Asn Val Leu Leu Met Leu Ala Gly		
225			230			235	Ala Gly Leu Leu Trp Met Gly Trp Ser Gly Phe Asn Gly Gly Ala Pro		240
245			250			255	Tyr Ala Ala Asn Leu Thr Ser Ser Ile Ala Val Leu Asn Thr Asn Leu		
260			265			270	Ser Ala Ala Thr Ser Leu Leu Val Trp Thr Thr Leu Asp Val Ile Phe		
275			280			285	Phe Gly Lys Pro Ser Val Ile Gly Ala Ile Gln Gly Met Val Thr Gly		
290			295			300	Leu Ala Gly Val Thr Pro Gly Ala Gly Leu Ile Gln Thr Trp Ala Ala		
305			310			315	Ile Ile Ile Gly Val Val Ser Gly Thr Ala Pro Trp Ala Ser Met Met		320
325			330			335	Ile Ile His Lys Lys Ser Ala Leu Leu Gln Lys Val Asp Asp Thr Leu		
340			345			350	Ala Val Phe Tyr Thr His Ala Val Ala Gly Leu Leu Gly Gly Ile Met		
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405			410			415	Ser Thr Thr Ile Ile Leu Leu Ala Ile Arq Val Phe Ile Pro Leu Arq		
420			425			430	Met Ala Glu Glu Glu Leu Gly Ile Gly Asp Asp Ala Ala His Gly Glu		
435			440			445	Glu Ala Tyr Ala Leu Trp Gly Asp Gly Glu Lys Phe Asp Ala Thr Arg		
450			455			460	His Val Gln Gln Phe Glu Arg Asp Gln Glu Ala Ala His Pro Ser Tyr		
465			Val His Gly Ala Arg Gly Val Thr Ile Val Leu 470			475			

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- Continued

65			70	Ala Met Leu Cys Ala Gly Ser Val Arg Ala Lys Asn Thr Met Asn Ile		75			80
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100			105	Leu Phe Gly Tyr Ala Phe Ala Phe Gly Glu Ser Ser Asp Gly Phe Ile		110			
115			120	Gly Arg His Asn Phe Gly Leu Gln Asn Phe Pro Thr Leu Thr Ser Asp		125			
130			135	Tyr Ser Phe Phe Leu Tyr Gln Trp Ala Phe Ala Ile Ala Ala Ala Gly		140			
145			150	Ile Thr Ser Gly Ser Ile Ala Glu Arg Thr Lys Phe Val Ala Tyr Leu		155			160
165			170	Ile Tyr Ser Ser Phe Leu Thr Gly Phe Val Tyr Pro Val Val Ser His		175			
180			185	Trp Phe Trp Ser Pro Asp Gly Trp Ala Ser Pro Phe Arg Ser Glu Asp		190			
195			200	Arg Leu Phe Gly Thr Gly Ala Ile Asp Phe Ala Gly Ser Gly Val Val		205			
210			215	His Met Val Gly Gly Ile Ala Gly Leu Trp Gly Ala Leu Ile Glu Gly		220			
225			230	Pro Arg Ile Gly Arg Phe Pro Asp Gly Gly His Ala Ile Ala Leu Arg		235			240
245			250	Gly His Ser Ala Ser Leu Val Val Leu Gly Thr Phe Leu Leu Trp Phe		255			
260			265	Gly Trp Tyr Gly Phe Asn Pro Gly Ser Phe Thr Lys Ile Leu Ile Pro		270			
275			280	Tyr Asn Ser Gly Ser Asn Tyr Gly Gln Trp Ser Gly Ile Gly Arg Thr		285			
290			295	Ala Val Thr Thr Thr Leu Ser Gly Cys Thr Ala Ala Leu Thr Thr Leu		300			
305			310	Phe Gly Lys Arg Leu Leu Ser Gly His Trp Asn Val Thr Asp Val Cys		315			320
325			330	Asn Gly Leu Leu Gly Gly Phe Ala Ala Ile Thr Ala Gly Cys Ser Val		335			
340			345	Val Asp Pro Trp Ala Ala Ile Val Cys Gly Phe Val Ala Ser Leu Val		350			
355			360	Leu Ile Gly Cys Asn Lys Leu Ala Glu Leu Leu Lys Tyr Asp Asp Pro		365			
370			375	Leu Glu Ala Ala Gln Leu His Gly Gly Cys Gly Ala Trp Gly Leu Ile		380			
				Phe Val Gly Leu Phe Ala Lys Glu Lys Tyr Ile Asn Glu Val Tyr Gly		395			
385			390	Ala Ser Pro Gly Arq His Tyr Gly Leu Phe Met Gly Gly Gly Gly Lys					400
405			410	Leu Leu Gly Ala Gln Leu Val Gln Ile Ile Val Ile Val Gly Trp Val		415			
420			425	Ser Ala Thr Met Gly Thr Leu Phe Phe Ile Leu Lys Lys Leu Asn Leu		430			
435			440	Leu Arg Ile Ser Glu Gln His Glu Met Arg Gly Met Asp Leu Ala Gly		445			
450			455	His Gly Gly Phe Ala Tyr Ile Tyr His Asp Asn Asp Asp Asp Ser Ile		460			

42

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50 60 60 Asn Thr Met Asn Ile Met Leu. Thr Asn. Val Leu. Asp Ala Ala Ala Gly 65 70 70 75 75 80 Ala Leu Phe Tyr Tyr Leu Phe Gly Phe Ala Phe Ala Phe Gly Thr Pro 85 90 95 Ser Asn Gly Phe Ile Gly Lys Gln Phe Phe Gly Leu Lys His Leu Pro 100 105 Arg Thr Gly Phe Asp Tyr Asp Phe Phe Leu Tyr Gln Trp Ala Phe Ala
115 20 125 Ile Ala Ala Ala Gly Ile Thr Ser Gly Ser Ile Ala Glu Arg Thr Gln 130 135 Phe Val Ala Tyr Leu Ile Tyr Ser Ala Phe Leu Thr Gly Phe Val Tyr 145 160 Pro Val Val Ser His Trp Phe Trp Ser Ala Asp Gly Trp Ala Gly Ala
165 170 175 Ser Arg Thr Ser Gly Pro Leu Leu Phe Gly Ser Gly Val Ile Asp Phe 180 190 Ala Gly Ser Gly Val Val His Met Val Gly Gly Ile Ala Gly Leu Trp 200 205 Gly Ala Leu Ile Glu Gly Pro Arg Ile Gly Arg Phe Asp His Ala Gly 210 215 220 Arg Ser Val Ala Leu Lys Gly His Ser Ala Ser Leu Val Val Leu Gly 225 230 230 Thr Phe Leu Leu Trp Phe Gly Trp Tyr Gly Phe Asn Pro Gly Ser Phe 245 250 255 Thr Thr Ile Leu Lys Ser Tyr Gly Pro Ala Gly Thr Val His Gly Gln

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265 260	270	
275 280	Trp Ser Ala Val Gly Arg Thr Ala Val Thr Thr Thr Leu Ala Gly Ser 285	
290 295	Val Ala Ala Leu Thr Thr Leu Phe Gly Lys Arg Leu Gln Thr Gly His 300	
305 310	Trp Asn Val Val Asp Val Cys Asn Gly Leu Leu Gly Gly Phe Ala Ala 315 320	
325 330	Ile Thr Ala Gly Cys Ser Val Val Glu Pro Trp Ala Ala Val Ile Cys 335	
340 345	Gly Phe Val Ser Ala Trp Val Leu Ile Gly Ala Asn Ala Leu Ala Ala 350	
355 360	Arg Phe Arg Phe Asp Asp Pro Leu Glu Ala Ala Gln Leu His Gly Gly 365	
370 375	Cys Gly Ala Trp Gly Val Leu Phe Thr Gly Leu Phe Ala Arg Arg Lys 380	
385 390	Tyr Val Glu Glu Ile Tyr Gly Ala Gly Arg Pro Tyr Gly Leu Phe Met 395 400	
405 410	Gly Gly Gly Gly Lys Leu Leu Ala Ala Gln Ile Ile Gln Ile Leu Val 415	
420 425	Ile Ala Gly Trp Val Ser Cys Thr Met Gly Pro Leu Phe Tyr Ala Leu 430	
435 440	Lys Lys Leu Gly Leu Leu Arg Ile Ser Ala Asp Asp Glu Met Ser Gly 445	
450 455	Met Asp Leu Thr Arg His Gly Gly Phe Ala Tyr Val Tyr His Asp Glu 460	
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	180 ctaccagage tegteggegt egeeggaetg getgaacaag ggegacaatg egtggeagat	
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	540 ctacceggee gecaccatgg tgtactteca gtgegtgtte gecageatea eegteateat	
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Thr Arg His Gly Gly Phe Ala Tyr Ala Tyr His Asp Asp Asp Leu Ser 260	265	270		
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ctcttctact acctattcgg cttcgccttc gcgtacggga ccccgtccaa cggcttcatc				300
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cgcacgcagt tegtggegta ecteatetae teegeettee teaeeggett egtgtaeeeg				480
gtggtgtece aetgggtetg gteegeegae ggetgggeet egeegteaeg gaegtegggg				540
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<u> 1980 - John Stein, amerikansk politiker (</u>

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85 90 95 Ala Arg Pro Ala Leu Ser Gln Gly Gly Leu Val Gly Gln Ala Gly Leu
100 0 105 110 Pro Ala Thr Ala His His Phe Ala Ser Gly Ala Leu Glu Thr Pro Ala
115 20 20 20 Ala Glu Pro Leu Tyr Pro Met Ala Thr Val Val Tyr Phe Gln Cys Val 130 Phe Ala Ala Ile Thr Leu Val Leu Val Ala Gly Ser Leu Leu Gly Arg 145 150 160 Met Ser Phe Ala Ala Trp Met Leu Phe Val Pro Leu Trp Leu Thr Phe
165 170 175 Ser Tyr Thr Val Gly Ala Phe Ser Val Trp Gly Gly Gly Phe Leu Phe 8O 85 90 Gln Trp Gly Val Ile Asp Tyr Cys Gly Gly Tyr Val Ile His Leu Ser 200 205 Ala Gly Phe Ala Gly Phe Thr Ala Ala Tyr Trp Val Gly Pro Arg Ala
210 215 220 Gln Lys Asp Arg Glu Arg Phe Pro Pro Asn Asn Ile Leu Phe Thr Leu. 225 230 230 Thr Gly Ala Gly Leu Leu Trp Met Gly Trp Ala Gly Phe Asn Gly Gly 245 250 255 Gly Pro Tyr Ala Ala Asn Val Val Ala Ser Met Ser Val Leu Asn Thr 260 265 270 Asn Ile Cys Thr Ala Met Ser Leu Leu Val Trp Thr Cys Leu Asp Val 275 280 285 Val Phe Phe Lys Lys Pro Ser Val Val Gly Ala Val Gln Gly Met Ile 290 295 300

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Thr Gly Leu Val Cys Ile Thr Pro Ala Ala
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405	410	Gly Gly Gly Gly Lys Leu Leu Ala Ala His Val Ile Gln Ile Leu Val 415			
420	425	Ile Phe Gly Trp Val Ser Cys Thr Met Gly Pro Leu Phe Tyr Gly Leu 430			
435	440	Lys Lys Leu Gly Leu Leu Arg Ile Ser Ala Glu Asp Glu Thr Ser Gly 445			
450	455	Met Asp Leu Thr Arg His Gly Gly Phe Ala Tyr Val Tyr His Asp Glu 460			
465	470	Asp Glu His Asp Lys Ser Gly Val Gly Gly Phe Met Leu Arg Ser Ala 475		480	
485	490	Gln Thr Arg Val Glu Pro Ala Ala Ala Gly Cys Leu Gln Gln Gln Gln 495			
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		agccgtgcag ctgtggtgga gtgaccacgg ccacgactcc gtgcgcgcgg gtggacgtaa			180
		gcgttgggcc ctcggctcgc gcgcgcggcc gcatccggcg atgcatcggt cgcgttcgcg			240
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		cttgttgacg tccacgagtt ggcgagttgc tctgttcctc tctcgcgcgc gccgcagata			360
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		cccgtgaaaa gtttcagaaa agcattacaa agcttcagat aagttcaggg gtgactgaaa			540
		tacacataca acaagtaacg tagagagatc cccaaatcag ctgcggcaga aggcagaaac			600
		cgtgactagt acatctcata aacttaacga gcagtacaat ttctgtacat tggtttatca			660 720
		ataagtcaag agtagcattt gggtaagaag agaaaaaaaa tcttttacgg tggcgtttat tgacatttga teeetggage egagaagaet agtttatete ateegtgaaa aetatttgte			780
		actagacatc aacgtetege tgaggacace eggtttgeaa tttgetaata agaaacacte			840
		gtttccgtcc aatggcgatt cgtttactag agatccgtcc attctctgaa cttctgaagg			900
		tcaaccttct gatatgcata caggtgtggt agcaggcacg acaaaagtat aaaacaatag			960
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60

62

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245	Phe Leu Leu Trp Phe Gly Trp Tyr Gly Phe Asn Pro Gly Ser Phe Leu 250 255	
260	Thr Ile Leu Lys Ser Tyr Gly Pro Pro Gly Ser Ile His Gly Gln Trp 265 270	
275	Ser Ala Val Gly Arg Thr Ala Val Thr Thr Thr Leu Ala Gly Ser Thr 280 285	
290	Ala Ala Leu Thr Thr Leu Phe Gly Lys Arg Leu Gln Thr Gly His Trp 295 300	
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340	Phe Val Ser Ala Trp Val Leu Ile Gly Leu Asn Ala Leu Ala Ala Arg 345 350	
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385	Val Asp Gln Ile Phe Gly Gln Pro Gly Arg Pro Tyr Gly Leu Phe Met 390 395	400
405	Gly Gly Gly Gly Arg Leu Leu Gly Ala His Ile Val Val Ile Leu Val 410 415	
420	Ile Ala Ala Trp Val Ser Phe Thr Met Ala Pro Leu Phe Leu Val Leu 425 430	
435	Asn Lys Leu Gly Leu Leu Arg Ile Ser Ala Glu Asp Glu Met Ala Gly 440 445	
450	Met Asp Gln Thr Arg His Gly Gly Phe Ala Tyr Ala Tyr His Asp Asp 455 460	
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	ggcgcggtcg gtccgaggac ggagaaggac agggaggcgt tcccgccgaa caacgtcctg	180
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	gcgccgtacg ccgccaacgt cgacgcgtcg gtcaccgtcg tgaacacgca cctctgcacg	300

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66
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35 40 45 Val Lys Lys Lys Trp Ala Val Asn. Ser Ala Phe Met Ala Leu. Tyr Ala 50 Tyr Ala Ser Ser Leu Leu Val Trp Val Leu Val Gly Phe Arg Met Ala
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100 0 105 110 Thr Ala His Tyr Gly Lys Asp Gly Ala Leu Glu Ser Pro Arg Thr Glu 115 20 Pro Phe Tyr Pro Glu Ala Ser Met Val Leu Phe Gln Phe Glu Leu Ala
130 3140 35 Ala Ile Thr Leu Val Leu Leu Ala Gly Ser Leu Leu Gly Arg Met Asn 145 55 160 Ile Lys Ala Trp Met Ala Phe Thr Pro Leu Trp Leu Leu Phe Ser Tyr 165 170 175 Thr Val Cys Ala Phe Ser Leu Trp Gly Gly Gly Phe Leu Tyr Gln Trp 180 190 Gly Val Ile Asp Tyr Ser Gly Gly Tyr Val Ile His Leu Ser Ser Gly
200 205 Ile Ala Gly Phe Thr Ala Ala Tyr Trp Val Gly Pro Arg Leu Lys Ser 210 215 Asp Arg Glu Arg Phe Ser Pro Asn Asn Ile Leu Leu Met Ile Ala Gly 225 230 230 Gly Gly Leu Leu. Trp Leu. Gly Trp Ala Gly Phe Asn Gly Gly Ala Pro 245 250 255 Tyr Ala Pro Asn Ile Thr Ala Ser Ile Ala Val Leu Asn. Thr Asn. Val 260 265 270

 $72\,$

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165		170			175				
Leu Trp Leu Thr Phe Ser Tyr Thr Val Gly Ala Phe Ser Leu Trp Gly 180		185			190				
Gly Gly Phe Leu Phe His Trp Gly Val Met Asp Tyr Ser Gly Gly Tyr 195		200			205				
Val Ile His Leu Ser Ser Gly Val Ala Gly Phe Thr Ala Ala Tyr Trp 210		215			220				
Val Gly Pro Arg Ser Thr Lys Asp Arg Glu Arg Phe Pro Pro Asn Asn 225		230			235			240	
Val Leu Leu Met Leu Thr Gly Ala Gly Ile Leu Trp Met Gly Trp Ala 245		250			255				
Gly Phe Asn Gly Gly Asp Pro Tyr Ser Ala Asn Ile Asp Ser Ser Leu 260		265			270				
Ala Val Leu Asn Thr Asn Ile Cys Ala Ala Thr Ser Leu Leu Val Trp 275		280			285				
Thr Cys Leu Asp Val Ile Phe Phe Lys Lys Pro Ser Val Ile Gly Ala 290		295			300				
Val Gln Gly Met Ile Thr Gly Leu Val Cys Ile Thr Pro Gly Ala Gly 305		310			315			320	
Leu Val Gln Gly Trp Ala Ala Ile Val Met Gly Ile Leu Ser Gly Ser 325		330			335				
Ile Pro Trp Phe Thr Met Met Val Val His Lys Arg Ser Arg Leu Leu 340		345			350				
Gln Gln Val Asp Asp Thr Leu Gly Val Phe His Thr His Ala Val Ala 355		360			365				
Gly Phe Leu Gly Gly Ala Thr Thr Gly Leu Phe Ala Glu Pro Val Leu 370		375			380				
Cys Ser Leu Phe Leu Pro Val Thr Asn Ser Arg Gly Ala Phe Tyr Pro 385		390			395			400	
Gly Arg Gly Gly Gly Leu Gln Phe Val Arg Gln Val Ala Gly Ala Leu 405		410			415				
Phe Ile Ile Cys Trp Asn Val Val Val Thr Ser Leu Val Cys Leu Ala 420		425			430				
Val Arg Ala Val Val Pro Leu Arg Met Pro Glu Glu Glu Leu Ala Ile 435		440			445				
Gly Asp Asp Ala Val His Gly Glu Glu Ala Tyr Ala Leu Trp Gly Asp 450		455			460				
Gly Glu Lys Tyr Asp Ser Thr Lys His Gly Trp Tyr Ser Asp Asn Asn 465		470			475			480	
Asp Thr His His Asn Asn Asn Lys Ala Ala Pro Ser Gly Val Thr Gln 485		490			495				
Asn Val									
<210> SEO ID NO 47 <211> LENGTH: 1497 <212> TYPE: DNA <213> ORGANISM: Oryza sativa									
<400> SEQUENCE: 47									
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gactacctgt gcaaccggtt cgccgacacg acgtcggcgg tggacgcgac gtacctgctc								120	

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115			120			125			
130	Ile Ala Ala Ala Gly Ile Thr Ser Gly Ser Ile Ala Glu Arg Thr Gln		135			140			
145	Phe Val Ala Tyr Leu Ile Tyr Ser Ala Phe Leu Thr Gly Phe Val Tyr		150			155			160
165	Pro Val Val Ser His Trp Ile Trp Ser Ala Asp Gly Trp Ala Ser Ala		170			175			
180	Ser Arg Thr Ser Gly Pro Leu Leu Phe Gly Ser Gly Val Ile Asp Phe		185			190			
195	Ala Gly Ser Gly Val Val His Met Val Gly Gly Val Ala Gly Leu Trp		200			205			
210	Gly Ala Leu Ile Glu Gly Pro Arg Ile Gly Arg Phe Asp His Ala Gly		215			220			
225	Arg Ser Val Ala Leu Lys Gly His Ser Ala Ser Leu Val Val Leu Gly		230			235			240
245	Thr Phe Leu Leu Trp Phe Gly Trp Tyr Gly Phe Asn Pro Gly Ser Phe		250			255			
260	Thr Thr Ile Leu Lys Thr Tyr Gly Pro Ala Gly Gly Ile Asn Gly Gln		265			270			
275	Trp Ser Gly Val Gly Arg Thr Ala Val Thr Thr Thr Leu Ala Gly Ser		280			285			
290	Val Ala Ala Leu Thr Thr Leu Phe Gly Lys Arg Leu Gln Thr Gly His		295			300			
305	Trp Asn Val Val Asp Val Cys Asn Gly Leu Leu Gly Gly Phe Ala Ala		310			315			320
325	Ile Thr Ala Gly Cys Ser Val Val Asp Pro Trp Ala Ala Ile Ile Cys		330			335			
340	Gly Phe Val Ser Ala Trp Val Leu Ile Gly Leu Asn Ala Leu Ala Ala		345			350			
355	Arg Leu Lys Phe Asp Asp Pro Leu Glu Ala Ala Gln Leu His Gly Gly		360			365			
370	Cys Gly Ala Trp Gly Ile Leu Phe Thr Ala Leu Phe Ala Arg Gln Lys		375			380			
385	Tyr Val Glu Glu Ile Tyr Gly Ala Gly Arg Pro Tyr Gly Leu Phe Met		390			395			400
405	Gly Gly Gly Gly Lys Leu Leu Ala Ala His Val Ile Gln Ile Leu Val		410			415			
420	Ile Phe Gly Trp Val Ser Cys Thr Met Gly Pro Leu Phe Tyr Gly Leu		425			430			
435	Lys Lys Leu Gly Leu Leu Arg Ile Ser Ala Glu Asp Glu Thr Ser Gly		440			445			
450	Met Asp Leu Thr Arg His Gly Gly Phe Ala Tyr Val Tyr His Asp Glu		455			460			
465	Asp Glu His Asp Lys Ser Gly Val Gly Gly Phe Met Leu Arg Ser Ala		470			475			480
485	Gln Thr Arq Val Glu Pro Ala Ala Ala Ala Ala Ser Asn Ser Asn Asn		490			495			
	Gln Val								
	<210> SEO ID NO 49								

 $<$ 211> LENGTH: 438

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 $80\,$

SEQ ID NO 53
LENGTH: 1853 TYPE: DNA ORGANISM: Oryza sativa 1853

SEQUENCE: 53

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325	330	335	
340	345	Leu His Lys Lys Trp Ser Phe Met Gln Arg Ile Asp Asp Thr Leu Gly 350	
355	360	Val Phe His Thr His Ala Val Ala Gly Phe Leu Gly Gly Ala Thr Thr 365	
370	375	Gly Leu Phe Ala Glu Pro Ile Leu Cys Ser Leu Phe Leu Ser Ile Pro 380	
385	390	Asp Ser Lys Gly Ala Phe Tyr Gly Gly Pro Gly Gly Ser Gln Phe Gly 400 395	
405	410	Lys Gln Ile Ala Gly Ala Leu Phe Val Thr Ala Trp Asn Ile Val Ile 415	
420	425	Thr Ser Ile Ile Cys Val Ile Ile Ser Leu Ile Leu Pro Leu Arg Ile 430	
435	440	Ala Asp Gln Glu Leu Leu Ile Gly Asp Asp Ala Val His Gly Glu Glu 445	
450	455	Ala Tyr Ala Ile Trp Ala Glu Gly Glu Leu Asn Asp Met Thr His His 460	
465	470	Asn Glu Ser Thr His Ser Gly Val Ser Val Gly Val Thr Gln Asn Val 475 480	
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		gteeeeegea agtgggeget eaeeteegea tteatggege tetaegeeat ggeegeeaee	180
		atgeegtget gggegetetg ggegeaeaae atggeetteg geegeegeet eeteeeette	240
		gteggeegee eegeeeegge getegeeeag gaetaeatge teageeagge getgeteeee	300
		tecaecetee aeeteegete caaeggegag gttgagaegg eegeggtgge geegetgtae	360
		ccgtcggcga gcatggtgtt cttccagtgg gccttcgccg gcgtcaccgt ggggctggtc	420
		gccggcgccg tgctcgggcg catgagcgtc aaggcgtgga tggcgttcgt gccgctgtgg	480
		acgacgctgt cctacacggt gggagcgtac agcatctggg gcggaggctt cctcttccac	540
		tggggggtca tggactacte eggeggetae gtegtgetee tegeegeegg egteteegge	600
		tacacggccg cgtactgggt gggacccagg aggaaggagg aggacgagga ggaaatggca	660
		acggcgagtg gtggcaacct ggtggtgatg gtggccggcg cgggcatcct gtggatgggg	720
		tggaccgget teaaeggegg egaceeette teegeeaaea eegactegte ggtggeggtg	780
		ctcaacacgc acatctgcgc caccaccagc atcgtcgctt gggtttgctg cgacgtcgcc	840
		gtccgcggga ggccgtcggt ggtgggcgcg gtgcagggca tgatcaccgg cctggtgtgc	900
		atcactccaa ggtcaaacat caagtacagc tttcttctag tagtaatttc tgatgagatg	960
cctgttcctg atctgagcta g			981
<210> SEQ ID NO 58 2211 T.FNCTH. 226			

<211> LENGTH: 326
<212> TYPE: PRT
<213> ORGANISM: Oryza sativa

<4 OO SEQUENCE: 59

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65				70			75			80
85				90	Phe Gly Glu Glu Leu Leu Pro Phe Trp Gly Lys Gly Ala Pro Ala Leu		95			
	100			105	Gly Gln Lys Phe Leu Thr Lys Arg Ala Val Val Asn Glu Thr Ile His		110			
	115			120	His Phe Asp Asn Gly Thr Val Glu Ser Pro Pro Glu Glu Pro Phe Tyr		125			
	130			135	Pro Met Ala Ser Leu Val Tyr Phe Gln Phe Thr Phe Ala Ala Ile Thr		140			
	145			150	Leu Ile Leu Leu Ala Gly Ser Val Leu Gly Arg Met Asn Ile Lys Ala		155			160
	165			170	Trp Met Ala Phe Val Pro Leu Trp Leu Ile Phe Ser Tyr Thr Val Gly		175			
	180			185	Ala Phe Ser Leu Trp Gly Gly Gly Phe Leu Tyr Gln Trp Gly Val Ile		190			
	195			200	Asp Tyr Ser Gly Gly Tyr Val Ile His Leu Ser Ser Gly Ile Ala Gly		205			
	210			215	Phe Thr Ala Ala Tyr Trp Val Gly Pro Arg Leu Lys Ser Asp Arg Glu		220			
	225			230	Arg Phe Pro Pro Asn Asn Val Leu Leu Met Leu Ala Gly Ala Gly Leu		235			240
	245			250	Leu Trp Met Gly Trp Ser Gly Phe Asn Gly Gly Ala Pro Tyr Ala Ala		255			
	260			265	Asn Ile Ala Ser Ser Ile Ala Val Leu Asn Thr Asn Ile Cys Ala Ala		270			
	275			280	Thr Ser Phe Leu Val Trp Thr Thr Leu Asp Val Ile Phe Phe Gly Lys		285			
	290			295	Pro Ser Val Ile Gly Ala Val Gln Gly Met Met Thr Gly Leu Val Cys		300			
	305			310	Ile Thr Pro Gly Ala Gly Leu Val His Ser Trp Ala Val Ile Val Met		315			320
	325			330	Gly Ile Leu Phe Gly Ser Ile Pro Trp Val Thr Met Met Ile Leu His		335			
	340			345	Lys Lys Ser Thr Leu Leu Gln Lys Val Asp Asp Thr Leu Gly Val Phe		350			
	355			360	His Thr His Ala Val Ala Gly Leu Leu Gly Gly Leu Leu Thr Gly Leu		365			
	370			375	Leu Ala Glu Pro Ala Leu Cys Arg Leu Leu Leu Pro Val Thr Asn Ser		380			
					Arg Gly Ala Phe Tyr Gly Gly Gly Gly Gly Val Gln Phe Phe Lys Gln					
	385			390	Leu Val Ala Ala Met Phe Val Ile Gly Trp Asn Leu Val Ser Thr Thr		395			400
	405			410	Ile Ile Leu Leu Val Ile Lys Leu Phe Ile Pro Leu Arq Met Pro Asp		415			
	420			425	Glu Gln Leu Glu Ile Gly Asp Asp Ala Val His Gly Glu Glu Ala Tyr		430			
	435			440	Ala Leu Trp Gly Asp Gly Glu Lys Tyr Asp Pro Thr Arg His Gly Ser		445			
	450			455	Leu Gln Ser Gly Asn Thr Thr Val Ser Pro Tyr Val Asn Gly Ala Arg		460			
	465			470			475			480

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Gly Val Thr Ile Asn Leu
485

<210> SEQ ID NO 63
<211> LENGTH: 2191
<212> TYPE: DNA
<212> ORGANISM: Glycine max

<400> SEQUENCE: 63

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305 310 315 320	
Asp Val Cys Asn Gly Leu Leu Gly Gly Phe Ala Ala Ile Thr Ala Gly 330 325 335	
Cys Ser Val Val Glu Pro Trp Ala Ala Ile Val Cys Gly Phe Val Ala 345 350 340	
Ser Ile Val Leu Ile Ala Cys Asn Lys Leu Ala Glu Lys Val Lys Phe 355 360 365	
Asp Asp Pro Leu Glu Ala Ala Gln Leu His Gly Gly Cys Gly Thr Trp 375 380 370	
Gly Val Ile Phe Thr Ala Leu Phe Ala Lys Lys Glu Tyr Val Lys Glu 395 390 400 385	
Val Tyr Gly Leu Gly Arg Ala His Gly Leu Leu Met Gly Gly Gly Gly 415 405 410	
Lys Leu Leu Ala Ala His Val Ile Gln Ile Leu Val Ile Ala Gly Trp 425 430 420	
Val Ser Ala Thr Met Gly Pro Leu Phe Trp Gly Leu Asn Lys Leu Lys 440 435 445	
Leu Leu Arg Ile Ser Ser Glu Asp Glu Leu Ala Gly Met Asp Met Thr 450 455 460	
Arq His Gly Gly Phe Ala Tyr Ala Tyr Glu Asp Asp Glu Thr His Lys 465 470 475 480	
His Gly Met Gln Leu Arg Arg Val Gly Pro Asn Ala Ser Ser Thr Pro 485 490 495	
Thr Thr Asp Glu 500	
<210> SEQ ID NO 65 <211> LENGTH: 800 <212> TYPE: DNA <213> ORGANISM: Glycine max	
<400> SEQUENCE: 65	
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aaccatgteg etgeeagatt gteeegeegt eeaacttgee eaacteetgg geecaaatac	120
cacaaacgct gccgccgccg cctccttcat ctgcgaccgg ttcaccgccg tggacaacaa	180
gttegtegae aeggeetteg eggtegaeaa eaettaeete etetteteeg eetaeetegt	240
cttctcgatg cagctcggct tcgccatgct ctgcgccggc tccgtccgcg ccaagaacac	300
catgaacatc atgeteacca acgtectega egeegeegee ggeggeetet tetactacet	360
ctteggette geettegeet teggeteeee etecaaegge tteattggea aacaettett	420
cggcctcaag gaactcccct cccaaagctt cgactacagc aactttctct atcaatgggc	480
cttegecate geegeegeeg geateaceag eggetecate geegaaegea eacagttegt	540
ggcctatctc atctactcct ccttcctcac eggcttegtc tacccegteg teteccactg	600
gttetggtee geagaegget gggettetge eattteeeee ggagaeegge tatttteeae	660
cggcgtgata gacttcgccg gctccggcgt agtccacatg gttggtggag tagccggctt	720
ctggggcgca ctgatagaag gcccgagaat cggacgcttc gaccacgcgg gacgcgccgt	780
tgccctcaga ggccacagcg	800

<210> SEQ ID NO 66

agaaaaaatg ggcagtgaac tcagctttca tggctctcta cgcctttgcg gcggttctaa 480 tatgttgggt gettgtgtgt tacegcatgg ectttggaga aaaactttta ecettetggg 540 ggaagggtgc tcccagactt aggccagaat tcgtcacaaa acgagccgga gtcaatgaaa 600 cgctgcacca ctttgatagt ggcactgtag aatcccctcg cgaagagcca ctttacccta 660 atggcgtact tgtgtatgtc cgattgactt ttgctgctat gtaccatata gtgatggctg 720 gctctgtgct gccacgaaga acatcgaag 749 <210> SEQ ID NO 70 $<$ 211> LENGTH: 159 $<$ 212> TYPE: PRT <213> ORGANISM: Glycine max <400> SEQUENCE: 70 Met Ala Thr Pro Leu Ala Tyr Gln Glu His Leu Pro Ala Ala Pro Glu 10 15 Trp Leu Asn Lys Gly Asp Asn Ala Trp Gln Leu Thr Ala Ala Thr Leu 20 25 30 Val Gly Leu Gln Ser Met Pro Gly Leu Val Ile Leu Tyr Ala Ser Ile 35 45 40 Val Lys Lys Lys Trp Ala Val Asn Ser Ala Phe Met Ala Leu Tyr Ala 50 55 60 Phe Ala Ala Val Leu Ile Cys Trp Val Leu Val Cys Tyr Arg Met Ala 70 75 65 80 Phe Gly Glu Lys Leu Leu Pro Phe Trp Gly Lys Gly Ala Pro Arg Leu 85 90 Arg Pro Glu Phe Val Thr Lys Arg Ala Gly Val Asn Glu Thr Leu His 100 105 110 His Phe Asp Ser Gly Thr Val Glu Ser Pro Arg Glu Glu Pro Leu Tyr 115 120 125 Pro Asn Gly Val Leu Val Tyr Val Arg Leu Thr Phe Ala Ala Met Tyr 130 135 140 His Ile Val Met Ala Gly Ser Val Leu Pro Arg Arg Thr Ser Lys 145 150 155 <210> SEQ ID NO 71 <211> LENGTH: 1871 $<$ 212> TYPE: DNA <213> ORGANISM: Glycine max <400> SEOUENCE: 71 ctctaacage caaagcatgg cttctctctc ttgctccgcc aacgaccttg ccccactctt 60 caacgacacc geegeegeea actacetetg egeecaatte gattecattt etagaaaget 120 cgccgaaaca acctacgccg tcgacaacac ctaccttctg ttttcagcgt atcttgtctt 180 cgccatgcag ctcggcttcg ccatgctctg cgccggctcc gtcagagcca aaaacaccat 240 gaacatcatg ctcaccaacg tectegaege egeegeegge ggteteteet actacctatt 300 eggetttgea ttegeetteg geggeeeete eaaeggette ateggeegee aettettegg 360 cctacgagat tacccaatgg getectetec etceggegae tacagettet tectetacca 420 480 gtgggccttc gccatcgccg ccgcaggaat caccagcggc tccatcgccg agagaacaca gttegtgget taeettatet aetettettt ettaaeeggt ttegtttaee eeategttte 540


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130 135 <210> SEO ID NO 75 <211> LENGTH: 799 $<$ 212> TYPE: DNA <213> ORGANISM: Glycine max <400> SEOUENCE: 75 gtgtgtggtt ttgtcgcttc agtgtttctg atagcgtgca acaaattagc agagaaggtt 60 aagttcgatg atcctttgga agcggcgcag ttacacggtg ggtgtggcgc gtggggggtg 120 180 gcgcacgggt tgttcatgag gggtggaggg aagttgctgg cggcgcacgt gattcagatt 240 ttggttattg ttgggtgggt gagtgcgacc atgggaccct tgttttgggg gttgaataaa 300 ttgaaattgt tgaggattte tteegaggat gagettgegg ggatggatet taeeegteat 360 ggaggatttg cttatgctta tgaggatgat gagtcgcaca agcatgggat tcagctgagg 420 aaggttgggc ccaacgcgtc gtccacaccc accactgatg aatgattacg atcacgatta 480 atteggeeee gacagtatta tetteaattg aaattaegtg tgaettagaa gaagaaaaaa 540 agatgatgat gattttgttt gtaatttatt ttatttgttt tgggtttttt ttttaatttt 600 gtagattttt ctttttatga tgggtaagta gggattttaa tttgtaattg ttattggccg 660 tatattggta gatgctggaa attgaagatt ctgctggaag atgcgaacgt ttctgaaaat 720 gatagatggc tgtggaaaat gaaaatattt tatttgtggg atttaatttt cgtagttttc 780 799 qccaaaaaaq aaqqaaqaq <210> SEQ ID NO 76 $<$ 211> LENGTH: 154 $<$ 212> TYPE: PRT <213> ORGANISM: Glycine max <400> SEOUENCE: 76 Val Cys Gly Phe Val Ala Ser Val Phe Leu Ile Ala Cys Asn Lys Leu $\mathbf 1$ - 5 10 15 Ala Glu Lys Val Lys Phe Asp Asp Pro Leu Glu Ala Ala Gln Leu His 20 25 30 Gly Gly Cys Gly Ala Trp Gly Val Ile Phe Thr Ala Leu Phe Ala Lys 35 40 45 Lys Glu Tyr Val Ser Gln Val Tyr Gly Glu Gly Arg Ala His Gly Leu 50 55 60 Phe Met Arg Gly Gly Gly Lys Leu Leu Ala Ala His Val Ile Gln Ile 70 75 65 80 Leu Val Ile Val Gly Trp Val Ser Ala Thr Met Gly Pro Leu Phe Trp 85 90 95 Gly Leu Asn Lys Leu Lys Leu Leu Arg Ile Ser Ser Glu Asp Glu Leu 100 105 110 Ala Gly Met Asp Leu Thr Arg His Gly Gly Phe Ala Tyr Ala Tyr Glu 115 120 125 Asp Asp Glu Ser His Lys His Gly Ile Gln Leu Arg Lys Val Gly Pro 130 135 140 Asn Ala Ser Ser Thr Pro Thr Thr Asp Glu 145 150

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What is claimed is:

1. An isolated polynucleotide selected from the group consisting of:

- a. a polynucleotide having at least 70% sequence identity, as determined by the GAP algorithm under default parameters, to the full length sequence of a polynucleotide selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81; wherein the polynucleotide encodes a polypeptide that functions as a modifier of AMT;
- b. a polynucleotide encoding a polypeptide selected from the group consisting of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80 or 82;
- c. a polynucleotide selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25,

27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81; and

d. A polynucleotide which is complementary to the polynucleotide of (a) , (b) or (c) .

2. A recombinant expression cassette, comprising the polynucleotide of claim 1, wherein the polynucleotide is operably linked, in sense or anti-sense orientation, to a promoter.

3. A host cell comprising the expression cassette of claim 2.

4. A transgenic plant comprising the recombinant expression cassette of claim 2.

5. The transgenic plant of claim 4, wherein said plant is a monocot.

6. The transgenic plant of claim 4, wherein said plant is a dicot.

7. The transgenic plant of claim 4, wherein said plant is selected from the group consisting of: maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, peanut, switchgrass, myscanthus, triticale and cocoa.

8. A transgenic seed from the transgenic plant of claim 4.

- 9. A method of modulating the AMT in plants, comprising:
- a. introducing into a plant cell a recombinant expression cassette comprising the polynucleotide of claim 1 operably linked to a promoter; and
- b. culturing the plant under plant cell growing conditions; wherein the
- AMT in said plant cell is modulated.

10. The method of claim 9, wherein the plant cell is from a plant selected from the group consisting of: maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, peanut, switchgrass, myscanthus, triticale and cocoa.

11. A method of modulating the AMT in a plant, comprising.

- a. introducing into a plant cell a recombinant expression cassette comprising the polynucleotide of claim 1 operably linked to a promoter;
- b. culturing the plant cell under plant cell growing conditions; and
- c. regenerating a plant form said plant cell; wherein the AMT in said plant is modulated.

12. The method of claim 11, wherein the plant is selected from the group consisting of: maize, soybean, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, peanut, switchgrass, myscanthus, triticale and cocoa.

13. A method of decreasing the AMT transporter polypeptide activity in a plant cell, comprising:

- a. providing a nucleotide sequence comprising at least 15 consecutive nucleotides of the complement of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81;
- b. providing a plant cell comprising an mRNA having the sequence set forth in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15,

17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79 or 81; and

c. introducing the nucleotide sequence of step (a) into the plant cell, wherein the nucleotide sequence inhibits expression of the mRNA in the plant cell.

14. The method of claim 13, wherein said plant cell is from a monocot.

15. The method of claim 14, wherein said monocot is maize, wheat, rice, barley, sorghum, switchgrass, myscanthus, triticale or rye.

16. The method of claim 13, wherein said plant cell is from a dicot.

17. The transgenic plant of claim 4, wherein the AMT transporter activity in said plant is decreased.

18. The transgenic plant of claim 17, wherein the plant has enhanced root growth.

19. The transgenic plant of claim 17, wherein the plant has increased seed size.

20. The transgenic plant of claim 17, wherein the plant has increased seed weight.

21. The transgenic plant of claim 17, wherein the plant has seed with increased embryo size.

22. The transgenic plant of claim 17, wherein the plant has increased leaf size.

23. The transgenic plant of claim 17, wherein the plant has increased seedling vigor.

24. The transgenic plant of claim 17, wherein the plant has enhanced silk emergence.

25. The transgenic plant of claim 17, wherein the plant has increased ear size.

26. The transgenic plant of claim 4, wherein the AMT transporter activity in said plant is increased.

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