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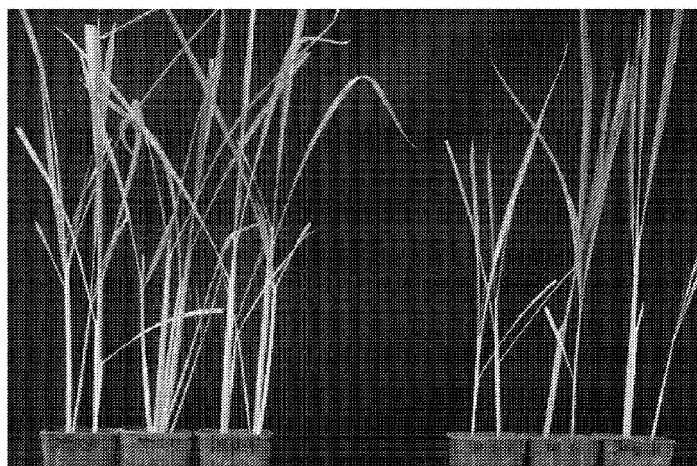
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[Continued on next page]

(54) Title: CONSTRUCTS AND METHODS INVOLVING GENES ENCODING GLUTAMATE RECEPTOR POLYPEPTIDES



DP0015

CK

Figure 5

(57) Abstract: The invention discloses isolated polynucleotides and polypeptides, and recombinant DNA constructs useful for conferring drought tolerance and/or improved nitrogen use efficiency; compositions (such as plants or seeds) comprising these recombinant DNA constructs; and methods utilizing these recombinant DNA constructs. The recombinant DNA constructs comprise a polynucleotide operably linked to a promoter that is functional in a plant, wherein said polynucleotides encode glutamate receptor polypeptides.



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CONSTRUCTS AND METHODS INVOLVING GENES ENCODING GLUTAMATE RECEPTOR POLYPEPTIDES

FIELD OF THE INVENTION

5 The field of the invention relates to plant breeding and genetics and, in particular, relates to recombinant DNA constructs useful in plants for conferring tolerance to abiotic stress, such as drought, and for improving nitrogen use efficiency.

BACKGROUND OF THE INVENTION

10 Abiotic stress is the primary cause of crop loss worldwide, causing average yield losses more than 50% for major crops (Boyer, J.S. (1982) *Science* 218:443-448; Bray, E.A. et al. (2000) In *Biochemistry and Molecular Biology of Plants*, edited by Buchannan, B.B. et al., Amer. Soc. Plant Biol., pp. 1158-1249). Among the various
15 abiotic stresses, drought is the major factor that limits crop productivity worldwide, and exposure of plants to a water-limiting environment during various developmental stages appears to activate various physiological and developmental changes. Although many reviews on molecular mechanisms of abiotic stress responses and genetic regulatory networks of drought stress tolerance have been published
20 (Valliyodan, B., and Nguyen, H.T. (2006) *Curr. Opin. Plant Biol.* 9:189-195; Wang, W., et al. (2003) *Planta* 218:1-14; Vinocur, B., and Altman, A. (2005) *Curr. Opin. Biotechnol.* 16:123-132; Chaves, M.M., and Oliveira, M.M. (2004) *J. Exp. Bot.* 55:2365-2384; Shinozaki, K., et al. (2003) *Curr. Opin. Plant Biol.* 6:410-417; Yamaguchi-Shinozaki, K., and Shinozaki, K. (2005) *Trends Plant Sci.* 10:88-94), it is
25 also a major challenge in biology to understand the basic biochemical and molecular mechanisms for drought stress perception, transduction and tolerance.

 Earlier work on molecular aspects of abiotic stress responses was accomplished by differential and/or subtractive analysis (Bray, E.A. (1993) *Plant Physiol.* 103:1035-1040; Shinozaki, K., and Yamaguchi-Shinozaki, K. (1997) *Plant Physiol.* 115:327-
30 334; Zhu, J.-K. et al. (1997) *Crit. Rev. Plant Sci.* 16:253-277; Thomashow, M.F. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50:571-599); and other methods which include selection of candidate genes and analysis of expression of such a gene or its active product under stresses, or by functional complementation in a

stressor system that is well defined (Xiong, L. and Zhu, J.-K. (2001) *Physiologia Plantarum* 112:152-166). Additionally, forward and reverse genetic studies involving the identification and isolation of mutations in regulatory genes have been used to provide evidence for observed changes in gene expression under stress (Xiong, L. and Zhu, J.-K. (2001) *Physiologia Plantarum* 112:152-166).

The absorption of nitrogen by plants plays an important role in their growth (Gallais et al. (2004), *J. Exp. Bot.* 55(396):295-306). Plants synthesize amino acids from inorganic nitrogen absorbed from environment, so, nitrogen fertilization has been a powerful tool for increasing yield of cultivated plants, such as maize. Lack of sufficient plant-available nitrogen for optimum growth and development may be considered as an abiotic stress. In order to avoid pollution by nitrates and to maintain a sufficient profit margin, today farmers desire to reduce the use of nitrogen fertilizer. If a plant variety has increased nitrogen assimilation capacity, it would also be expected to have increased growth and yield. In summary, plant varieties that have better nitrogen use efficiency (NUE) are desirable.

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

Glutamate receptors (GLR) can bind glutamate and function through ligand gated ion channels or G-protein coupled receptors in the major excitatory neurotransmitter in mammals. GLR-like genes have been identified in plants (Davenport R. (2002) *Annals of Botany* 90:549-557). GLRs may function in nutrient uptake, transport, and signaling (Davenport, *supra*), so manipulation of GLR genes may improve plant tolerance to stresses, particularly abiotic stress such as drought, cold, or low nitrogen.

SUMMARY OF THE INVENTION

The following embodiments are among those encompassed by the invention:

1. An isolated polynucleotide comprising: (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity, based on either Clustal V or GAP

method of alignment, to SEQ ID NO: 3; (b) a polynucleotide with nucleotide sequence of at least 85% sequence identity, based on either Clustal V or Gap method of alignment, to SEQ ID NO: 5; (c) a polynucleotide encoding a polypeptide with amino acid sequence of at least of 90% sequence identity, based on either
5 Clustal V or GAP method of alignment, to SEQ ID NO:4; (d) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO:6; or (e) the full complement of the nucleotide sequence of (a) or (b) or (c) or (d).

10 2. A recombinant DNA construct comprising the isolated polynucleotide of embodiment 1 operably linked to at least one regulatory sequence.

3. A plant or seed comprising a recombinant DNA construct, wherein the recombinant DNA construct comprises the polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes GLR protein having amino acid sequence of at least 90% sequence identity, based on either Clustal V or
15 GAP method of alignment, to SEQ ID NO:4 or SEQ ID NO:6.

4. A plant comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes GLR protein having amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID
20 NO:4 or SEQ ID NO:6, wherein said plant exhibits increased drought tolerance and/or improved nitrogen use efficiency (NUE) when compared to a control plant, and wherein said plant exhibits an increase in grain yield, biomass, or both, when compared to a control plant.

5. The plant of embodiment 4, wherein said plant exhibits said increase in grain
25 yield, biomass, or both when compared, under water limiting conditions or nitrogen limiting conditions, to said control plant.

6. The plant of embodiment 4, wherein said plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

30 7. Seed of the plant of embodiment 4, wherein said seed comprises in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes GLR protein having amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO:4 or SEQ ID NO:6, and

wherein a plant produced from said seed exhibits an increase in at least one trait selected from the group consisting of drought tolerance, grain yield and biomass, when compared to a control plant.

8. A rice plant comprising a recombinant transcriptional activator element enhancing the expression of an endogenous polynucleotide, wherein the polynucleotide encodes an amino acid sequence that is 90% identical to SEQ ID NO: 4 or SEQ ID NO: 6.

9. The rice plant of embodiment 8, wherein the transcriptional activator element is between positions 15760218 and 15760298 in Chromosome 9 of the rice genome.

10. The rice plant of embodiment 8, wherein the endogenous polynucleotide encodes the polypeptide of SEQ ID NO: 4 or SEQ ID NO: 6.

11. A method of improving drought tolerance or NUE comprising: (a) crossing the plant of embodiment 8 with a second rice plant to produce progeny seed; (b) harvesting and planting the progeny seed to produce at least one progeny plant of a subsequent generation which comprises the insertion between positions 15760218 and 15760298 in Chromosome 9 of the rice genome; (c) crossing the progeny plant with the second rice plant to produce at least one backcross progeny seed; and optionally (d) repeating steps (b) and (c) for additional generations to produce a plant with improved drought tolerance or NUE when compared to the second rice plant.

12. A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or SEQ ID NO: 6; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) obtaining a progeny plant derived from the transgenic plant of step (b), wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant.

13. A method of evaluating drought tolerance in a plant, comprising: (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein the polynucleotide encodes a polypeptide having an

amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or SEQ ID NO: 6; (b) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (c) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

14. A method of determining an alteration of grain yield, biomass, or both in a plant, comprising: (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 90% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or SEQ ID NO: 6; (b) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (c) measuring the grain yield and/or biomass of the progeny plant and comparing said measurement to the grain yield and/or biomass of a control plant.

15. The method of embodiment 14, wherein grain yield and/or biomass of the progeny plant and control plant are measured under water-limiting conditions, and wherein said grain yield and/or biomass of the progeny plant is increased relative to that of the control plant.

16. The method of any one of embodiment 11 to 15, wherein said plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

In another embodiment, the present disclosure concerns a recombinant DNA construct comprising any of the isolated polynucleotides of the present disclosure operably linked to at least one regulatory sequence, and a cell, a plant, or a seed comprising the recombinant DNA construct. The cell may be eukaryotic, e.g., a yeast, insect or plant cell; or prokaryotic, e.g., a bacterial cell.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTING

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

Figure 1 provides a schematic of the A-vector used to generate the rice activation-tagged population. Four copies of the CaMV 35S enhancer (4X CaMV 35S enhancer) are positioned next to the right border (RB), LTP2::RFP (red fluorescent protein) is positioned next to the left border (LB), UBI::hygromycin resistance gene (Hyg^r) is used as a selection marker, a 15-kb “spacer” is positioned between the 4X CaMV35S enhancer and the Hyg^r. Amp^r, ampicillin resistance gene; Hyg^r, hygromycin resistance gene; UBI, maize ubiquitin promoter; and LTP2, barley lipid transfer protein 2 promoter.

Figures 2A and 2B show the activated expression levels of GLR1 and GLR2 genes in different tissues of line AH01486 plants as revealed by real-time PCR analyses. Zhonghua11-WT, wild type of Zhonghua11; Zhonghua11-TC, Zhonghua11 from tissue culture without a construct; R, roots; S, stems; L, leaves. The numbers on top of the columns are the fold-changes compared to the control leaves. 2A. for GLR1 gene, AH01486 fold-changes in expression levels are 17.74 for roots, 3.95 for stems, and 53.83 for leaves; 2B. for GLR2 gene, AH01486 fold-changes in expression levels are 1.00 for roots, 1.45 for stems, and 38.45 for leaves.

Figure 3 provides GLR2 transgene expression levels in leaves of eight separate transgenic rice events compared to control (CK) by real-time PCR analyses. The base level of expression in CK event was set at 1.00, and the expression levels in other GLR2 events are shown as fold-increases compared to control.

Figure 4 provides GLR1 transgene expression levels in leaves of transgenic rice events compared to control (CK) by real-time PCR analyses. The base level of expression in CK event was set at 1.00, and the expression levels in other GLR1 events are shown as fold-increases compared to control.

Figure 5 provides photograph indicating more tillers of rice plants over-expressing GLR2 under CaMV 35S promoter (DP0015, T1 generation) than T1 rice plants introduced with vector control (CK, T1 generation), under low nitrogen conditions.

Figure 6A-C provides photographs and figures indicating that over-expression of rice GLR2 gene under CaMV 35S promoter in *Arabidopsis* (AtDP0015.05, AtDP0015.06) can significantly increase drought tolerance. A. GLR2 transgene expression levels in event AtDP0015.05 and AtDP0015.06; B. Survival rate on the 3rd day after re-watering; and C. photographs taken on the 3rd day after re-watering. WT, wild type Columbia; VC, vector control DP0009 transformant.

Figure 7A-C provides photographs and figures indicating that over-expression of rice GLR1 gene under CaMV 35S promoter in *Arabidopsis* (AtDP0025.13, AtDP0025.16) can significantly increase drought tolerance. A. GLR1 transgene expression levels in event AtDP0025.13 and AtDP0025.16; B. Survival rate on the 3rd day after re-watering; and C, photographs taken on the 3rd day after re-watering. WT, wild type Columbia; VC, vector control DP0009 transformant.

Table 1. SEQ ID NOs for nucleotide and amino acid sequences provided in the sequence listing

Table 2. Increased drought tolerance assays of AH01486 at T2 generation under greenhouse conditions

Table 3. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T0 generation under greenhouse conditions

Table 4. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse conditions

Table 5. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T2 generation under greenhouse conditions

Table 6. Enhanced drought tolerance of CaMV 35S Pro: GLR1-rice plants at T1 generation under greenhouse conditions

Table 7. Increased tiller number of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse low nitrogen conditions

Table 8. Increased SPAD value of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse low nitrogen conditions

Table 1. SEQ ID NOs for nucleotide and amino acid sequences provided in the sequence listing

Source species	Clone Designation	SEQ ID NO: (Nucleotide)	SEQ ID NO: (Amino Acid)
Oryza sativa	T-DNA flanking sequence in AH01486 (RB)	1	n/a
Oryza sativa	T-DNA flanking sequence in AH01486 (LB)	2	n/a
Oryza sativa	GLR1	3	4
Oryza sativa	GLR2	5	6
Artificial	Primers	7 - 28	n/a

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

DETAILED DESCRIPTION

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

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

As used herein:

“GLR polypeptide” refers to a glutamate receptor polypeptide. GLR1 and GLR2 are examples of GLR polypeptides. In this invention, GLR1 refers to the protein (SEQ ID NO: 4) encoded by SEQ ID NO: 3, and GLR2 refers to the protein (SEQ ID NO: 6) encoded by SEQ ID NO: 5.

The terms “monocot” and “monocotyledonous plant” are used interchangeably herein. A monocot of the current invention includes plants of the Gramineae family.

The terms “dicot” and “dicotyledonous plant” are used interchangeably herein. A dicot of the current invention includes the following families: Brassicaceae, Leguminosae, and Solanaceae.

The terms “full complement” and “full-length complement” are used interchangeably herein, and refer to a complement of a given nucleotide sequence, wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

An “Expressed Sequence Tag” (“EST”) is a DNA sequence derived from a cDNA library and therefore represents a sequence which has been transcribed. An EST is typically obtained by a single sequencing pass of a cDNA insert. The sequence of an entire cDNA insert is termed the “Full-Insert Sequence” (“FIS”). A “Contig” sequence is a sequence assembled from two or more sequences that can

be selected from, but not limited to, the group consisting of an EST, FIS and PCR sequence. A sequence encoding an entire or functional protein is termed a "Complete Gene Sequence" ("CGS") and can be derived from an FIS or a contig.

The term "trait" refers to a physiological, morphological, biochemical, or physical characteristics of a plant or particular plant material or cell. In some instances, this characteristics is visible to the human eye, such as seed or plant size, or can be measured by biochemical techniques, such as detecting the protein, starch, or oil content of seed or leaves, or by observation of a metabolic or physiological process, e.g. by measuring tolerance to water deprivation or particular salt or sugar or nitrogen concentrations, or by the observation of the expression level of a gene or genes, or by agricultural observations such as osmotic stress tolerance or yield.

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

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

The ability to increase the biomass or size of a plant would have several important commercial applications. Crop cultivars may be developed to produce higher yield of the vegetative portion of the plant, to be used in food, feed, fiber, and/or biofuel.

Increased leaf size may be of particular interest. Increased leaf biomass can be used to increase production of plant-derived pharmaceutical or industrial products. Increased tiller number may be of particular interest and can be used to increase yield. An increase in total plant photosynthesis is typically achieved by increasing leaf area of the plant. Additional photosynthetic capacity may be used to increase the yield derived from particular plant tissue, including the leaves, roots, fruits or seed, or

permit the growth of a plant under decreased light intensity or under high light intensity.

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

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

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

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

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

interest; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

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

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

“Progeny” comprises any subsequent generation of a plant.

“Transgenic plant” includes reference to a plant which comprises within its genome a heterologous polynucleotide. For example, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct. A T₀ plant is directly recovered from the transformation and regeneration process. Progeny of T₀ plants are referred to as T₁ (first progeny generation), T₂ (second progeny generation), etc.

“Heterologous” with respect to sequence means a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention.

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

“Polypeptide”, “peptide”, “amino acid sequence” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to

naturally occurring amino acid polymers. The terms “polypeptide”, “peptide”, “amino acid sequence”, and “protein” are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, and sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation.

5 “Messenger RNA (mRNA)” refers to the RNA which has no intron and can be translated into protein by the cell.

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

10 polymerase I.

“Mature” protein refers to a post-translationally processed polypeptide; i.e., any pre- or pro-peptides present in the primary translation product has been removed.

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

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

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

“Recombinant DNA construct” refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences

derived from the same source, but arranged in a manner different than that normally found in nature.

The terms “entry clone” and “entry vector” are used interchangeably herein.

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

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

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

15 “Tissue-specific promoter” and “tissue-preferred promoter” may refer to a promoter that is expressed predominantly but not necessarily exclusively in one tissue or organ, but that may also be expressed in one specific cell or cell type.

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

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

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

“Phenotype” means the detectable characteristics of a cell or organism.

30 “Introduced” in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct) into a cell, means “transfection” or “transformation” or “transduction” and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or

mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

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

5 “Transformation” as used herein refers to both stable transformation and transient transformation.

“Stable transformation” refers to the introduction of a nucleic acid fragment into a genome of a host organism resulting in genetically stable inheritance. Once stably transformed, the nucleic acid fragment is stably integrated in the genome of the host
10 organism and any subsequent generation.

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

An “allele” is one of two or more alternative forms of a gene occupying a given
15 locus on a chromosome. When the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant are the same, that plant is homozygous at that locus. If the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant differ, that plant is heterozygous at that locus. If a transgene is present on one of a pair of homologous chromosomes in a diploid plant,
20 that plant is hemizygous at that locus.

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

Methods to determine the relationship of various polynucleotide and polypeptide sequences are known. As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence, such as a segment of a full-length cDNA or gene sequence, or may be the complete cDNA or gene sequence. As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide or polypeptide sequence, wherein the 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 or amino acids in length, and optionally can be 30, 40, 50, 100 or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the sequence, a gap penalty is typically introduced and is subtracted from the number of matches.

The determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Examples of such mathematical algorithms for sequence comparison include the algorithm of Myers and Miller. (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith, *et al.* (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch. (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman. (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul. (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul. (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA); and the Megalign® program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, WI).

Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins, *et al.* (1988) *Gene* 73:237-244; Higgins, *et al.* (1989) *CABIOS* 5:151-153; Corpet, *et al.* (1988) *Nucleic*

Acids Res. 16:10881-10890; Huang, *et al.* (1992) *CABIOS* 8:155-165 and Pearson, *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller, (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul. (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the inventions. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the inventions. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul, *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules (Altschul, *et al.* (1997) *supra*). When utilizing BLAST, Gapped BLAST, PSI-BLAST and the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used (the National Center for Biotechnology Information of the National Library of Medicine of the National Institutes of Health of the U.S. government). Alignment may also be performed by manual inspection.

Paired sequence identity/similarity values can be obtained using GAP Version 10 with the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3 and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

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

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

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

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

percent identities and divergences provided and claimed herein were calculated in this manner.

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" 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.

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

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

Isolated Polynucleotides and Polypeptides:

The present disclosure includes the following isolated polynucleotides and polypeptides:

An isolated polynucleotide comprising: (i) a nucleic acid sequence encoding a polypeptide having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6; or (ii) a full complement of the nucleic acid sequence of (i), wherein the full complement and the nucleic acid sequence of (i) consist of the same number of nucleotides and are 100% complementary. Any of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs (including suppression DNA constructs) of the present disclosure. Over-expression of the encoded polypeptide preferably increases plant drought tolerance activity and/or NUE.

An isolated polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6. The polypeptide is preferably a GLR polypeptide. Over-expression of the polypeptide preferably increases plant drought tolerance activity and/or NUE.

An isolated polynucleotide comprising (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 3 or 5; or (ii) a full complement of the nucleic acid sequence of (i). Any of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs (including suppression DNA constructs) of the present disclosure. The isolated polynucleotide

preferably encodes a GLR polypeptide. Over-expression of the GLR polypeptide preferably improves plant drought tolerance activity and/or NUE.

Recombinant DNA Constructs and Suppression DNA Constructs:

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

In one embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein the polynucleotide comprises (i) a nucleic acid sequence encoding an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide comprises (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 3 or 5; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide encodes a GLR polypeptide. The GLR polypeptide preferably has drought tolerance activity and/or improved NUE. The GLR polypeptide may be from, for example, *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.

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

A suppression DNA construct may comprise at least one regulatory sequence (e.g., a promoter functional in a plant) operably linked to (a) all or part of: (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%,

64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6, or (ii) a full

5 complement of the nucleic acid sequence of (a)(i); or (b) a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region

having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%,

10 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, when compared to said all or part of a sense strand or antisense strand

from which said region is derived, and wherein said target gene of interest encodes a GLR polypeptide; or (c) all or part of: (i) a nucleic acid sequence of at least 50%,

15 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 3 or 5, or (ii) a full

20 complement of the nucleic acid sequence of (c)(i). The suppression DNA construct may comprise a cosuppression construct, antisense construct, viral-suppression construct, hairpin suppression construct, stem-loop suppression construct, double-stranded RNA-producing construct, RNAi construct, or small RNA construct (e.g., an siRNA construct or an miRNA construct).

25 It is understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences. Alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. For example, a codon for the amino acid alanine, a

30 hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be

expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is

determination of retention of biological activity of the encoded products.

“Suppression DNA construct” is a recombinant DNA construct which when transformed or stably integrated into the genome of the plant, results in “silencing” of a target gene in the plant. The target gene may be endogenous or transgenic to the plant. “Silencing,” as used herein with respect to the target gene, refers generally to the suppression of levels of mRNA or protein/enzyme expressed by the target gene, and/or the level of the enzyme activity or protein functionality. The terms “suppression”, “suppressing” and “silencing”, used interchangeably herein, include lowering, reducing, declining, decreasing, inhibiting, eliminating or preventing.

“Silencing” or “gene silencing” does not specify mechanism and is inclusive of, and not limited to, anti-sense, cosuppression, viral-suppression, hairpin suppression, stem-loop suppression, RNAi-based approaches, and small RNA-based approaches.

A suppression DNA construct may comprise a region derived from a target gene of interest and may comprise all or part of the nucleic acid sequence of the sense strand (or antisense strand) of the target gene of interest. Depending upon the approach to be utilized, the region may be 100% identical or less than 100% identical (e.g., at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) to all or part of the sense strand (or antisense strand) of the gene of interest.

Suppression DNA constructs are well-known in the art, are readily constructed once the target gene of interest is selected, and include, without limitation, cosuppression constructs, antisense constructs, viral-suppression constructs, hairpin suppression constructs, stem-loop suppression constructs, double-stranded RNA-producing constructs, and more generally, RNAi (RNA interference) constructs and small RNA constructs such as siRNA (short interfering RNA) constructs and miRNA (microRNA) constructs.

“Antisense inhibition” refers to the production of antisense RNA transcripts capable of suppressing the expression of the target gene or gene product.

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

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

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

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

Small RNAs play an important role in controlling gene expression, for example, small RNAs regulate many developmental processes which include flowering. It is now possible to engineer changes in gene expression of plant genes by using transgenic constructs which produce small RNAs in the plant.

Small RNAs appear to function by base-pairing to complementary RNA or DNA target sequences. When bound to RNA, small RNAs trigger either RNA cleavage or translational inhibition of the target sequence. When bound to DNA target sequences, it is thought that small RNAs can mediate DNA methylation of the target

sequence. The consequence of these events, regardless of the specific mechanism, is that gene expression is inhibited.

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

miRNAs appear to regulate target genes by binding to complementary sequences located in the transcripts produced by these genes. It seems likely that miRNAs can enter at least two pathways of target gene regulation: (1) translational inhibition; and (2) RNA cleavage. miRNAs entering the RNA cleavage pathway are analogous to the 21-25 nt siRNAs generated during RNAi in animals and PTGS in plants, and likely are incorporated into an RNA-induced silencing complex (RISC) that is similar or identical to that seen for RNAi.

Regulatory Sequences:

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

A regulatory sequence may be a promoter.

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

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

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

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

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

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

Many leaf-preferred promoters are known in the art (Yamamoto et al. (1997) *Plant J.* 12(2):255-265; Kwon et al. (1994) *Plant Physiol.* 105:357-367; Yamamoto et al. (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor et al. (1993) *Plant J.* 3:509-518; Orozco et al. (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka et al. (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590).

Promoters which are seed or embryo-specific and may be useful in the invention include soybean Kunitz trypsin inhibitor (Kti3, Jofuku and Goldberg. (1989) *Plant Cell* 1:1079-1093), convicilin, vicilin, and legumin (pea cotyledons) (Rerie, W.G., et al. (1991) *Mol. Gen. Genet.* 259:149-157; Newbigin, E.J., et al. (1990) *Planta* 180:461-470; Higgins, T.J.V., et al. (1988) *Plant. Mol. Biol.* 11:683-695), zein (maize endosperm) (Schemthaner, J.P., et al. (1988) *EMBO J.* 7:1249-1255), phaseolin (bean cotyledon) (Segupta-Gopalan, C., et al. (1985) *Proc. Natl. Acad. Sci.* 82:3320-3324), phytohemagglutinin (bean cotyledon) (Voelker, T. et al. (1987) *EMBO J.* 6:3571-3577), B-conglycinin and glycinin (soybean cotyledon) (Chen, Z-L, et al. (1988) *EMBO J.* 7:297-302), glutelin (rice endosperm), hordein (barley endosperm)

(Marris, C., et al. (1988) *Plant Mol. Biol.* 10:359-366), glutenin and gliadin (wheat endosperm) (Colot, V., et al. (1987) *EMBO J.* 6:3559-3564). Promoters of seed-specific genes operably linked to heterologous coding regions in chimeric gene constructions maintain their temporal and spatial expression pattern in transgenic plants. Such examples include *Arabidopsis* 2S seed storage protein gene promoter to express enkephalin peptides in *Arabidopsis* and *Brassica napus* seeds (Vanderkerckhove et al. (1989) *Bio/Technology* 7:L929-932), bean lectin and bean beta-phaseolin promoters to express luciferase (Riggs et al. (1989) *Plant Sci.* 63:47-57), and wheat glutenin promoters to express chloramphenicol acetyl transferase (Colot et al. (1987) *EMBO J* 6:3559-3564).

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

Promoters for use in certain embodiments include the following: 1) the stress-inducible promoter RD29A (Kasuga et al. (1999) *Nature Biotechnol.* 17:287-291); 2) the stress-inducible promoter Rab17 (Vilardell et al. (1991) *Plant Mol. Bio.* 17:985-993; Kamp Busk et al. (1997) *Plant J* 11(6):1285-1295); 3) the barley promoter B22E whose expression is specific to the pedicel in developing maize kernels ("Primary Structure of a Novel Barley Gene Differentially Expressed in Immature Aleurone Layers". Klemsdal, S.S. et al. (1991) *Mol. Gen. Genet.* 228(1/2):9-16); and 4) maize promoter Zag2 ("Identification and molecular characterization of ZAG1, the maize homolog of the *Arabidopsis* floral homeotic gene AGAMOUS", Schmidt, R.J. et al. (1993) *Plant Cell* 5(7):729-737; "Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of AGAMOUS-like *MADS-box* genes from maize", Theissen et al. (1995) *Gene* 156(2):155-166; NCBI GenBank Accession No. X80206)). Zag2 transcripts can be detected 5 days prior to pollination to 7 to 8 days after pollination ("DAP"), and directs expression in the carpel of developing female inflorescences and Ciml which is specific to the nucleus of developing maize kernels. Ciml transcript is detected 4 to 5 days before pollination to

6 to 8 DAP. Other useful promoters include any promoter which can be derived from a gene whose expression is maternally associated with developing female florets.

For the expression of a polynucleotide in developing seed tissue, promoters of particular interest include seed-preferred promoters, particularly early kernel/embryo promoters and late kernel/embryo promoters. Kernel development post-pollination is divided into approximately three primary phases. The lag phase of kernel growth occurs from about 0 to 10-12 DAP. During this phase the kernel is not growing significantly in mass, but rather important events are being carried out that will determine kernel vitality (e.g., number of cells established). The linear grain fill stage begins at about 10-12 DAP and continues to about 40 DAP. During this stage of kernel development, the kernel attains almost all of its final mass, and various storage products (i.e., starch, protein, oil) are produced. Finally, the maturation phase occurs from about 40 DAP to harvest. During this phase of kernel development the kernel becomes quiescent and begins to dry down in preparation for a long period of dormancy prior to germination. As defined herein "early kernel/embryo promoters" are promoters that drive expression principally in developing seed during the lag phase of development (i.e., from about 0 to about 12 DAP). "Late kernel/embryo promoters", as defined herein, drive expression principally in developing seed from about 12 DAP through maturation. There may be some overlap in the window of expression. The choice of the promoter will depend on the ABA-associated sequence utilized and the phenotype desired.

Early kernel/embryo promoters include, for example, *Cim1* that is active 5 DAP in particular tissues (WO 00/11177), which is herein incorporated by reference. Other early kernel/embryo promoters include the seed-preferred promoters *end1* which is active 7-10 DAP, and *end2*, which is active 9-14 DAP in the whole kernel and active 10 DAP in the endosperm and pericarp. (WO 00/12733), herein incorporated by reference. Additional early kernel/embryo promoters that find use in certain methods of the present disclosure include the seed-preferred promoter *ltp2* (U.S. Pat. No. 5,525,716); maize *Zm40* promoter (U.S. Pat. No. 6,403,862); maize *nuc1c* (U.S. Pat. No. 6,407,315); maize *ckx1-2* promoter (U.S. Pat. No. 6,921,815 and US Patent Application Publication Number 2006/0037103); maize *lec1* promoter (U.S. Pat. No. 7,122,658); maize *ESR* promoter (U.S. Pat. No. 7,276,596); maize *ZAP* promoter (U.S. Patent Application Publication Numbers 20040025206 and 20070136891); maize promoter *eep1* (U.S. Patent Application Publication Number 20070169226);

and maize promoter *ADF4* (U.S. Patent Application No. 60/963,878, filed 7 Aug. 2007).

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

Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments.

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

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

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

Any plant can be selected for the identification of regulatory sequences and GLR polypeptide genes to be used in recombinant DNA constructs of the present

disclosure. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, *Arabidopsis*, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, switchgrass, tangerine, tea, tobacco, tomato, triticale, turf, turnip, vine, watermelon, wheat, yams, and zucchini.

Compositions:

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

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

The plant may be a monocotyledonous or dicotyledonous plant, for example, a rice or maize or soybean plant, such as a maize hybrid plant or a maize inbred plant.

The plant may also be sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, sugar cane or switchgrass.

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

5 Particular embodiments include but are not limited to the following:

1. A plant (for example, a rice or maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%,
10 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6, and wherein said plant exhibits
15 increased drought tolerance when compared to a control plant. The plant may further exhibit an alteration of at least one agronomic characteristic when compared to the control plant.

2. A plant (for example, a rice or maize or soybean plant) comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked
20 to at least one regulatory sequence, wherein said polynucleotide encodes a GLR polypeptide, and wherein said plant exhibits increased drought tolerance when compared to a control plant. The plant may further exhibit an alteration of at least one agronomic characteristic when compared to the control plant.

3. A plant (for example, a rice or maize or soybean plant) comprising in its
25 genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein said polynucleotide encodes a GLR polypeptide, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant.

4. A plant (for example, a rice or maize or soybean plant) comprising in its
30 genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%,

83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO: 4 or 6, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant.

5 5. A plant (for example, a rice or maize or soybean plant) comprising in its genome a suppression DNA construct comprising at least one regulatory element operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%,
10 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target
15 gene of interest encodes a GLR polypeptide, and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant.

6. A plant (for example, a rice or maize or soybean plant) comprising in its genome a suppression DNA construct comprising at least one regulatory element operably linked to all or part of (a) a nucleic acid sequence encoding a polypeptide
20 having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of
25 alignment, to SEQ ID NO: 4 or 6, or (b) a full complement of the nucleic acid sequence of (a), and wherein said plant exhibits an alteration of at least one agronomic characteristic when compared to a control plant.

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

In any of the foregoing embodiments 1-7 or other embodiments, the GLR polypeptide may be from *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.

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

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

In any of the foregoing embodiments 1-7 or other embodiments, the at least one agronomic characteristic may be selected from the group consisting of greenness, grain yield, growth rate, biomass, fresh weight at maturation, dry weight at maturation, fruit yield, seed yield, total plant nitrogen content, fruit nitrogen content, 10 seed nitrogen content, nitrogen content in a vegetative tissue, total plant free amino acid content, fruit free amino acid content, seed free amino acid content, free amino acid content in a vegetative tissue, total plant protein content, fruit protein content, seed protein content, protein content in a vegetative tissue, drought tolerance, nitrogen uptake, root lodging, harvest index, stalk lodging, plant height, ear height, 15 ear length, salt tolerance, tiller number, panicle size, early seedling vigor and seedling emergence under low temperature stress. For example, the alteration of at least one agronomic characteristic may be an increase in grain yield, greenness or biomass.

In any of the foregoing embodiments 1-7 or other embodiments, the plant may 20 exhibit the alteration of at least one agronomic characteristic when compared, under water limiting conditions, to a control plant.

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

25 “Drought tolerance” reflects a plant’s ability to survive under drought without exhibiting substantial physiological or physical deterioration, and/ or its ability to recover when water is restored following a period of drought.

“Drought tolerance activity” of a polypeptide indicates that over-expression of the polypeptide in a transgenic plant confers increased drought tolerance of the 30 transgenic plant relative to a reference or control plant.

“Increased drought tolerance” of a plant is measured relative to a reference or control plant, and reflects ability of the plant to survive under drought conditions with less physiological or physical deterioration than a reference or control plant grown under similar drought conditions, or ability of the plant to recover more substantially

and/or more quickly than would a control plant when water is restored following a period of drought.

“NUE” is nitrogen utilization efficiency and refers to a plant’s ability to utilize nitrogen in low or high levels of fertilizer. It reflects plant ability to uptake, assimilate, and/or otherwise utilize nitrogen.

Soil plant analyses development (SPAD) value is SPAD reading which is measured by SPAD-502 plus (a chlorophyll meter, made by KONICA MINOLTA). The SPAD value is relative content of leaf chlorophyll and an important indicator of plant health. Many studies indicated that a significant and positive correlation was observed between leaf nitrogen content and SPAD value (Swain D.K. and Sandip S.J. (2010) *Journal of Agronomy* 9 (2):38-44), and leaf SPAD value is used as index of nitrogen status diagnosis in crops (Cai H.-G. et al. (2010) *Acta metallurgica sinica* 16 (4): 866-873).

The SPAD value may be measured during low nitrogen treatment.

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

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

Parameters such as gene expression level, water use efficiency, level or activity of an encoded protein, and others are typically presented with reference to a control cell or control plant. A “control” or “control plant” or “control plant cell” provides a reference point for measuring changes in phenotype of a subject plant or plant cell in which genetic alteration, such as transformation, has been effected as to a gene of interest. A subject plant or plant cell may be descended from a plant or cell so altered and will comprise the alteration. One of ordinary skill in the art would readily recognize a suitable control or reference plant to be utilized when assessing or measuring an agronomic characteristic or phenotype of a transgenic plant using

compositions or methods as described herein. For example, by way of non-limiting illustrations:

1. Progeny of a transformed plant which is hemizygous with respect to a recombinant DNA construct (or suppression DNA construct), such that the progeny are segregating into plants either comprising or not comprising the recombinant DNA construct (or suppression DNA construct): the progeny comprising the recombinant DNA construct (or suppression DNA construct) would be typically measured relative to the progeny not comprising the recombinant DNA construct (or suppression DNA construct). The progeny not comprising the recombinant DNA construct (or the suppression DNA construct) is the control or reference plant.

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

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

4. A plant comprising a recombinant DNA construct (or suppression DNA construct): the plant may be assessed or measured relative to a control plant not comprising the recombinant DNA construct (or suppression DNA construct) but otherwise having a comparable genetic background to the plant (e.g., sharing at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity of nuclear genetic material compared to the plant comprising the recombinant DNA construct (or suppression DNA construct)). There are many laboratory-based techniques available for the analysis, comparison and characterization of plant genetic backgrounds; among these are Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA

Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Amplified Fragment Length Polymorphisms (AFLP®s), and Simple Sequence Repeats (SSRs) which are also referred to as Microsatellites.

5 A control plant or plant cell may comprise, for example: (a) a wild-type (WT) plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (i.e., with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a
10 non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to conditions or stimulus that would induce expression of the gene of interest or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed. A control may comprise numerous individuals
15 representing one or more of the categories above; for example, a collection of the non-transformed segregants of category "c" is often referred to as a bulk null.

Methods:

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

Methods include but are not limited to the following:

30 A method for transforming a cell comprising transforming a cell with any one or more of the isolated polynucleotides of the present disclosure, wherein, in particular embodiments, the cell is a eukaryotic cell, e.g., a yeast, insect or plant cell; or a prokaryotic cell, e.g., a bacterial cell.

A method for producing a transgenic plant comprising transforming a plant cell with any of the isolated polynucleotides or recombinant DNA constructs (including

suppression DNA constructs) of the present disclosure and regenerating a transgenic plant from the transformed plant cell, wherein, the transgenic plant and the transgenic seed obtained by this method may be used in other methods of the present disclosure.

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

10 A method for altering the level of expression of a polypeptide of the invention in a host cell comprising: (a) transforming a host cell with a recombinant DNA construct of the present disclosure; and (b) growing the transformed host cell under conditions that are suitable for the expression of the recombinant DNA construct, wherein the expression of the recombinant DNA construct results in production of altered levels
15 of the polypeptide of the invention in the transformed host cell.

 A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein the polynucleotide encodes a polypeptide
20 having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of
25 alignment, to SEQ ID NO: 4 or 6; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant; and further (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the
30 recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant.

 A method of evaluating drought tolerance in a plant comprising (a) obtaining a transgenic plant, which comprises in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for

example, a promoter functional in a plant), wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, to SEQ ID NO:4 or 6; (b) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (c) evaluating the progeny plant for drought tolerance compared to a control plant.

A method of evaluating drought tolerance in a plant comprising (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, when compared to SEQ ID NO:4 or 6, or (ii) a full complement of the nucleic acid sequence of (a)(i); (b) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (c) evaluating the progeny plant for drought tolerance compared to a control plant.

A method of evaluating drought tolerance in a plant comprising (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of

alignment, when compared to said all or part of a sense strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a GLR polypeptide; (b) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (c) evaluating the progeny plant for drought tolerance compared to a control plant.

A method of determining an alteration of an agronomic characteristic in a plant comprising (a) obtaining a transgenic plant which comprises in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, when compared to SEQ ID NO:4 or 6; (b) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (c) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant.

A method of determining an alteration of an agronomic characteristic in a plant comprising (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to all or part of (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, when compared to SEQ ID NO: 4 or 6, or (ii) a full complement of the nucleic acid sequence of (i); (b) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (c) determining

whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally under water limiting conditions, to a control plant.

5 A method of determining an alteration of an agronomic characteristic in a plant comprising (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a suppression DNA construct comprising at least one regulatory sequence (for example, a promoter functional in a plant) operably linked to a region derived from all or part of a sense strand or antisense strand of a target gene of interest, said region having a nucleic acid sequence of at least 50%, 51%,
10 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on either Clustal V or GAP method of alignment, when compared to said all or part of a sense
15 strand or antisense strand from which said region is derived, and wherein said target gene of interest encodes a GLR polypeptide; (b) obtaining a progeny plant derived from said transgenic plant, wherein the progeny plant comprises in its genome the suppression DNA construct; and (c) determining whether the progeny plant exhibits an alteration in at least one agronomic characteristic when compared, optionally
20 under water limiting conditions, to a control plant.

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

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

30 In any of the preceding methods or any other embodiments of methods of the present disclosure, said regenerating step may comprise the following: (i) culturing said transformed plant cell or cells in a medium comprising an embryogenic promoting hormone until callus organization is observed; (ii) transferring said transformed plant cell or cells of step (i) to a first medium which includes a tissue

organization promoting hormone; and (iii) subculturing said transformed plant cell or cells after step (ii) onto a second medium, to allow for shoot elongation, root development or both.

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

In any of the preceding methods or any other embodiments of methods of the present disclosure, the plant may exhibit the alteration of at least one agronomic characteristic when compared, under water limiting conditions, to a control plant.

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

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

In addition, methods to modify or alter the host endogenous genomic DNA are available. This includes altering the host native DNA sequence or a pre-existing transgenic sequence including regulatory elements, coding and non-coding sequences. These methods are also useful in targeting nucleic acids to pre-engineered target recognition sequences in the genome. As an example, the genetically modified cell or plant described herein, is generated using "custom" meganucleases produced to modify plant genomes (e.g., WO 2009/114321; Gao et al. (2010) *Plant Journal* 1:176-187). Another site-directed engineering is through the use of zinc finger domain recognition coupled with the restriction properties of restriction enzyme (e.g., Urnov, et al. (2010) *Nat Rev Genet.* 11(9):636-46; Shukla, et al. (2009) *Nature* 459 (7245):437-41).

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

EXAMPLES

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

EXAMPLE 1

Creation of a Rice Population with an Activation-Tagging Construct

5 In this research, a 4 X CaMV 35S enhancer- based binary construct was used, and the rice activation tagging population was developed from Zhonghua11 (*Oryza sativa* L. Zhonghua11) which was transformed by *Agrobacteria*-mediated transformation. Zhonghua11 was cultivated by institute of crop sciences, Chinese Academy of Agricultural Sciences. The first batch of seeds used in this research was
10 provided by Beijing Weiming Kaituo Agriculture Biotech Co., Ltd. Calli induced from embryos was transformed with *Agrobacteria* with the vector. The events generated with the T-vector were developed and the transgenic seeds were harvested to form the rice activation tagging population.

EXAMPLE 2

Seedling Screens to Identify Lines with Enhanced Drought Tolerance in Greenhouse

Seedling screens for drought tolerance and nitrogen use efficiency (NUE) were carried out in greenhouse. Two types of lamps were provided as light source, i.e. sodium lamp and metal halide lamp, the ratio is 1:1. Lamps provided the 16 h/8 h
20 period of day/night, and were placed approximately 1.5 m above the seedbed. The light intensity 30 cm above the seedbed was measured as 10,000-20,000 lx in sunny day, while 6,000-10,000 lx in cloudy day; the relative humidity ranged from 30% to 90%, and the temperature ranged from 20 to 35 °C.

The T2 (or T1) seeds which showed red color under green fluorescent light were
25 used for greenhouse screens including drought tolerance and NUE except as otherwise specifically noted.

Thirty-two uniform seedlings from each line, 1 positive control (a drought tolerant variety, Mianhui 501), 1 negative control (a drought sensitive variety, Dongbeiyin 2) and 1 wild-type control (Zhonghua11) from tissue culture procedure were used for
30 the drought tolerance screens. The seedlings were planted in one tray filled with mixture of organic soil (FangJie soil from Beijing HuiYeShengDa Center), vermiculite (Beijing QingYuanShiJi Garden Center) and sand (Beijing Shuntun Construction Material Market) (V: V: V= 3:3:2). For each line, the total 32 plants grew in different positions of the tray. After all the seedlings grew to the 3-leaf stage, watering was

stopped and the trays were kept in a dry place until the leaves became curved (approximately 9-15 days depending on the season). The trays were transferred into water pool to recover the seedlings for 5-7 days, and then plants were scored for the degree of recovery. The following scoring system was used: one green stem = 1, one green leaf = 1, a half green stem or leaf = 0.5, one third green leaf=0.2, no green leaf or stem = 0. The recovery degree is the sum of the score of the green tissues, and the data are statistically analyzed using SAS-ANOVA. The lines which showed significant difference from controls and other mutant plants were considered as primary positive lines.

The primary positives were randomly inserted into the unscreened pools and screened again and the data were analyzed using SAS-ANOVA.

The tagged lines which passed the second screens were rescreened again. In the third screen, each tagged line had 60 plants and was analyzed only with respect to the wild type plants, using SAS-ANOVA ($P < 0.05$).

Survival rate was also used as a parameter for the screens, which is the percentage of survived plants over the total plant number.

Lines which passed the third screen were considered positive lines and moved into the molecular cloning stage.

EXAMPLE 3

Seedling Screens to Identify Lines with Enhanced Nitrogen Use Efficiency (NUE) in Greenhouse

For each line to be tested, 6 uniform seedlings were planted, 2 plants were planted in one pot filled with vermiculite. In each tray, there were 24 mutant lines plus 1 positive control (Liutiaonuo), 1 negative control (Liaojing 294) and 1 transformed wild type control from tissue culture procedure. After all the seedlings grew to 3-leaf stage, water was replaced by Hoagland solution containing 0.75 mM nitrogen (KNO_3). To make aerobic conditions, the nutrition solution was drained off daily for 2 h, and then new solution was added. When the positive control plants differentiated tillers, SPAD value was measured by a SPAD meter (SPAD 502 Plus, made by KONICA MINOLTA) with the second leaf from the top, and tiller numbers were counted. After statistical analysis of these data (SPAD value and tiller number), the positive lines were selected by $P < 0.05$.

EXAMPLE 4

Field Drought Screens of Mature Plants

Field drought screens of mature rice were carried out in Hainan Province. For each tagged line to be tested, about 100 positive seeds were soaked in water for 16 h at room temperature, germinated for 36 h at 35-37 °C in an incubator, and planted in a bedded field. At 3-leaf stage, the seedlings were transplanted into the testing field, with 4 replicates and 10 plants per replicate for each tagged line, and the 4 replicates were planted in the same block. The wild type Zhonghua11 plants which were from the tissue culture procedure, were nearby the tagged lines in the same block, and were used as control in the statistical analysis.

The rice plants were managed by normal practice using pesticides and fertilizers. Watering was stopped at the booting stage, so as to give drought stress at flowering stage depending on the weather conditions (temperature and humidity). In case the drought was too severe for the plants to survive, they were re-watered one or two times in order to keep a reasonable yield. The soil water content was measured every 3 days at about 10 sites per block using TDR30 (Spectrum Technologies, Inc.).

Plant phenotypes were observed and recorded during the experiments. The phenotypes include heading time, leaf rolling, drought sensitive and drought tolerant. Special attention was paid to leaf rolling at noontime. At the end of the season, 5 representative plants were harvested from the middle of the row per line, and grain weight per plant was measured.

The grain weight data were statistically analyzed using SAS-ANOVA-mixed model by ASReml program. Positive lines were selected based on the analysis ($P < 0.05$). Similar to the greenhouse screens, the primary positives were rescreened twice and only those lines which passed all three screens were considered as positive lines.

All lines which passed the third screens were planted in greenhouse soil or in field (depending on the season) for harvesting leaf materials and molecular cloning of the T-DNA-flanking sequences and candidate genes. In general, 20-30 g of fresh leaf tissues were harvested from 30 uniform seedlings of the same line, frozen in liquid nitrogen, and stored in -80 °C freezer.

EXAMPLE 5**Results for Line AH01486**

In repeated greenhouse drought screening assays, line AH01486 consistently showed enhanced drought tolerance compared to Zhonghua11 control generated from tissue culture procedure.

For T1 seeds from line AH01486, 10 of 13 plants (77%) were recovered or survived under greenhouse drought stress condition; while only 33% of the Zhonghua11 control plants survived. These results indicate that AH01486 plants have significantly enhanced drought tolerance compared to Zhonghua11 control.

Observation at the T2 generation, as shown in Table 2, confirmed the drought-tolerance findings. In the first round screen, 85% of AH01486 plants survived, while only 35% of Zhonghua11 control survived. The average recovery degree of AH01486 plants was higher than that of Zhonghua11 control, and there was significant difference between AH01486 plants and the control. In another two repeated screens, both survival rate and recovery degree of AH01486 plants consistently exhibited significantly enhanced drought tolerance compared to Zhonghua11 control. These results demonstrate that AH01486 plant has enhanced drought tolerance under greenhouse drought condition at seedling stages.

Table 2. Drought Tolerance Assays of AH01486 at T2 generation under greenhouse conditions

Line	Screen round	# Survived plants	# Total plants	Survival rate (%)	Average recovery degree	p-value	P<0.05
CK	1 st	7	20	35	0.26		
AH01486		17	20	85	1.09	0.0001	Y
CK	2 nd	17	26	65	0.35		
AH01486		29	32	91	1.01	0.0198	Y
CK	3 rd	19	60	32	0.61		
AH01486		27	60	45	1.12	0.0171	Y

In field experiments under drought conditions in Hainan province, Line AH01486 gave an average grain yield of 23.93 g /plant (20.72, 18.38, and 32.70 g/plant for three replicates) compared to the Zhonghua11 control grain yield of 17.70 g/plant (17.80, 18.29, and 17.00 g/plant for three replicates). On the average AH01486 exhibited 35% grain yield advantage over the control ($p < 0.05$).

In light of these results, further work was carried out to identify the gene(s) affected by the activation tagging of Line AH01486.

EXAMPLE 6

Identification of Activation-Tagged Genes

Genes flanking the T-DNA insertion locus in the drought-tolerant lines AH01486 are identified using one or both of the following two standard procedures: (1) Plasmid Rescue (Friedrich J. Behringer and June I. Medford. (1992), *Plant Molecular Biology Reporter* Vol. 10, 2:190-198); and (2) Inverse PCR (M. J. McPherson and Philip Quirke. (1991), *PCR: a practical approach*, 137-146). In lines with complex multimerized T-DNA inserts, plasmid rescue and inverse PCR may both prove insufficient to identify candidate genes. In these cases, other procedures, including TAIL PCR (Liu et al. (1995), *Plant J.* 8:457-463) can be employed.

A successful result is one where a single DNA fragment contains a T-DNA border sequence and flanking genomic sequence. When a tag of genomic sequence flanking a T-DNA insert is obtained, candidate genes are identified by alignment to publicly available rice genome sequence. Specifically, the annotated genes nearest the CaMV 35S enhancer elements/T-DNA RB and LB are candidates for genes that are activated.

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

Genomic DNA was isolated from the leaf tissues of the AH01486 line using CTAB method (Murray, M.G. and W.F. Thompson. (1980) *Nucleic Acids Res.* 8: 4321-4326), and 10 µg of this genomic DNA from AH01486 leaves was digested by

2 μ L *Bgl*II (NEB, Ipswich, MA). After self-ligation and transformation into competent *E. coli* DH5 α through electroporation, colonies surviving on an ampicillin-containing plate were colony-PCR analyzed using the following primers:

P2up-5389: 5'-ACCCCAGGCTTTACACTTTATGCTTCC-3' (SEQ ID NO: 7)

5 P2down-3534: 5'-AACCCACTCGTGCACCCAACTGATC-3' (SEQ ID NO: 8)

PCR Reaction mixture and procedure:

Reaction mix (25 μ L):

Template	2 μ L
Primer (10 μ M)	each 1 μ L
dNTPs (2.5 mM each)	2 μ L
La Taq (TaKaRa)	0.2 μ L
10 $\times$ LA PCR buffer II	1.25 μ L
2 $\times$ GC bufferI	6.25 μ L
ddH ₂ O	11.3 μ L

PCR cycle:

94°C	3 min	
98°C	20 s	} 30 cycles
68°C	15 min	
72°C	10 min	

20 A total of 16 colonies were analyzed and all produced a band approximately 4 kb in size on agarose gel electrophoresis. Clones with this ~4 kb DNA fragment were sequenced by primer M13R-46 (primer) and determined the RB-flanking sequence of T-DNA in AH01486. This sequence is provided as SEQ ID NO: 1. According to the rice genomic annotation of the U.S. government's National Center for Biotechnology Information of the National Library of Medicine, National Institutes of Health, and the website for the rice genome annotation project at Michigan State University, Blast search revealed that the right border (RB) of the T-DNA was inserted at 15760218 of Chromosome 9. Based on the information, primers were designed to further define and confirm the T-DNA insertion site on both RB and LB (left border) sides. These primers are as follows:

RB out down; primer to confirm RB flanking sequence

5'-TGAAAGCGACGTTGGATGTTTCATTGCG-3' (SEQ ID NO: 9)

AH01486 checkup; primer for RB flanking sequence

5'-CCTAATTGATTGGCCCAAGAAGATATGAC-3' (SEQ ID NO: 10)

LB out up; primer for LB flanking sequence

5'-CTTCAACGTTGCGGTTCTGTCAGTTC-3' (SEQ ID NO: 11)

AH01486 check down; primer for LB flanking sequence

5'-CTGTCGCAGTAGGACTTGAGG-3' (SEQ ID NO: 12).

5 The LB flanking sequence of the T-DNA in AH01486 is listed in SEQ ID NO: 2.

The right border (RB) of T-DNA is inserted in the 15760218 bp in rice

Chromosome 9, and the left border (LB) in the 15760298 bp in Chromosome 9. The T-DNA insertion resulted in 79 bp rice genomic DNA deletion (based on rice genome database in June-July, 2009). The T-DNA in AH01486 has the LB::LB serial

10 construction. The T-DNA has 22 bp deletion at RB end. On the left border end, there is the Ubi1ZM promoter with 65 bp deletion at the 3' end. The T-DNA inserted in the second intron of a glutamate receptor gene (GLR1, LOC_Os09g26144.1). Upstream of the T-DNA RB, there are two retrotransposon protein genes. Downstream of the T-DNA LB, there are another glutamate receptor gene (GLR2, LOC_Os09g26160.1),
15 a MYB family transcription factor gene, and a transcription initiation factor TFIID gene.

A functional activation-tagged allele should result in up-regulation of the candidate gene in tissues where it is normally expressed, ectopic expression in tissues that do not normally express that gene, or both.

20 Expression levels of the candidate gene in selected tissues of the cognate mutant line vs. wild-type are compared. A standard RT-PCR or a real-time PCR procedure, such as the QuantiTect® Reverse Transcription Kit from Qiagen® and Real Time-PCR(SYBR^RPremix Ex TaqTM, TaKaRa), is used. EF-1 gene is used as a control to show that the amplification and loading of samples from the mutant line
25 and wild-type are similar. Assay conditions are optimized for each gene. If the activation-tagged allele results in ectopic expression in other tissues, it is not detected by this assay. As such, a positive result is useful but a negative result does not eliminate a gene from further analysis.

To understand which genes are activated in AH01486, RT-PCR and real-time
30 PCR analyses were carried out. The semi-quantitative RT-PCR assays indicated that MYB transcription factor gene did not change its expression level in the leaf tissues of AH01486, and transcription initiation factor TFIID was slightly down regulated in AH01486. Both GLR1 and GLR2 were significantly activated in AH01486 root, stem, and leaf. The total RNA was extracted from about 50 mg leaf, or stem, or root tissues

of AH01486 plants at 4-leaf-stage using RNAiso Plus kit (TaKaRa) according to manufacturer's instruction. The cDNA were prepared by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) and from 500 ng total RNA. The real-time-PCR (SYBR^RPremix Ex Taq™, TaKaRa) was conducted using 7,500 Fast real-time PCR equipment and according to the manual (ABI). The primers for real time PCR for the GLR1 gene and GLR2 gene are listed below:

GLR1-5'RT-F: 5'-GTCAAGGTCCGGCAAGAAG-3' (SEQ ID NO: 13)

GLR1-5'RT-R: 5'-CTTCCCTCTGTCCATGATGTTC-3' (SEQ ID NO: 14)

GLR2-RT-F: 5'-CTCCTAAAGATATTCCACG-3' (SEQ ID NO: 15)

GLR2-RT-R: 5'-ACGCTCTCCACATCACTGC-3' (SEQ ID NO: 16)

As shown in Figure 2A and 2B, the GLR1 gene and GLR2 gene were significantly activated in AH01486 plants (root, stem, and leaf tissues) compared to both wild-type control and tissue-cultured Zhonghua11 control. In leaf tissues, the GLR1 gene and GLR2 gene expression levels were increased 53- and 38-fold compared to the control, respectively. Based on these results, GLR1 gene (SEQ ID NO: 3) and GLR2 gene (SEQ ID NO: 5) were cloned and validated as to their functions in drought tolerance and other agronomic trait improvement.

EXAMPLE 7

GLR gene Cloning and Over-expression Vector Construction

Based on the sequence information of gene ID LOC_Os09g26144.1, two primers were designed for cloning GLR1 (glutamate receptor) cDNA. The primers are as follows:

56-26144-F: 5'-TCTGCCTGCAGGATGGCTGGTCACACCCCGAATC-3' (SEQ ID NO: 17)

56-26144 R: 5'-TAACCTGCAGGTTACTGGCCTCTGCCATTCTCT-3' (SEQ ID NO: 18)

PCR reaction was carried out using these two primers and pooled cDNA from leaf, stem, root tissues of Zhonghua11 plants. A ~2.8 kb PCR fragment was obtained, purified, and then digested by *Sbf*I. This DNA fragment was ligated with *Pst*I-digested vector DP0005 to generate an over-expression vector DP0025 (CaMV35S Pro::GLR1::Nos). The GLR1 gene sequence and orientation in DP0025 were confirmed by sequencing. The sequence of this DNA fragment is provided as

SEQ ID NO: 3, the encoded amino acid sequence is provided as SEQ ID NO: 4, and this gene is named GLR1 (glutamate receptor 1).

The GLR2 (glutamate receptor 2) (LOC_Os09g26160.1) gene was amplified from Zhonghua11 cDNA pool using the following primers which were designed based on the sequence of LOC_Os09g26160.1 in the rice genbank:

56-26160-F: 5'-TTTAGGATCCTTGTCCATGGAAGCTG-3' (SEQ ID NO: 19)

56-26160-R: 5'-ACTCTCGAGTTATTGCCCCCTCTCTGAATG-3' (SEQ ID NO: 20)

*Bam*HI restriction site was added in primer 56-26160-F, and *Xho*I in primer 56-26160-R, as indicated in the underlined areas. A ~2.8 kb PCR fragment was purified, and then digested by *Bam*HI and *Xho*I. This DNA was ligated with *Bgl*II-*Sal*I-digested DP0005 and GLR2 over-expression vector DP0015 was obtained (CaMV35S Pro::GLR2::Nos). The nucleotide sequence and orientation of GLR2 gene in DP0015 were confirmed by sequencing. The nucleotide sequence of this cDNA is listed as SEQ ID NO: 5, and the encoded amino acid sequence is listed as SEQ ID NO: 6.

EXAMPLE 8

Vector Construction of DP0005 and DP0009

DP0005 was constructed from pCAMBIA1300 (Cambia, Brisbane, Australia). The CaMV 35S promoter was amplified by PCR using template of pCAMBIA1300 and primers of 35sF and 35sR. The restriction enzyme sites *Hind*III and *Pst*I were added in 35sF primer (forward primer of CaMV 35S promoter) and 35sR primer (reverse primer of CaMV 35S promoter), respectively.

35sF 5'-AAGCTTAGAGCAGCTTGGCAACATGGTGGAGCAC-3' (SEQ ID NO: 21)

35sR 5'-CTGCAGAGAGATAGATTTGTAGAGAGAG-3' (SEQ ID NO: 22).

This amplified CaMV 35S promoter fragment was digested by *Hind*III and *Pst*I and ligated into *Hind*III-*Pst*I-digested pCAMBIA1300 to produce pCAMBIA1300-35s.

The Terminator NOS polyA was amplified by PCR using template of pCAMBIA1303 and primers of TnosF and TnosR. The restriction enzymes sites *Pst*I+*Bgl*II+*Kpn*I+*Nco*I+*sal*I and *Bam*HI were designed in Terminator NOS polyA 5' and 3' ends of the primers, respectively.

TnosF: 5'-CTGCAGAGATCTGGTACCGGTCGCCACCATGGAAGTCG
ACGTTTCTTGTTTCTTAAGATTGAATCCTGTTG-3' (SEQ ID NO: 23)

TnosR: 5'-GGATCCTCTAGTCCCGATCTAGTAACATAG-3' (SEQ ID NO: 24)

Terminator NOS polyA fragment was digested with *Pst*I and *Bam*HI and ligated into *Pst*I –*Bam*HI-digested pCAMBIA1300-35s to pCAMBIA1300-35s-Tnos.

The AsRED gene was isolated from pAsRED2 (Clontech Laboratories Inc.) by *Not*I digestion, blunt-ended by T4 polymerase, and then *Nco*I digestion. The 703 bp-
5 DNA fragment containing the AsRED gene was ligated with pCAMBIA1300-35s – Tnos which was digested by *Sa*II, blunt-ended by T4 polymerase, and then *Nco*I digested. The resulting construct was designated pCAMBIA1300-35s -AsRED-Tnos.

For convenient construction of gene expression vectors, the new Terminator NOS polyA fragment has *Bam*HI+*Nru*I+*Sa*II sites at the 5' end and an *Eco*RI at the 3' end. The primers are:

TnosF2: 5'-CCGGGATCCTCGCGAGTCGACCTCCAAGCTGGGCCACAACACTGAAG-3' (SEQ ID NO: 25)

TnosR2: 5'-CGAGAATTCTCTAGTCCCGATCTAGTAACATAG-3' (SEQ ID NO: 26).

The new Terminator NOS polyA fragment was amplified by PCR using
15 pCAMBIA1300-35s-AsRED-Tnos as a template. This DNA fragment was digested by *Bam*HI and *Eco*RI, and ligated to pCAMBIA1300-35s-AsRED-Tnos which was digested by *Bam*HI and *Eco*RI to produce DP0005, an over-expression vector backbone for the candidate genes of GLR1 and GLR2. DP0005 was digested by *Bgl*II and *Bam*HI, and then self-ligated for eliminating the AsRED gene from DP0005
20 to produce DP0009.

EXAMPLE 9

Functional Validation of GLR1 and GLR2 Genes in Transgenic Rice Events

Both empty vectors (DP0005) and over-expression vectors (DP0015 and
25 DP0025) were transformed into Zhonghua11 using Agrobacteria rice transformation methods as known to those of skill in the art.

More than 30 independent transgenic events were generated for both empty vector (DP0005) and over-expression vector (DP0015 and DP0025). The functional assays (drought tolerance and NUE) were carried out at T0, and/or T1 and/or T2
30 generations.

The T0 plants were cultured in greenhouse to 3-leaf stage. Uniform healthy plants were selected for drought screens as described above. For the T1 seeds, the first selection was for hygromycin-resistance, by culturing the seedling (from 1-2 cm in height) in 50 mg/L hygromycin solution. Only the surviving plants (hygromycin-

resistant) were used in the drought and NUE assays as described above (examples 2, 3, and 4). Leaf samples were collected for real-time PCR analysis to verify that the events from DP0015 and DP0025 were transgenic and over-expressed the GLR2 or GLR1 transgene.

5 The total RNA was extracted and the real-time PCR analysis was carried out as described in example 6. For GLR2-transgenic rice plants, Primer DP0018-F1 and DP0018-R1 were used, primers for detecting the expression levels of GLR1 transgene are as follow:

DP0018-F1: 5'-GACGCCGTACCTGAGGATCTTC-3' (SEQ ID NO: 27)

10 DP0018-R1: 5'-CACTTGCGCTCGATGAGGTTC-3' (SEQ ID NO: 28)

As shown in Figure 3 and Figure 4, compared to the transgene expression level in the control (CK), GLR2 transgene overexpressed in the GLR2-transgenic rice, and the GLR1 transgene overexpressed in the GLR1-transgenic rice.

15 EXAMPLE 10

Enhanced Drought Tolerance by Over-expressing GLR1 or GLR2 gene

T0, T1 and T2 DP0015 transgenic seedlings were planted in greenhouse and treated as described in example 2.

Constitutive (CaMV 35S-driven) over-expression of the GLR2 gene resulted in
20 significantly enhanced drought tolerance under greenhouse conditions (Table 3-5). In this validation experiments, empty vector (DP0005) transformed rice plants were used as control. As shown in Table 3, at T0 generation, over-expression of GLR2 gene significantly enhanced survival rate and recovery degree after the drought treatments; 48% of GLR2-transgenic rice survived, while 23% of control survived
25 after drought treatment, and the average recovery degree is 0.62 for GLR2-transgenic rice and 0.28 for the control, which indicates the GLR2-transgenic plants have enhanced drought tolerance. The transgenic events were further evaluated at T1 and T2 generations. As indicated in Table 4 and 5, multiple events which showed enhanced expression levels of GLR2 transgene (FIG. 3) consistently exhibited
30 increased drought tolerance under the greenhouse conditions; the average recovery degree are two times more than that of the control. These results demonstrate that enhanced GLR2 expression level contributed to the enhanced drought tolerance of AH01486.

Table 3. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T0 generation under greenhouse conditions

Line	# Survived plants	# Total plants	Survival rate (%)	Average recovery degree	p-value	P<0.05
CK	11	48	23	0.28		
DP0015	23	48	48	0.62	0.0127	Y

5

Table 4. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse conditions

Line	# Survived plants	# Total plants	Survival rate (%)	Average recovery degree	p-value	P<0.05
CK	4	16	25	0.25		
DP0015.05	9	16	56	0.96	0.0123	Y
CK	4	16	25	0.25		
DP0015.10	11	16	69	1.22	0.0010	Y
CK	4	16	25	0.25		
DP0015.17	9	16	56	0.98	0.0100	Y
CK	6	16	38	0.39		
DP0015.21	12	16	75	0.93	0.0178	Y
CK	4	16	25	0.25		
DP0015.30	8	16	50	0.94	0.0210	Y

10

Table 5. Enhanced drought tolerance of CaMV 35S Pro: GLR2-rice plants at T2 generation under greenhouse conditions

Line	# Survived plants	# Total plants	Survival rate (%)	Average recovery degree	p-value	P<0.05
CK	4	8	50	0.50		
DP0015.10	7	8	88	1.35	0.0167	Y
CK	0	8	0	0.00		
DP0015.17	4	8	50	0.50	0.0192	Y
CK	4	8	50	0.56		
DP0015.21	8	8	100	1.28	0.0200	Y

Arabidopsis GLR studies demonstrated that plant GLR proteins contain “three-plus-one” transmembrane domains (M1 to M4) and the putative ligand-binding domains (GlnH1 and GlnH2) (Lam, H.-M. et al. (1998) *Nature* 396:125-126), and functional analysis indicated rice GLR1 and GLR2 have same domain, so GLR1 gene was cloned and an over-expression vector was constructed.

As indicated in Figure 6 and 7, over-expression of rice GLR1 or GLR2 genes confers enhanced drought tolerance in *Arabidopsis*.

Therefore, the function of GLR1 in rice in drought tolerance is studied. As shown in Table 6, the GLR1-transgenic rice plants show higher survival rate and recovery degree compared to the control under moderate drought stress in the greenhouse assays. These results show that over-expression of rice GLR1 enhanced the drought tolerance of the GLR1-transgenic rice. An RNAi construct (DP0018) of GLR1 gene was constructed, and the transgenic rice (DP0018) was obtained. The gene validation revealed that the survival rate and the recovery degree of the DP0018 rice were lower than that of the control, which is correlated with the expression levels of GLR1 gene in the DP0018 transgenic events by real-time analysis (data not shown). Decrease of the GLR1 gene expression reduced the drought tolerance of the transgenic rice.

Table 6. Enhanced drought tolerance of CaMV 35S Pro: GLR1-rice plants at T1 generation under greenhouse conditions

Line	# Survived plants	# Total plants	Survival rate (%)	Average recovery degree	p-value	P<0.05
CK	5	8	63	0.71		
DP0025.05	8	8	100	1.20	0.075	
CK	6	8	75	1.13		
DP0025.11	8	8	100	1.83	0.035	Y
CK	6	8	75	1.13		
DP0025.15	8	8	100	2.30	0.001	Y

EXAMPLE 11

Enhanced Nitrogen Use Efficiency (NUE) by Over-expressing GLR2 gene

To understand if over-expression of GLR2 gene improves NUE under low nitrogen conditions, some events were evaluated in a greenhouse NUE assay. As

shown in Table 7 and 8, the average tiller number of the transgenic events are two times that of the control, and the average SPAD values are 40% more than that of the control, which demonstrate over-expression of GLR2 gene could promote tiller formation (FIG. 5) and/or increase SPAD value under greenhouse condition at a limited nitrogen level. These results indicate that over-expression of GLR2 gene can increase NUE.

Table 7. Increased tiller number of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse low nitrogen conditions

Line	Average tiller number	p-value	P<0.05
CK	1.0		
DP0015.11	2.0	0.0493	Y
CK	1.2		
DP0015.14	2.2	0.0384	Y
CK	1.0		
DP0015.24	2.2	0.0157	Y
CK	1.0		
DP0015.30	2.5	0.0050	Y

Table 8. Increased SPAD value of CaMV 35S Pro: GLR2-rice plants at T1 generation under greenhouse low nitrogen conditions

Line	Average SPAD value	p-value	P<0.05
CK	26.10		
DP0015.10	36.82	0.0407	Y
CK	26.10		
DP0015.14	37.32	0.0427	Y
CK	26.10		
DP0015.21	38.60	0.0220	Y

EXAMPLE 12

Transformation of *Arabidopsis* with GLR1 or GLR2

Soybean, sorghum and *Arabidopsis* homologs to rice GLR1 and/or GLR2 can be identified (for example based on homology searches), and transformed into *Arabidopsis* and/or soybean under control of the CaMV 35S promoter for assessment of their ability to enhance drought tolerance. Vector construction, plant

transformation and phenotypic analysis will be similar to that in previously described examples.

To understand if rice GLR genes can improve dicot plants' drought tolerance, NUE or other traits, rice GLR1 or GLR2 gene (vector DP0025 or DP0015) was transformed into *Arabidopsis* using standard known protocols.

To identify the transgenic progeny, the *Arabidopsis* seeds were planted onto MS selection plates containing appropriate antibiotics. The seeds were vernalized by placing at 4 °C for 3 days; and then were moved to a growth chamber under long-day conditions. After growing on MS selection plates for 7-10 days, seedlings with healthy green cotyledons and true leaves and roots extend into the selection medium were considered as transformants. The transformants were then transplanted in planting soil to produce *Arabidopsis* seeds, and the transgenic plants were further confirmed by PCR analysis. The expression levels of GLR1 and GLR2 transgene in transgenic *Arabidopsis* were analyzed. The total RNA of the transformed *Arabidopsis* seedlings were extracted as described in example 6. Primers of GLR1-5'RT-F and GLR1-5'RT-R; and GLR2-RT-F and GLR2-RT-R are used for real-time PCR analysis.

As shown in Figure 6 A and Figure 7 A, GLR2 transgene over-expressed in the transgenic AtDP0015 plants, GLR1 transgene over-expressed in the transgenic AtDP0025 plants, and the expression levels of GLR2 transgene and GLR1 transgene are not detectable in the wild-type *Arabidopsis*.

To characterize the performance of CaMV 35S Pro:: GLR2 (AtDP0015) and CaMV 35S Pro::GLR1 (AtDP0025) transgenic *Arabidopsis* plants under drought stress, wild-type (Columbia), vector control plants (AtDP0009), two AtDP0015 events, and two AtDP0025 events were planted in planting soil to grow for 4 weeks under normal watering condition. The planting soils were watered to saturation before drought treatment, and the *Arabidopsis* plants grew without watering for about 12 days. When the relative water content of the soil decreased to about 3%, the *Arabidopsis* plants were rewatered for 3 days. Three measurements were performed and 12 plants were used. During drought treatment, the wilting levels of wild-type plants and vector control plants were more apparent than those of the AtDP0015 events and AtDP0025 events, and the GLR1 gene and the GLR2 gene transgenic *Arabidopsis* grew better than control at the 3rd day after re-watering (FIG. 6, 7). After re-watering, more than 60% of transgenic events survived, whereas the corresponding survival rates were less than 20% for wild-type plants and vector

control plants.

These results demonstrate that over-expression of rice GLR1 or GLR2 gene in *Arabidopsis* can confer enhanced drought tolerance. In addition, these results further show that a monocot gene (rice GLR) can function in a dicot plant (*Arabidopsis*).

5

10

CLAIMS

1. An isolated polynucleotide comprising (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity, based on GAP method of alignment, to the full length of SEQ ID NO: 3, (b) a polynucleotide with nucleotide sequence of at least 85% sequence identity, based on GAP method of alignment, to the full length of SEQ ID NO: 5; (c) a polynucleotide encoding a polypeptide with amino acid sequence of at least of 90% sequence identity, based on GAP method of alignment, to the full length of SEQ ID NO:4; (d) a polynucleotide encoding a polypeptide with amino acid sequence of at least of 90% sequence identity, based on GAP method of alignment, to the full length of SEQ ID NO:6;or (e) the full complement of the nucleotide sequence of (a) or (b) or (c) or (d).
2. The polynucleotide of Claim 1, wherein the nucleotide sequence comprises SEQ ID NO: 3 or SEQ ID NO: 5.
3. The polynucleotide of Claim 1, wherein the amino acid sequence of the polypeptide comprises SEQ ID NO: 4 or SEQ ID NO: 6.
4. A vector comprising the polynucleotide of claim 1.
5. A recombinant DNA construct comprising the isolated polynucleotide of Claim 1 operably linked to at least one regulatory sequence.
6. A plant or seed comprising a recombinant DNA construct, wherein the recombinant DNA construct comprises the polynucleotide of Claim 1 operably linked to at least one regulatory sequence.
7. A plant comprising in its genome a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked to at least one regulatory element, wherein said plant exhibits increased drought tolerance and/or improved nitrogen use efficiency (NUE) when compared to a control plant.
8. A plant comprising in its genome a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked to at least one regulatory element, wherein said plant exhibits an increase in grain yield, biomass, or both, when compared to a control plant.
9. The plant of Claim 7 or 8, wherein said plant exhibits said increase in grain yield, biomass, or both when compared, under water limiting conditions or nitrogen limiting conditions, to said control plant.

10. The plant of Claim 7 or 8, wherein said plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

5 11. Seed of the plant of Claim 7 or 8, wherein said seed comprises in its genome a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked to at least one regulatory element, wherein a plant produced from said seed exhibits an increase in at least one trait selected from the group consisting of drought tolerance, grain yield and biomass, when compared to a control plant.

10 12. A method of increasing drought tolerance in a plant, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked to at least one regulatory sequence; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) obtaining a progeny plant derived from the transgenic plant of step (b), wherein
15 said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased drought tolerance when compared to a control plant.

13. A method of evaluating drought tolerance in a plant, comprising: (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked
20 to at least one regulatory element; (b) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (c) evaluating the progeny plant for drought tolerance compared to a control plant not comprising the recombinant DNA construct.

25 14. A method of determining an alteration of grain yield, biomass, or both in a plant, comprising: (a) obtaining a transgenic plant, wherein the transgenic plant comprises in its genome a recombinant DNA construct comprising a polynucleotide of Claim 1 operably linked to at least one regulatory element; (b) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its
30 genome the recombinant DNA construct; and (c) measuring the grain yield and/or biomass of the progeny plant and comparing said measurement to the grain yield and/or biomass of a control plant.

15. The method of Claim 14, wherein grain yield and/or biomass of the progeny plant and control plant are measured under water-limiting conditions.

16. The method of Claim 14, wherein said grain yield and/or biomass of the progeny plant is increased relative to that of the control plant.

17. The method of any one of Claim 12 to 14, wherein said plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola,
5 wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

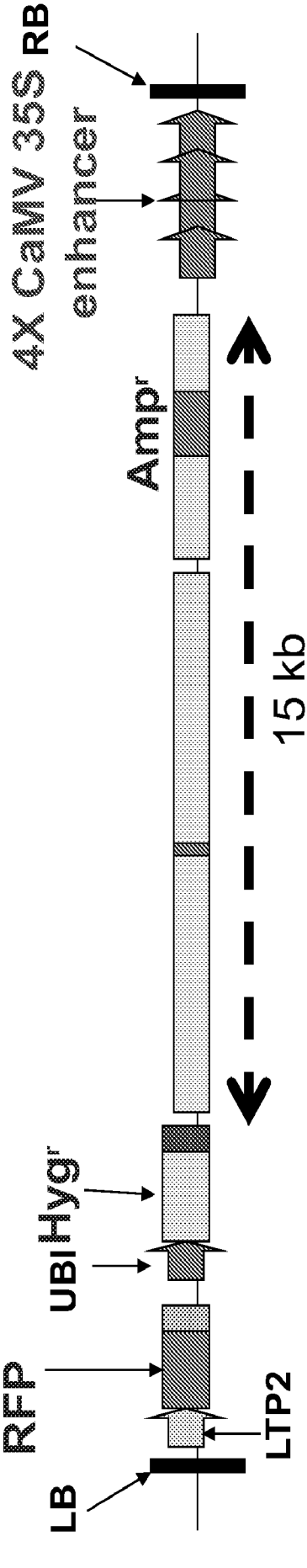


Figure 1

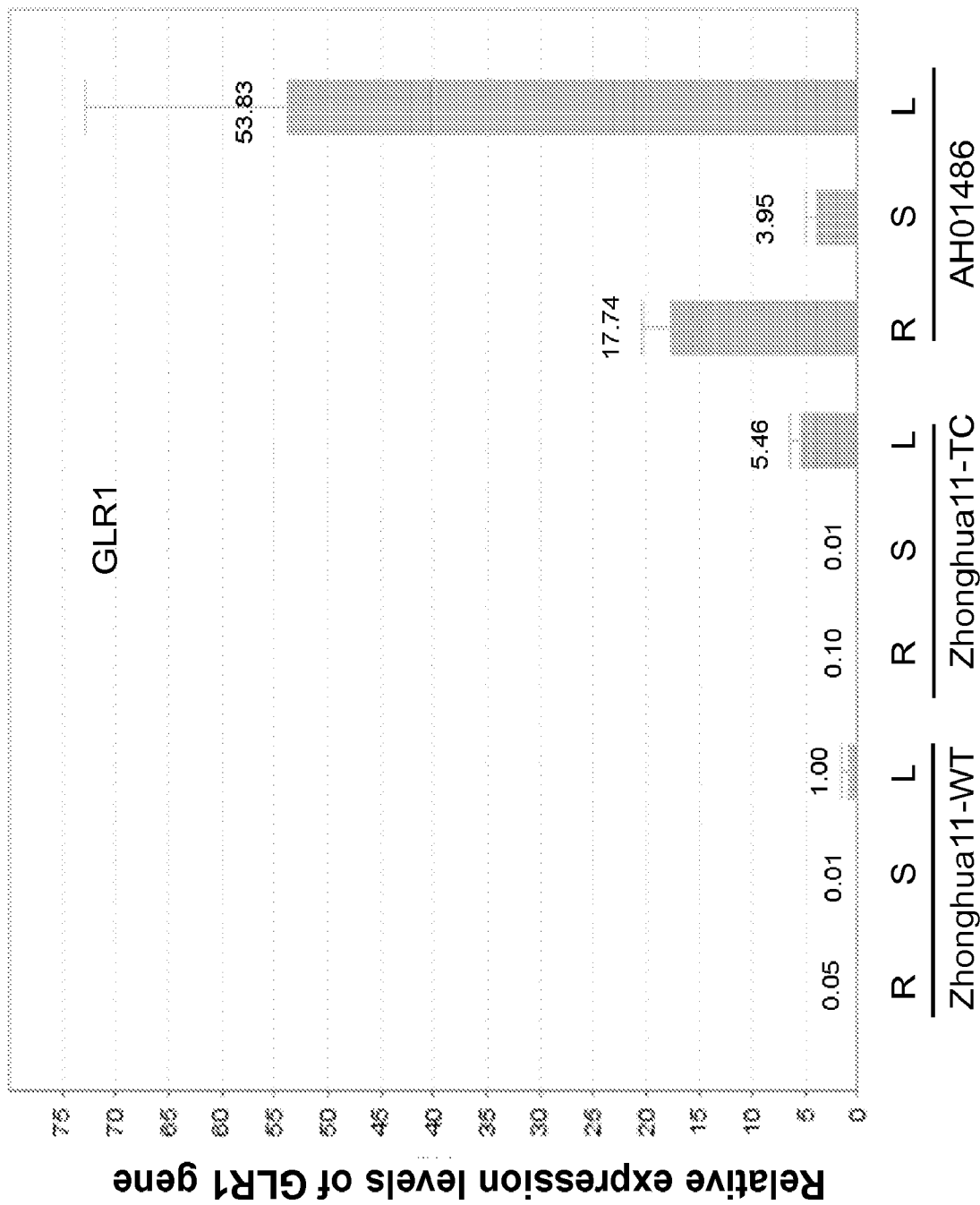


Figure 2A

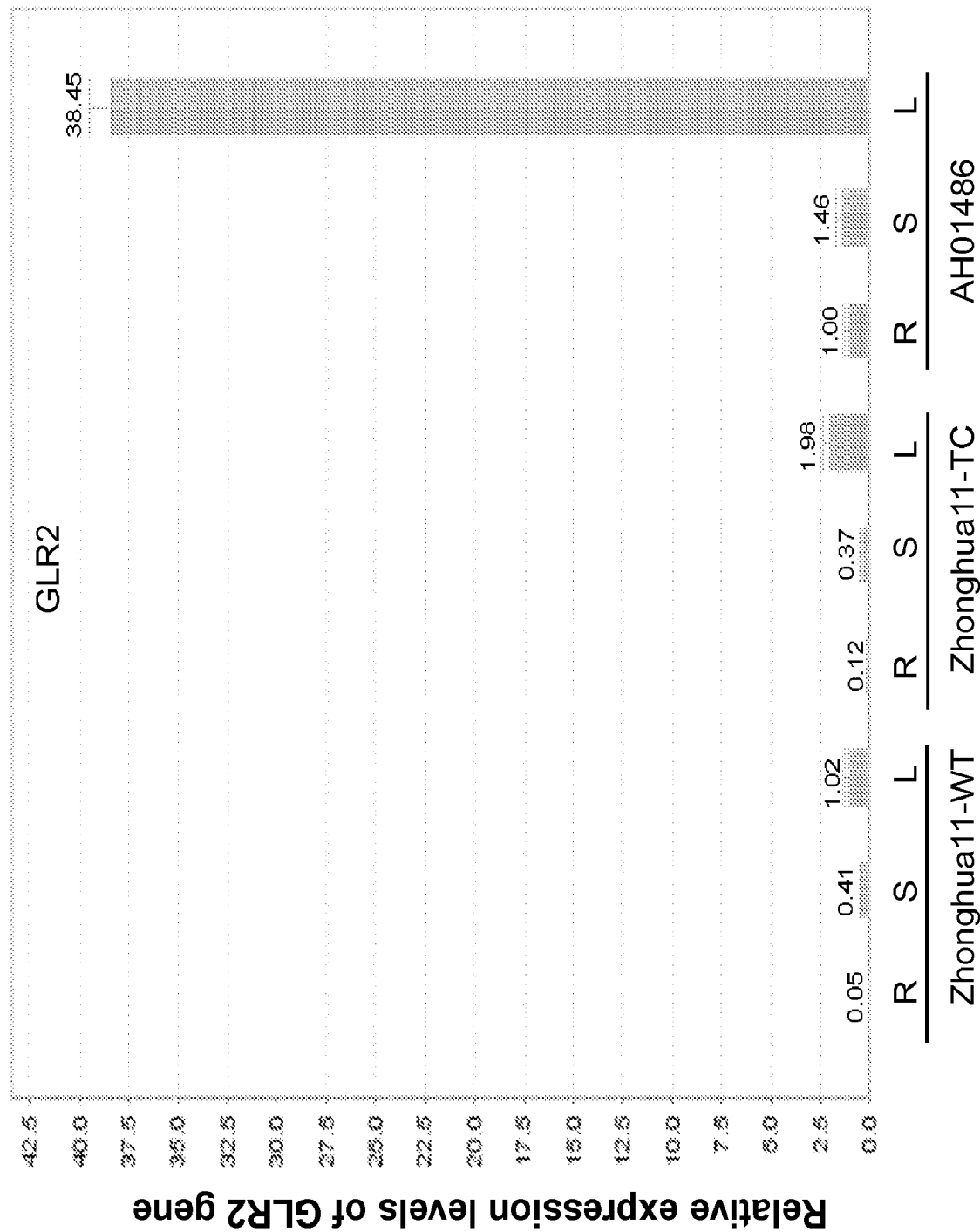


Figure 2B

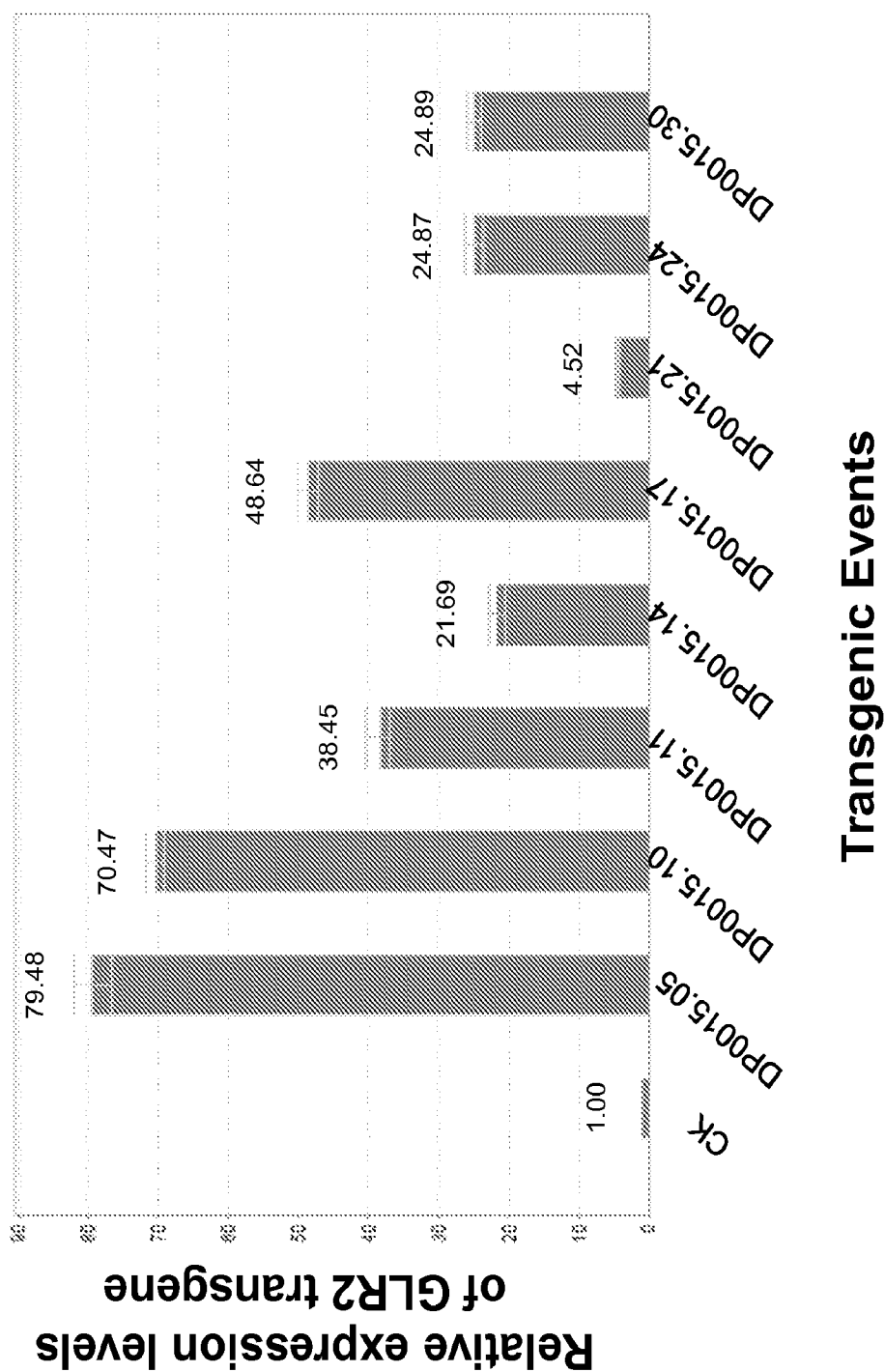


Figure 3

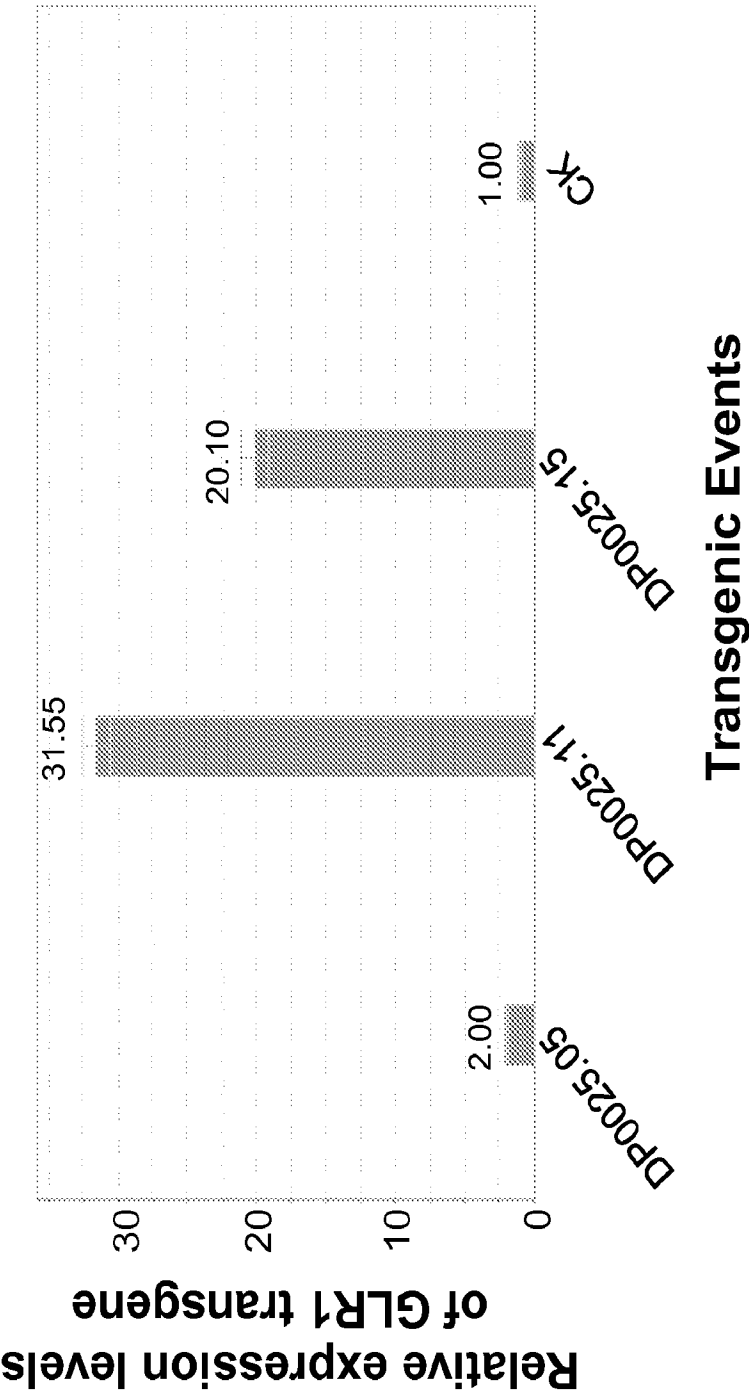


Figure 4

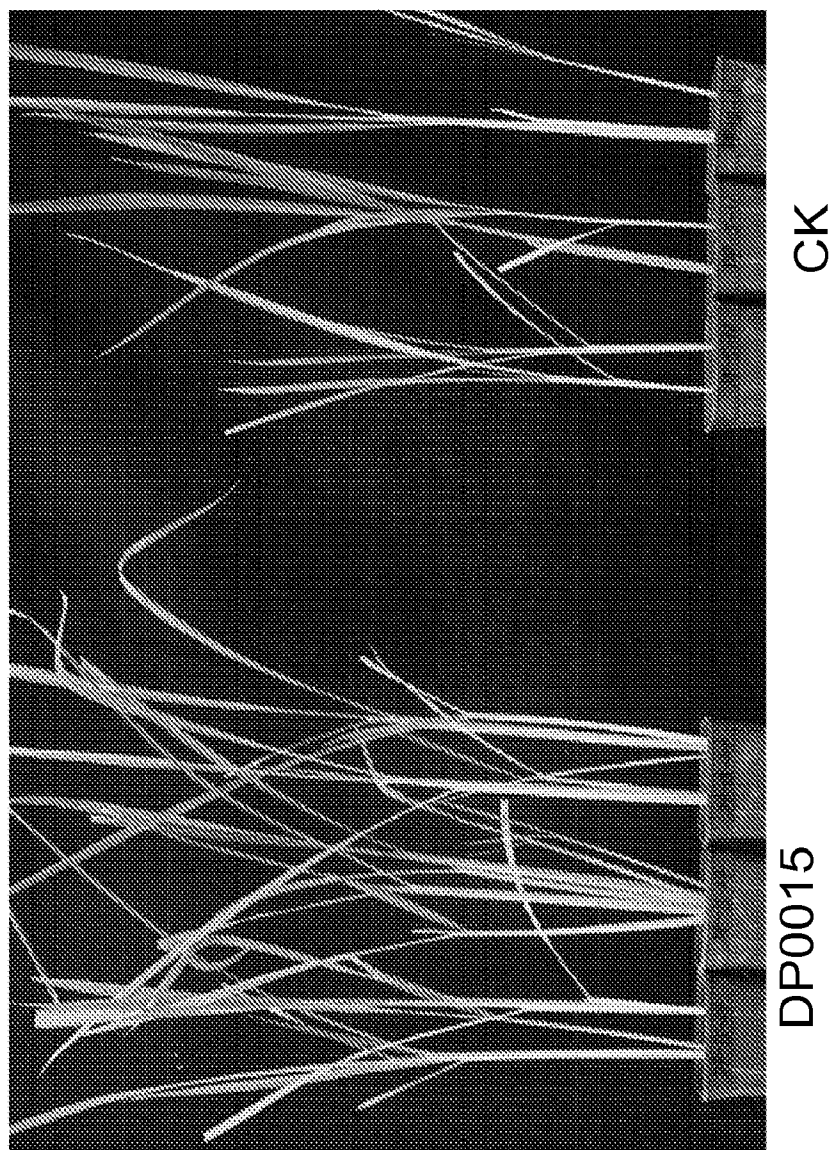


Figure 5

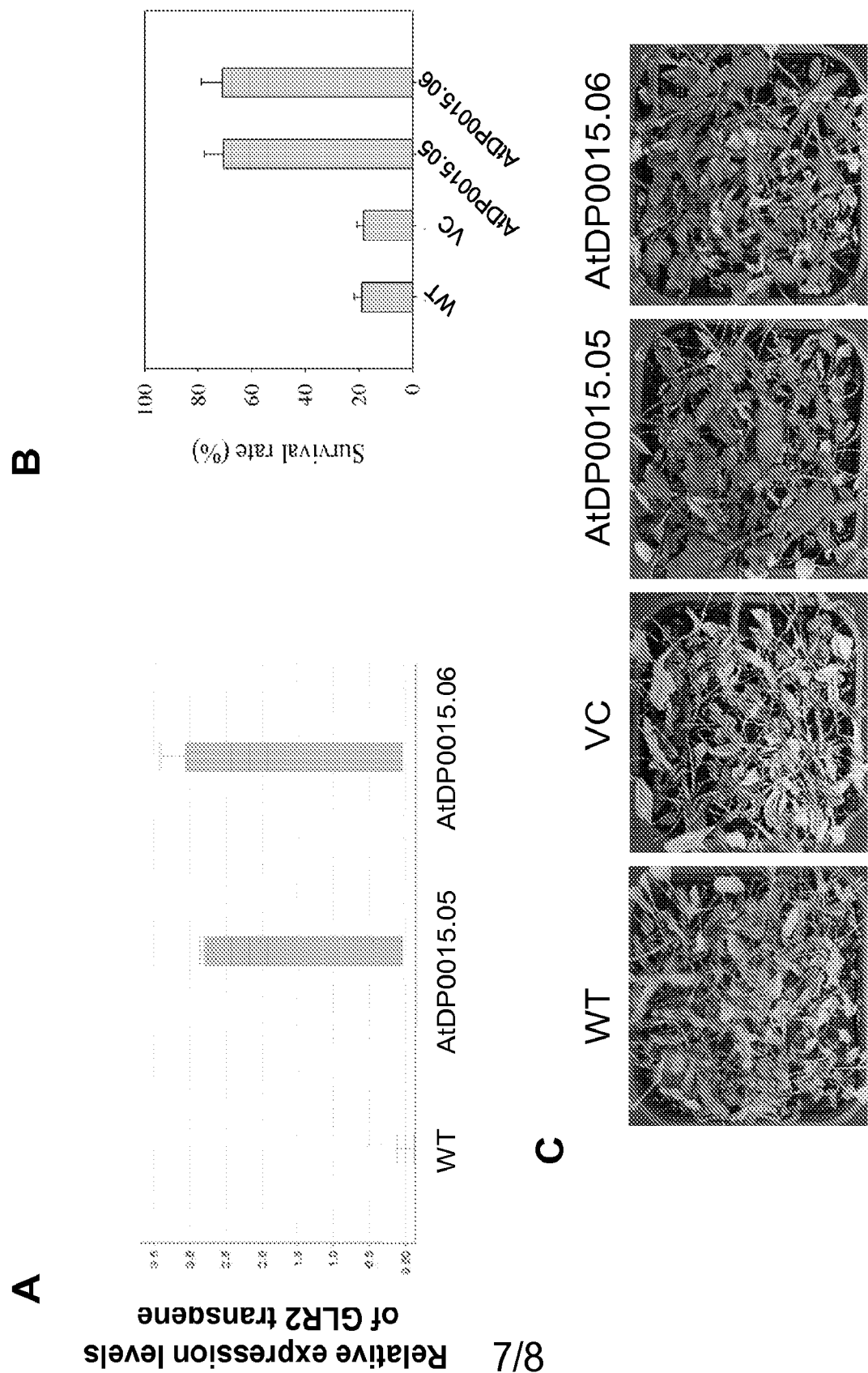


Figure 6

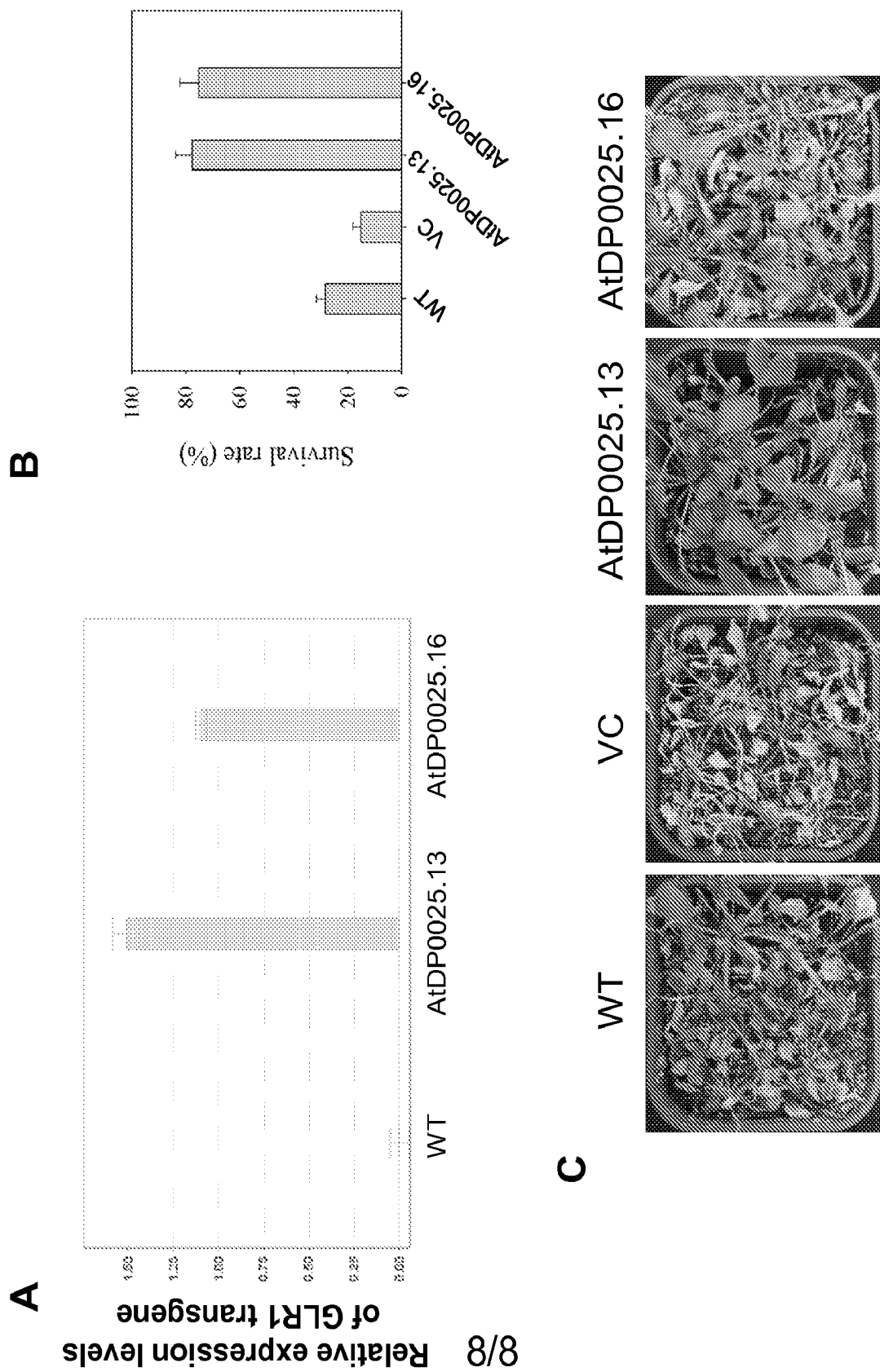


Figure 7