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(54) Title: TRANSGENIC PLANTS WITH ENHANCED TRAITS

(57) Abstract: This disclosure provides recombinant DNA constructs and transgenic plants having enhanced traits such as increased yield, increased nitrogen use efficiency and enhanced drought tolerance; propagules, progeny and field crops of such transgenic plants; and methods of making and using such transgenic plants. This disclosure also provides methods of producing seed from such transgenic plants, growing such seed and selecting progeny plants with enhanced traits. Also disclosed are transgenic plants with altered phenotypes which are useful for screening and selecting transgenic events for the desired enhanced trait.

Transgenic Plants with Enhanced Traits

Cross Reference to Related Application

[0001] This application claims the benefit under 35USC §119(e) of US provisional application Serial No. 62/086,918 filed on December 3, 2014 herein incorporated by reference in its entirety.

Incorporation of Sequence Listing

[0002] The sequence listing file named "60803WO0000_ST25.txt", which is 270 kilobytes (measured in MS-WINDOWS) and was created on December 2, 2014, is filed herewith and incorporated herein by reference in its entirety.

Field of the Invention

[0003] Disclosed herein are recombinant DNA constructs, plants having enhanced traits such as increased yield, increased nitrogen use efficiency and increased water use efficiency; propagules, progenies and field crops of such plants; and methods of making and using such plants. Also disclosed are methods of producing seed from such plants, growing such seed and/or selecting progeny plants with enhanced traits.

Summary of the Invention

[0004] In one aspect, the disclosure provides a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:

a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;

b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;

c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-96; or
d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.

[0005] In another aspect, the disclosure provides a suppression recombinant DNA construct that transcribes into a double-stranded RNA, an antisense RNA, a miRNA or a ta-siRNA.

[0006] In another aspect, the disclosure provides a suppression recombinant DNA construct that transcribes into a miRNA precursor that produces a mature miRNA having a nucleic acid sequence with at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to a fragment of at least 19, 20, 21, 22, 23, 24, 25, 26 or 27 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60.

[0007] In another aspect, the disclosure provides a suppression recombinant DNA construct that transcribes into a miRNA precursor that produces a mature miRNA having a nucleic acid sequence with at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complimentarity to a fragment of at least 19, 20, 21, 22, 23, 24, 25, 26 or 27 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60.

[0008] In another aspect, the disclosure provides a suppression recombinant DNA construct that transcribes into a miRNA precursor that produces a mature miRNA having a nucleic acid sequence with 100% identity or 100% complementarity to a fragment of 21 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60.

[0009] In another aspect, the disclosure provides a suppression recombinant DNA construct comprising a sequence selected from the group consisting of SEQ ID NOs: 67-72.

[0010] In another aspect, the disclosure provides a plant comprising a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:

a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;

b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;

c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-96; or

d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.

[0011] In another aspect, the disclosure provides a plant comprising a recombinant DNA construct of the present disclosure, and having at least one altered phenotype or at least one enhanced trait as compared to a control plant. Such phenotype is characterized or measured by anthocyanin content, biomass, canopy area, chlorophyll content, plant height, water applied, water content or water use efficiency. Such enhanced trait is increased yield, increased nitrogen use efficiency, or increased water use efficiency.

[0012] In another aspect, the disclosure provides a plant comprising a recombinant DNA construct of the present disclosure, wherein the plant is a progeny, a propagule, or a field crop.

[0013] In another aspect, the disclosure provides a field crop comprising a recombinant DNA construct of the present disclosure, wherein the field crop is selected from the group consisting of

corn, soybean, cotton, canola, rice, barley, oat, wheat, turf grass, alfalfa, sugar beet, sunflower, quinoa and sugar cane.

[0014] In another aspect, the disclosure provides a propagule comprising a recombinant DNA construct the present disclosure, wherein the propagule is selected from the group consisting of cell, pollen, ovule, flower, embryo, leaf, root, stem, shoot, meristem, grain and seed.

[0015] In another aspect, the disclosure provides a plant comprising a recombinant DNA construct of the present disclosure, wherein the plant is a monocot plant or is a member of the family Poaceae, wheat plant, maize plant, sweet corn plant, rice plant, wild rice plant, barley plant, rye, millet plant, sorghum plant, sugar cane plant, turfgrass plant, bamboo plant, oat plant, brome-grass plant, *Miscanthus* plant, pampas grass plant, switchgrass (Panicum) plant, and/or teosinte plant, or is a member of the family Alliaceae, onion plant, leek plant, garlic plant; or wherein the plant is a dicot plant or is a member of the family Amaranthaceae, spinach plant, quinoa plant, a member of the family Anacardiaceae, mango plant, a member of the family Asteraceae, sunflower plant, endive plant, lettuce plant, artichoke plant, a member of the family Brassicaceae, *Arabidopsis thaliana* plant, rape plant, oilseed rape plant, broccoli plant, Brussels sprouts plant, cabbage plant, canola plant, cauliflower plant, kohlrabi plant, turnip plant, radish plant, a member of the family Bromeliaceae, pineapple plant, a member of the family Caricaceae, papaya plant, a member of the family Chenopodiaceae, beet plant, a member of the family Curcurbitaceae, melon plant, cantaloupe plant, squash plant, watermelon plant, honeydew plant, cucumber plant, pumpkin plant, a member of the family Dioscoreaceae, yam plant, a member of the family Ericaceae, blueberry plant, a member of the family Euphorbiaceae, cassava plant, a member of the family Fabaceae, alfalfa plant, clover plant, peanut plant, a member of the family Grossulariaceae, currant plant, a member of the family Juglandaceae, walnut plant, a member of the family Lamiaceae, mint plant, a member of the family Lauraceae, avocado plant, a member of the family Leguminosae, soybean plant, bean plant, pea plant, a member of the family Malvaceae, cotton plant, a member of the family Marantaceae, arrowroot plant, a member of the family Myrtaceae, guava plant, eucalyptus plant, a member of the family Rosaceae, peach plant, apple plant, cherry plant, plum plant, pear plant, prune plant, blackberry plant, raspberry plant, strawberry plant, a member of the family Rubiaceae, coffee plant, a member of the family Rutaceae, citrus plant, orange plant, lemon plant, grapefruit plant,

tangerine plant, a member of the family Salicaceae, poplar plant, willow plant, a member of the family Solanaceae, potato plant, sweet potato plant, tomato plant, *Capsicum* plant, tobacco plant, tomatillo plant, eggplant plant, *Atropa belladona* plant, *Datura stramonium* plant, a member of the family Vitaceae, grape plant, a member of the family Umbelliferae, carrot plant, or a member of the family Musaceae, banana plant; or wherein the plant is a member of the family Pinaceae, cedar plant, fir plant, hemlock plant, larch plant, pine plant, or spruce plant.

[0016] In another aspect, the disclosure provides a method for increasing yield, increasing nitrogen use efficiency, or increasing water use efficiency in a plant comprising producing a plant comprising a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:

a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;

b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;

c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-96; or

d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.

[0017] In another aspect, the disclosure provides a method for producing a plant by transforming a plant cell or tissue with the recombinant DNA construct of the present disclosure and regenerating a plant from said cell or tissue containing said recombinant DNA construct. In another aspect, the disclosure provides a method for producing a plant by crossing said plant through breeding with:

a) itself;
b) a second plant from the same plant line;
c) a wild type plant; or
d) a second plant from a different line of plants

to produce a seed, growing said seed to produce a plurality of progeny plants; and selecting a progeny plant with increased yield, increased nitrogen use efficiency, or increased water use efficiency as compared to a control plant.

Detailed Description of the Invention

[0018] In the attached sequence listing:

[0019] SEQ ID NOs 1 to 27 are nucleotide sequences of the coding strand of the DNA used in the recombinant DNA constructs imparting an enhanced trait in plants, each representing a coding sequence for a protein.

[0020] SEQ ID NOs 28 to 54 are amino acid sequences of the cognate proteins of the DNA molecules with nucleotide sequences of SEQ ID NOs 1 to 27 respectively in the same order.

[0021] SEQ ID NOs: 55 to 60 are nucleotide sequences, each representing a coding sequence of a suppression target gene.

[0022] SEQ ID NOs 61 to 66 are amino acid sequences of the cognate proteins of the DNA molecules with nucleotide sequences of SEQ ID NOs 55 to 60 respectively in the same order.

[0023] SEQ ID NOs 67 to 72 are nucleotide sequences of DNA molecules used in the recombinant DNA constructs imparting an enhanced trait or altered phenotype in plants, each representing an engineered miRNA precursor sequence.

[0024] SEQ ID NOs: 73 to 78 are nucleotide sequences of the target recognition sites of the engineered miRNA precursors with nucleotide sequences of SEQ ID NOs 67 to 72 respectively in the same order.

[0025] SEQ ID NOs 79 to 96 are amino acid sequences of proteins homologous to the proteins with amino acid sequences of SEQ ID NOs 28 to 54, and 61 to 66.

[0026] SEQ ID NOs 97 to 100 are nucleotide sequences of DNA molecules used in the recombinant DNA constructs imparting an enhanced trait or altered phenotype in plants, each representing a promoter with a specific expression pattern.

[0027] SEQ ID NOs 101 to 104 are nucleotide sequences of variants of a rice MIR gene.

[0028] Unless otherwise stated, nucleic acid sequences in the text of this specification are given, when read from left to right, in the 5' to 3' direction. One of skill in the art would be aware that a given DNA sequence is understood to define a corresponding RNA sequence which is identical to the DNA sequence except for replacement of the thymine (T) nucleotide of the DNA with uracil (U) nucleotide. Thus, providing a specific DNA sequence is understood to define the exact RNA equivalent. A given first polynucleotide sequence, whether DNA or RNA, further defines the sequence of its exact complement (which can be DNA or RNA), i. e., a second polynucleotide that hybridizes perfectly to the first polynucleotide by forming Watson-Crick base-pairs. By "essentially identical" or "essentially complementary" to a target gene or a fragment of a target gene is meant that a polynucleotide strand (or at least one strand of a double-stranded polynucleotide) is designed to hybridize (generally under physiological conditions such as those found in a living plant or animal cell) to a target gene or to a fragment of a target gene or to the transcript of the target gene or the fragment of a target gene; one of skill in the art would understand that such hybridization does not necessarily require 100% sequence identity or complementarity. A first nucleic acid sequence is "operably" connected or "linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For example, a promoter sequence is "operably linked" to DNA if the promoter provides for transcription or expression of the DNA. Generally, operably linked DNA sequences are contiguous.

[0029] As used herein, the term "expression" refers to the production of a polynucleotide or a protein by a plant, plant cell or plant tissue which can give rise to an altered phenotype or enhanced trait. Expression can also refer to the process by which information from a gene is used in the synthesis of functional gene products, which may include but are not limited to other polynucleotides or proteins which may serve, *e.g.,* an enzymatic, structural or regulatory function. Gene products having a regulatory function include but are not limited to elements that affect the occurrence or level of transcription or translation of a target protein. In some cases, the expression product is a non-coding functional RNA.

[0030] "Modulation" of expression refers to the process of effecting either overexpression or suppression of a polynucleotide or a protein.

[0031] The term "suppression" as used herein refers to a lower expression level of a target polynucleotide or target protein in a plant, plant cell or plant tissue, as compared to the expression in a wild-type or control plant, cell or tissue, at any developmental or temporal stage for the gene. The term "target protein" as used in the context of suppression refers to a protein which is suppressed; similarly, "target mRNA" refers to a polynucleotide which can be suppressed or, once expressed, degraded so as to result in suppression of the target protein it encodes. The term "target gene" as used in the context of suppression refers to either "target protein" or "target mRNA". In alternate non-limiting embodiments, the target protein or target polynucleotide is one the suppression of which can give rise to an enhanced trait or altered phenotype directly or indirectly. In one exemplary embodiment, the target protein is one which can indirectly increase or decrease the expression of one or more other proteins, the increased or decreased expression, respectively, of which is associated with an enhanced trait or an altered phenotype. In another exemplary embodiment, the target protein can bind to one or more other proteins associated with an altered phenotype or enhanced trait to enhance or inhibit their function and thereby affect the altered phenotype or enhanced trait indirectly.

[0032] Suppression can be applied using numerous approaches. Non limiting examples include: suppressing an endogenous gene(s) or a subset of genes in a pathway, suppressing one or more mutation that has resulted in decreased activity of a protein, suppressing the production of an inhibitory agent, to elevate, reducing or eliminating the level of substrate that an enzyme requires

for activity, producing a new protein, activating a normally silent gene; or accumulating a product that does not normally increase under natural conditions.

[0033] Conversely, the term "overexpression" as used herein refers to a greater expression level of a polynucleotide or a protein in a plant, plant cell or plant tissue, compared to expression in a wild-type plant, cell or tissue, at any developmental or temporal stage for the gene. Overexpression can take place in plant cells normally lacking expression of polypeptides functionally equivalent or identical to the present polypeptides. Overexpression can also occur in plant cells where endogenous expression of the present polypeptides or functionally equivalent molecules normally occurs, but such normal expression is at a lower level. Overexpression thus results in a greater than normal production, or "overproduction" of the polypeptide in the plant, cell or tissue.

[0034] The term "target protein" as used herein in the context of overexpression refers to a protein which is overexpressed; "target mRNA" refers to an mRNA which encodes and is translated to produce the target protein, which can also be overexpressed. The term "target gene" as used in the context of overexpression refers to either "target protein" or "target mRNA". In alternative embodiments, the target protein can effect an enhanced trait or altered phenotype directly or indirectly. In the latter case it may do so, for example, by affecting the expression, function or substrate available to one or more other proteins. In an exemplary embodiment, the target protein can bind to one or more other proteins associated with an altered phenotype or enhanced trait to enhance or inhibit their function.

[0035] Overexpression can be achieved using numerous approaches. In one embodiment, overexpression can be achieved by placing the DNA sequence encoding one or more polynucleotides or polypeptides under the control of a promoter, examples of which include but are not limited to endogenous promoters, heterologous promoters, inducible promoters and tissue specific promoters. In one exemplary embodiment, the promoter is a constitutive promoter, for example, the cauliflower mosaic virus 35S transcription initiation region. Thus, depending on the promoter used, overexpression can occur throughout a plant, in specific tissues of the plant, or in the presence or absence of different inducing or inducible agents, such as hormones or environmental signals.

[0036] Gene Suppression Elements: The gene suppression element can be transcribable DNA of any suitable length, and generally includes at least about 19 to about 27 nucleotides (for example 19, 20, 21, 22, 23, or 24 nucleotides) for every target gene that the recombinant DNA construct is intended to suppress. In many embodiments the gene suppression element includes more than 23 nucleotides (for example, more than about 30, about 50, about 100, about 200, about 300, about 500, about 1000, about 1500, about 2000, about 3000, about 4000, or about 5000 nucleotides) for every target gene that the recombinant DNA construct is intended to suppress.

[0037] Suitable gene suppression elements useful in the recombinant DNA constructs of the invention include at least one element (and, in some embodiments, multiple elements) selected from the group consisting of:

(a) DNA that includes at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one first target gene; (b) DNA that includes multiple copies of at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one first target gene; (c) DNA that includes at least one sense DNA segment that is at least one segment of the at least one first target gene; (d) DNA that includes multiple copies of at least one sense DNA segment that is at least one segment of the at least one first target gene; (e) DNA that transcribes to RNA for suppressing the at least one first target gene by forming double-stranded RNA and includes at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one target gene and at least one sense DNA segment that is at least one segment of the at least one first target gene; (f) DNA that transcribes to RNA for suppressing the at least one first target gene by forming a single double-stranded RNA and includes multiple serial anti-sense DNA segments that are anti-sense to at least one segment of the at least one first target gene and multiple serial sense DNA segments that are at least one segment of the at least one first target gene; (g) DNA that transcribes to RNA for suppressing the at least one first target gene by forming multiple double strands of RNA and includes multiple anti-sense DNA segments that are anti-sense to at least one segment of the at least one first target gene and multiple sense DNA segments that are at least one segment of the at least one first target gene, and wherein said multiple anti-sense DNA segments and the multiple sense DNA segments are arranged in a series of inverted repeats; (h) DNA that includes nucleotides derived from a miRNA, preferably a plant miRNA; (i) DNA that includes nucleotides of a siRNA; (j) DNA that transcribes to an RNA

aptamer capable of binding to a ligand; and (k) DNA that transcribes to an RNA aptamer capable of binding to a ligand, and DNA that transcribes to regulatory RNA capable of regulating expression of the first target gene, wherein the regulation is dependent on the conformation of the regulatory RNA, and the conformation of the regulatory RNA is allosterically affected by the binding state of the RNA aptamer.

[0038] Any of these gene suppression elements, whether transcribing to a single double-stranded RNA or to multiple double-stranded RNAs, can be designed to suppress more than one target gene, including, for example, more than one allele of a target gene, multiple target genes (or multiple segments of at least one target gene) from a single species, or target genes from different species.

[0039] Anti-Sense DNA Segments: In one embodiment, the at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one first target gene includes DNA sequence that is anti-sense or complementary to at least a segment of the at least one first target gene, and can include multiple anti-sense DNA segments, that is, multiple copies of at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one first target gene. Multiple anti-sense DNA segments can include DNA sequence that is anti-sense or complementary to multiple segments of the at least one first target gene, or to multiple copies of a segment of the at least one first target gene, or to segments of multiple first target genes, or to any combination of these. Multiple anti-sense DNA segments can be fused into a chimera, e.g., including DNA sequences that are anti-sense to multiple segments of one or more first target genes and fused together.

[0040] The anti-sense DNA sequence that is anti-sense or complementary to (that is, can form Watson-Crick base-pairs with) at least a segment of the at least one first target gene has at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% complementarity to at least a segment of the at least one first target gene. In one embodiment, the DNA sequence that is anti-sense or complementary to at least a segment of the at least one first target gene has between about 95% to about 100% complementarity to at least a segment of the at least one first target gene. Where the at least one anti-sense DNA segment includes multiple anti-sense DNA

segments, the degree of complementarity can be, but need not be, identical for all of the multiple anti-sense DNA segments.

[0041] Sense DNA Segments: In another embodiment, the at least one sense DNA segment that is at least one segment of the at least one first target gene includes DNA sequence that corresponds to (that is, has a sequence that is identical or substantially identical to) at least a segment of the at least one first target gene, and can include multiple sense DNA segments, that is, multiple copies of at least one sense DNA segment that corresponds to (that is, has the nucleotide sequence of) at least one segment of the at least one first target gene. Multiple sense DNA segments can include DNA sequence that is or that corresponds to multiple segments of the at least one first target gene, or to multiple copies of a segment of the at least one first target gene, or to segments of multiple first target genes, or to any combination of these. Multiple sense DNA segments can be fused into a chimera, that is, can include DNA sequences corresponding to multiple segments of one or more first target genes and fused together.

[0042] The sense DNA sequence that corresponds to at least a segment of the target gene has at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% sequence identity to at least a segment of the target gene. In one embodiment, the DNA sequence that corresponds to at least a segment of the target gene has between about 95% to about 100% sequence identity to at least a segment of the target gene. Where the at least one sense DNA segment includes multiple sense DNA segments, the degree of sequence identity can be, but need not be, identical for all of the multiple sense DNA segments.

[0043] Multiple Copies: Where the gene suppression element includes multiple copies of anti-sense or multiple copies of sense DNA sequence, these multiple copies can be arranged serially in tandem repeats. In some embodiments, these multiple copies can be arranged serially end-to-end, that is, in directly connected tandem repeats. In some embodiments, these multiple copies can be arranged serially in interrupted tandem repeats, where one or more spacer DNA segment can be located adjacent to one or more of the multiple copies. Tandem repeats, whether directly connected or interrupted or a combination of both, can include multiple copies of a single anti-sense or multiple copies of a single sense DNA sequence in a serial arrangement or can include

multiple copies of more than one anti-sense DNA sequence or of more than one sense DNA sequence in a serial arrangement.

[0044] Double-stranded RNA: In those embodiments wherein the gene suppression element includes either at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one target gene or at least one sense DNA segment that is at least one segment of the at least one target gene, RNA transcribed from either the at least one anti-sense or at least one sense DNA may become double-stranded by the action of an RNA-dependent RNA polymerase. See, for example, U.S. Pat. No. 5,283,184, which is incorporated by reference herein.

[0045] In yet other embodiments, the gene suppression element can include DNA that transcribes to RNA for suppressing the at least one first target gene by forming double-stranded RNA and includes at least one anti-sense DNA segment that is anti-sense to at least one segment of the at least one target gene (as described above under the heading "Anti-sense DNA Segments") and at least one sense DNA segment that is at least one segment of the at least one first target gene (as described above under the heading "Sense DNA Segments"). Such a gene suppression element can further include spacer DNA segments. Each at least one anti-sense DNA segment is complementary to at least part of a sense DNA segment in order to permit formation of double-stranded RNA by intramolecular hybridization of the at least one anti-sense DNA segment and the at least one sense DNA segment. Such complementarity between an anti-sense DNA segment and a sense DNA segment can be, but need not be, 100% complementarity; in some embodiments, this complementarity can be preferably at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% complementarity.

[0046] The double-stranded RNA can be in the form of a single dsRNA "stem" (region of base-pairing between sense and anti-sense strands), or can have multiple dsRNA "stems". In one embodiment, the gene suppression element can include DNA that transcribes to RNA for suppressing the at least one first target gene by forming essentially a single double-stranded RNA and includes multiple serial anti-sense DNA segments that are anti-sense to at least one segment of the at least one first target gene and multiple serial sense DNA segments that are at least one segment of the at least one first target gene; the multiple serial anti-sense and multiple serial sense segments can form a single double-stranded RNA "stem" or multiple "stems" in a

serial arrangement (with or without non-base paired spacer DNA separating the multiple "stems"). In another embodiment, the gene suppression element includes DNA that transcribes to RNA for suppressing the at least one first target gene by forming multiple dsRNA "stems" of RNA and includes multiple anti-sense DNA segments that are anti-sense to at least one segment of the at least one first target gene and multiple sense DNA segments that are at least one segment of the at least one first target gene, and wherein said multiple anti-sense DNA segments and the multiple sense DNA segments are arranged in a series of dsRNA "stems" (such as, but not limited to "inverted repeats"). Such multiple dsRNA "stems" can further be arranged in series or clusters to form tandem inverted repeats, or structures resembling "hammerhead" or "cloverleaf" shapes. Any of these gene suppression elements can further include spacer DNA segments found within a dsRNA "stem" (for example, as a spacer between multiple anti-sense or sense DNA segments or as a spacer between a base-pairing anti-sense DNA segment and a sense DNA segment) or outside of a double-stranded RNA "stem" (for example, as a loop region separating a pair of inverted repeats). In cases where base-pairing anti-sense and sense DNA segment are of unequal length, the longer segment can act as a spacer.

[0047] miRNAs: In a further embodiment, the gene suppression element can include DNA that includes nucleotides derived from a miRNA (microRNA), that is, a DNA sequence that corresponds to a miRNA native to a virus or a eukaryote of interest (including plants and animals, especially invertebrates), or a DNA sequence derived from such a native miRNA but modified to include nucleotide sequences that do not correspond to the native miRNA. While miRNAs have not to date been reported in fungi, fungal miRNAs, should they exist, are also suitable for use in the invention. An embodiment includes a gene suppression element containing DNA that includes nucleotides derived from a viral or plant miRNA.

[0048] In a non-limiting example, the nucleotides derived from a miRNA can include DNA that includes nucleotides corresponding to the loop region of a native miRNA and nucleotides that are selected from a target gene sequence. In another non-limiting example, the nucleotides derived from a miRNA can include DNA derived from a miRNA precursor sequence, such as a native pri-miRNA or pre-miRNA sequence, or nucleotides corresponding to the regions of a native miRNA and nucleotides that are selected from a target gene sequence number such that the overall structure (e.g., the placement of mismatches in the stem structure of the pre-miRNA)

is preserved to permit the pre-miRNA to be processed into a mature miRNA. In yet another embodiment, the gene suppression element can include DNA that includes nucleotides derived from a miRNA and capable of inducing or guiding in-phase cleavage of an endogenous transcript into trans-acting siRNAs, as described by Allen et al. (2005) Cell, 121:207-221, which is incorporated by reference in its entirety herein. Thus, the DNA that includes nucleotides derived from a miRNA can include sequence naturally occurring in a miRNA or a miRNA precursor molecule, synthetic sequence, or both.

[0049] siRNAs: In yet another embodiment, the gene suppression element can include DNA that includes nucleotides of a small interfering RNA (siRNA). The siRNA can be one or more native siRNAs (such as siRNAs isolated from a non-transgenic eukaryote or from a transgenic eukaryote), or can be one or more DNA sequences predicted to have siRNA activity (such as by use of predictive tools known in the art, see, for example, Reynolds et al. (2004) Nature Biotechnol., 22:326-330, which is incorporated by reference in its entirety herein). Multiple native or predicted siRNA sequences can be joined in a chimeric siRNA sequence for gene suppression. Such a DNA that includes nucleotides of a siRNA includes at least 19 nucleotides, and in some embodiments includes at least 20, at least 21, at least 22, at least 23, or at least 24 nucleotides. In other embodiments, the DNA that includes nucleotides of a siRNA can contain substantially more than 21 nucleotides, for example, more than about 50, about 100, about 300, about 500, about 1000, about 3000, or about 5000 nucleotides or greater.

[0050] Engineered miRNAs and trans-acting siRNAs (ta-siRNAs) are useful for gene suppression with increased specificity. The invention provides recombinant DNA constructs, each including a transcribable engineered miRNA precursor designed to suppress a target sequence, wherein the transcribable engineered miRNA precursor is derived from the fold-back structure of a MIR gene, preferably a plant MIR sequence. These miRNA precursors are also useful for directing in-phase production of siRNAs (e.g., heterologous sequence designed to be processed in a trans-acting siRNA suppression mechanism in planta). The invention further provides a method to suppress expression of a target sequence in a plant cell, including transcribing in a plant cell a recombinant DNA including a transcribable engineered miRNA precursor designed to suppress a target sequence, wherein the transcribable engineered miRNA precursor is derived from the fold-back structure of a MIR gene, preferably a plant MIR

sequence, whereby expression of the target sequence is suppressed relative to its expression in the absence of transcription of the recombinant DNA construct. In specifically claimed embodiments, the transcribable engineered miRNA precursor is derived from the fold-back structure of a rice MIR sequence selected from the group consisting of SEQ ID NOs. 101-104, and their complements.

[0051] The mature miRNAs produced, or predicted to be produced, from these miRNA precursors may be engineered for use in suppression of a target gene, e.g., in transcriptional suppression by the miRNA, or to direct in-phase production of siRNAs in a trans-acting siRNA suppression mechanism (see Allen et al. (2005) Cell, 121:207-221, Vaucheret (2005) Science STKE, 2005:pe43, and Yoshikawa et al. (2005) Genes Dev., 19:2164-2175, all of which are incorporated by reference herein). Plant miRNAs generally have near-perfect complementarity to their target sequences (see, for example, Llave et al. (2002) Science, 297:2053-2056, Rhoades et al. (2002) Cell, 110:513-520, Jones-Rhoades and Bartel (2004) Mol. Cell, 14:787-799, all of which are incorporated by reference herein). Thus, the mature miRNAs can be engineered to serve as sequences useful for gene suppression of a target sequence, by replacing nucleotides of the mature miRNA sequence with nucleotides of the sequence that is targeted for suppression; see, for example, methods disclosed by Parizotto et al. (2004) Genes Dev., 18:2237-2242 and especially U.S. Patent Application Publications US2004/0053411A1, US2004/0268441A1, US2005/0144669, and US2005/0037988 all of which are incorporated by reference herein. When engineering a novel miRNA to target a specific sequence, one strategy is to select within the target sequence a region with sequence that is as similar as possible to the native miRNA sequence. Alternatively, the native miRNA sequence can be replaced with a region of the target sequence, preferably a region that meets structural and thermodynamic criteria believed to be important for miRNA function (see, for example, U.S. Patent Application Publication US2005/0037988). Sequences are preferably engineered such that the number and placement of mismatches in the stem structure of the fold-back region or pre-miRNA is preserved. Thus, an engineered miRNA or engineered miRNA precursor can be derived from any of the mature miRNA sequences, or their corresponding miRNA precursors (including the fold-back portions of the corresponding MIR genes) disclosed herein. The engineered miRNA precursor can be cloned and expressed (transiently or stably) in a plant cell or tissue or intact plant.

[0052] The construction and description of recombinant DNA constructs to modulate small non-coding RNA activities are disclosed in US Patent Application Publication US 2009/0070898 A1, US2011/0296555 A1, US2011/0035839 A1, all of which are incorporated herein by reference in their entirety. In particular, with respect to US2011/0035839 A1, see e.g., sections under the headings “Gene Suppression Elements” in paragraphs 122 to 135, and “Engineered Heterologous miRNA for Controlling Gene Expression in paragraphs 188 to 190.

[0053] As used herein a “plant” includes a whole plant, a transgenic plant, meristematic tissue, a shoot organ/structure (for example, leaf, stem and tuber), a root, a flower, a floral organ/structure (for example, a bract, a sepal, a petal, a stamen, a carpel, an anther and an ovule), a seed (including an embryo, endosperm, and a seed coat) and a fruit (the mature ovary), plant tissue (for example, vascular tissue, ground tissue, and the like) and a cell (for example, guard cell, egg cell, pollen, mesophyll cell, and the like), and progeny of same. The classes of plants that can be used in the disclosed methods are generally as broad as the classes of higher and lower plants amenable to transformation and breeding techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, horsetails, psilophytes, lycophytes, bryophytes, and multicellular algae.

[0054] As used herein a “transgenic plant cell” means a plant cell that is transformed with stably-integrated, recombinant DNA, for example, by *Agrobacterium*-mediated transformation or by bombardment using microparticles coated with recombinant DNA or by other means. A plant cell of this disclosure can be an originally-transformed plant cell that exists as a microorganism or as a progeny plant cell that is regenerated into differentiated tissue, for example, into a transgenic plant with stably-integrated, recombinant DNA, or seed or pollen derived from a progeny transgenic plant.

[0055] As used herein a “control plant” means a plant that does not contain the recombinant DNA of the present disclosure that imparts an enhanced trait or altered phenotype. A control plant is used to identify and select a transgenic plant that has an enhanced trait or altered phenotype. A suitable control plant can be a non-transgenic plant of the parental line used to generate a transgenic plant, for example, a wild type plant devoid of a recombinant DNA. A suitable control plant can also be a transgenic plant that contains recombinant DNA that imparts

other traits, for example, a transgenic plant having enhanced herbicide tolerance. A suitable control plant can in some cases be a progeny of a hemizygous transgenic plant line that does not contain the recombinant DNA, known as a negative segregant, or a negative isogenic line.

[0056] As used herein a “propagule” includes all products of meiosis and mitosis, including but not limited to, plant, seed and part of a plant able to propagate a new plant. Propagules include whole plants, cells, pollen, ovules, flowers, embryos, leaves, roots, stems, shoots, meristems, grains or seeds, or any plant part that is capable of growing into an entire plant. Propagule also includes graft where one portion of a plant is grafted to another portion of a different plant (even one of a different species) to create a living organism. Propagule also includes all plants and seeds produced by cloning or by bringing together meiotic products, or allowing meiotic products to come together to form an embryo or a fertilized egg (naturally or with human intervention).

[0057] As used herein a “progeny” includes any plant, seed, plant cell, and/or regenerable plant part comprising a recombinant DNA of the present disclosure derived from an ancestor plant. A progeny can be homozygous or heterozygous for the transgene. Progeny can be grown from seeds produced by a transgenic plant comprising a recombinant DNA of the present disclosure, and/or from seeds produced by a plant fertilized with pollen or ovule from a transgenic plant comprising a recombinant DNA of the present disclosure.

[0058] As used herein a “trait” is a physiological, morphological, biochemical, or physical characteristic of a plant or particular plant material or cell. In some instances, this characteristic is visible to the human eye, such as seed or plant size, or can be measured by biochemical techniques, such as detecting the protein, starch, certain metabolites, or oil content of seed or leaves, or by observation of a metabolic or physiological process, for example, by measuring tolerance to water deprivation or particular salt or sugar concentrations, or by the measurement of the expression level of a gene or genes, for example, by employing Northern analysis, RT-PCR, microarray gene expression assays, or reporter gene expression systems, or by agricultural observations such as hyperosmotic stress tolerance or yield. Any technique can be used to measure the amount of, comparative level of, or difference in any selected chemical compound or macromolecule in the transgenic plants, however.

[0059] As used herein an “enhanced trait” means a characteristic of a transgenic plant as a result of stable integration and expression of a recombinant DNA in the transgenic plant. Such traits include, but are not limited to, an enhanced agronomic trait characterized by enhanced plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance. In some specific aspects of this disclosure an enhanced trait is selected from the group consisting of drought tolerance, increased water use efficiency, cold tolerance, increased nitrogen use efficiency and increased yield as shown in Tables 7 and 9, and altered phenotypes as shown in Tables 4-6. In another aspect of the disclosure the trait is increased yield under non-stress conditions or increased yield under environmental stress conditions. Stress conditions can include both biotic and abiotic stress, for example, drought, shade, fungal disease, viral disease, bacterial disease, insect infestation, nematode infestation, cold temperature exposure, heat exposure, osmotic stress, reduced nitrogen nutrient availability, reduced phosphorus nutrient availability and high plant density. “Yield” can be affected by many properties including without limitation, plant height, plant biomass, pod number, pod position on the plant, number of internodes, incidence of pod shatter, grain size, efficiency of nodulation and nitrogen fixation, efficiency of nutrient assimilation, resistance to biotic and abiotic stress, carbon assimilation, plant architecture, resistance to lodging, percent seed germination, seedling vigor, and juvenile traits. Yield can also be affected by efficiency of germination (including germination in stressed conditions), growth rate (including growth rate in stressed conditions), ear number, ear size, ear weight, seed number per ear or pod, seed size, composition of seed (starch, oil, protein) and characteristics of seed fill.

[0060] Also used herein, the term “trait modification” encompasses altering the naturally occurring trait by producing a detectable difference in a characteristic in a plant comprising a recombinant DNA of the present disclosure relative to a plant not comprising the recombinant DNA, such as a wild-type plant, or a negative segregant. In some cases, the trait modification can be evaluated quantitatively. For example, the trait modification can entail an increase or decrease, in an observed trait as compared to a control plant. It is known that there can be natural variations in a modified trait. Therefore, the trait modification observed entails a change of the normal distribution and magnitude of the trait in the plants as compared to a control plant.

[0061] The present disclosure relates to a plant with improved economically important characteristics, more specifically increased yield. More specifically the present disclosure relates to a plant comprising a polynucleotide of this disclosure, wherein the plant has increased yield as compared to a control plant. Many plants of this disclosure exhibited increased yield as compared to a control plant. In an embodiment, a plant of the present disclosure exhibited an improved trait that is related to yield, including but not limited to increased nitrogen use efficiency, increased nitrogen stress tolerance, increased water use efficiency and increased drought tolerance, as defined and discussed *infra.*

[0062] Yield can be defined as the measurable produce of economic value from a crop. Yield can be defined in the scope of quantity and/or quality. Yield can be directly dependent on several factors, for example, the number and size of organs, plant architecture (such as the number of branches, plant biomass, etc.), seed production and more. Root development, photosynthetic efficiency, nutrient uptake, stress tolerance, early vigor, delayed senescence and functional stay green phenotypes can be important factors in determining yield. Optimizing the above mentioned factors can therefore contribute to increasing crop yield.

[0063] Reference herein to an increase in yield-related traits can also be taken to mean an increase in biomass (weight) of one or more parts of a plant, which can include above ground and/or below ground (harvestable) plant parts. In particular, such harvestable parts are seeds, and performance of the methods of the disclosure results in plants with increased yield and in particular increased seed yield relative to the seed yield of suitable control plants. The term "yield" of a plant can relate to vegetative biomass (root and/or shoot biomass), to reproductive organs, and/or to propagules (such as seeds) of that plant.

[0064] Increased yield of a plant of the present disclosure can be measured in a number of ways, including test weight, seed number per plant, seed weight, seed number per unit area (for example, seeds, or weight of seeds, per acre), bushels per acre, tons per acre, or kilo per hectare. For example, corn yield can be measured as production of shelled corn kernels per unit of production area, for example in bushels per acre or metric tons per hectare. This is often also reported on a moisture adjusted basis, for example at 15.5 percent moisture. Increased yield can result from improved utilization of key biochemical compounds, such as nitrogen, phosphorous

and carbohydrate, or from improved responses to environmental stresses, such as cold, heat, drought, salt, shade, high plant density, and attack by pests or pathogens. This disclosure can also be used to provide plants with improved growth and development, and ultimately increased yield, as the result of modified expression of plant growth regulators or modification of cell cycle or photosynthesis pathways. Also of interest is the generation of plants that demonstrate increased yield with respect to a seed component that may or may not correspond to an increase in overall plant yield.

[0065] In an embodiment, “alfalfa yield” can also be measured in forage yield, the amount of above ground biomass at harvest. Factors leading contributing to increased biomass include increased vegetative growth, branches, nodes and internodes, leaf area, and leaf area index.

[0066] In another embodiment, “canola yield” can also be measured in pod number, number of pods per plant, number of pods per node, number of internodes, incidence of pod shatter, seeds per silique, seed weight per silique, improved seed, oil, or protein composition.

[0067] Additionally, “corn or maize yield” can also be measured as production of shelled corn kernels per unit of production area, ears per acre, number of kernel rows per ear and number of kernels per row, kernel number or weight per ear, weight per kernel, ear number, ear weight, fresh or dry ear biomass (weight).

[0068] In yet another embodiment, “cotton yield” can be measured as bolls per plant, size of bolls, fiber quality, seed cotton yield in g/plant, seed cotton yield in lb/acre, lint yield in lb/acre, and number of bales.

[0069] Specific embodiment for “rice yield” can also include panicles per hill, grain per hill, and filled grains per panicle.

[0070] Still further embodiment for “soybean yield” can also include pods per plant, pods per acre, seeds per plant, seeds per pod, weight per seed, weight per pod, pods per node, number of nodes, and the number of internodes per plant.

[0071] In still further embodiment, “sugarcane yield” can be measured as cane yield (tons per acre; kg/hectare), total recoverable sugar (pounds per ton), and sugar yield (tons/acre).

[0072] In yet still further embodiment, “wheat yield” can include: cereal per unit area, grain number, grain weight, grain size, grains per head, seeds per head, seeds per plant, heads per acre, number of viable tillers per plant, composition of seed (for example, carbohydrates, starch, oil, and protein) and characteristics of seed fill.

[0073] The terms “yield”, “seed yield” are defined above for a number of core crops. The terms “increased”, “improved”, “enhanced” are interchangeable and are defined herein.

[0074] In another embodiment, the present disclosure provides a method for the production of plants having increased yield; performance of the method gives plants increased yield. “Increased yield” can manifest as one or more of the following: (i) increased plant biomass (weight) of one or more parts of a plant, particularly aboveground (harvestable) parts, of a plant, increased root biomass (increased number of roots, increased root thickness, increased root length) or increased biomass of any other harvestable part; or (ii) increased early vigor, defined herein as an improved seedling aboveground area approximately three weeks post-germination. “Early vigor” refers to active healthy plant growth especially during early stages of plant growth, and can result from increased plant fitness due to, for example, the plants being better adapted to their environment (for example, optimizing the use of energy resources, uptake of nutrients and partitioning carbon allocation between shoot and root). Early vigor in corn, for example, is a combination of the ability of corn seeds to germinate and emerge after planting and the ability of the young corn plants to grow and develop after emergence. Plants having early vigor also show increased seedling survival and better establishment of the crop, which often results in highly uniform fields with the majority of the plants reaching the various stages of development at substantially the same time, which often results in increased yield. Therefore early vigor can be determined by measuring various factors, such as kernel weight, percentage germination, percentage emergence, seedling growth, seedling height, root length, root and shoot biomass, canopy size and color and others.

[0075] Further, increased yield can also manifest as (iii) increased total seed yield, which may result from one or more of an increase in seed biomass (seed weight) due to an increase in the seed weight on a per plant and/or on an individual seed basis an increased number of panicles per plant; an increased number of pods; an increased number of nodes; an increased number of

flowers ("florets") per panicle/plant; increased seed fill rate; an increased number of filled seeds; increased seed size (length, width, area, perimeter), which can also influence the composition of seeds; and/or increased seed volume, which can also influence the composition of seeds.

[0076] Increased yield can also (iv) result in modified architecture, or can occur because of modified plant architecture.

[0077] Increased yield can also manifest as (v) increased harvest index, which is expressed as a ratio of the yield of harvestable parts, such as seeds, over the total biomass

[0078] Increased yield can also manifest as (vi) increased kernel weight, which is extrapolated from the number of filled seeds counted and their total weight. An increased kernel weight can result from an increased seed size and/or seed weight, an increase in embryo size, increased endosperm size, aleurone and/or scutellum, or an increase with respect to other parts of the seed that result in increased kernel weight.

[0079] Increased yield can also manifest as (vii) increased ear biomass, which is the weight of the ear and can be represented on a per ear, per plant or per plot basis.

[0080] In one embodiment, increased yield can be increased seed yield, and is selected from one of the following: (i) increased seed weight; (ii) increased number of filled seeds; and (iii) increased harvest index.

[0081] The disclosure also extends to harvestable parts of a plant such as, but not limited to, seeds, leaves, fruits, flowers, bolls, stems, rhizomes, tubers and bulbs. The disclosure furthermore relates to products derived from a harvestable part of such a plant, such as dry pellets, powders, oil, fat and fatty acids, starch or proteins.

[0082] The present disclosure provides a method for increasing "yield" of a plant or "broad acre yield" of a plant or plant part defined as the harvestable plant parts per unit area, for example seeds, or weight of seeds, per acre, pounds per acre, bushels per acre, tones per acre, tons per acre, kilo per hectare.

[0083] This disclosure further provides a method of increasing yield in a plant by producing a plant comprising a polynucleic acid sequence of this disclosure where the plant can be crossed

with itself, a second plant from the same plant line, a wild type plant, or a plant from a different line of plants to produce a seed. The seed of the resultant plant can be harvested from fertile plants and be used to grow progeny generations of plant(s) of this disclosure. In addition to direct transformation of a plant with a recombinant DNA construct, transgenic plants can be prepared by crossing a first plant having a stably integrated recombinant DNA construct with a second plant lacking the DNA. For example, recombinant DNA can be introduced into a first plant line that is amenable to transformation to produce a transgenic plant which can be crossed with a second plant line to introgress the recombinant DNA into the second plant line.

[0084] Selected transgenic plants transformed with a recombinant DNA construct and having the polynucleotide of this disclosure provides the enhanced trait of increased yield compared to a control plant. Use of genetic markers associated with the recombinant DNA can facilitate production of transgenic progeny that is homozygous for the desired recombinant DNA. Progeny plants carrying DNA for both parental traits can be back-crossed into a parent line multiple times, for example usually 6 to 8 generations, to produce a progeny plant with substantially the same genotype as the one reoccurring original transgenic parental line but having the recombinant DNA of the other transgenic parental line. The term “progeny” denotes the offspring of any generation of a parent plant prepared by the methods of this disclosure containing the recombinant polynucleotides as described herein.

[0085] As used herein “nitrogen use efficiency” refers to the processes which lead to an increase in the plant’s yield, biomass, vigor, and growth rate per nitrogen unit applied. The processes can include the uptake, assimilation, accumulation, signaling, sensing, retranslocation (within the plant) and use of nitrogen by the plant.

[0086] As used herein “nitrogen limiting conditions” refers to growth conditions or environments that provide less than optimal amounts of nitrogen needed for adequate or successful plant metabolism, growth, reproductive success and/or viability.

[0087] As used herein the “increased nitrogen stress tolerance” refers to the ability of plants to grow, develop, or yield normally, or grow, develop, or yield faster or better when subjected to less than optimal amounts of available/applied nitrogen, or under nitrogen limiting conditions.

[0088] As used herein "increased nitrogen use efficiency" refers to the ability of plants to grow, develop, or yield faster or better than normal when subjected to the same amount of available/applied nitrogen as under normal or standard conditions; ability of plants to grow, develop, or yield normally, or grow, develop, or yield faster or better when subjected to less than optimal amounts of available/applied nitrogen, or under nitrogen limiting conditions.

[0089] Increased plant nitrogen use efficiency can be translated in the field into either harvesting similar quantities of yield, while supplying less nitrogen, or increased yield gained by supplying optimal/sufficient amounts of nitrogen. The increased nitrogen use efficiency can improve plant nitrogen stress tolerance, and can also improve crop quality and biochemical constituents of the seed such as protein yield and oil yield. The terms "increased nitrogen use efficiency", "enhanced nitrogen use efficiency", and "nitrogen stress tolerance" are used inter-changeably in the present disclosure to refer to plants with improved productivity under nitrogen limiting conditions.

[0090] As used herein "water use efficiency" refers to the amount of carbon dioxide assimilated by leaves per unit of water vapor transpired. It constitutes one of the most important traits controlling plant productivity in dry environments. "Drought tolerance" refers to the degree to which a plant is adapted to arid or drought conditions. The physiological responses of plants to a deficit of water include leaf wilting, a reduction in leaf area, leaf abscission, and the stimulation of root growth by directing nutrients to the underground parts of the plants. Plants are more susceptible to drought during flowering and seed development (the reproductive stages), as plant's resources are deviated to support root growth. In addition, abscisic acid (ABA), a plant stress hormone, induces the closure of leaf stomata (microscopic pores involved in gas exchange), thereby reducing water loss through transpiration, and decreasing the rate of photosynthesis. These responses improve the water-use efficiency of the plant on the short term. The terms "increased water use efficiency", "enhanced water use efficiency", and "increased drought tolerance" are used inter-changeably in the present disclosure to refer to plants with improved productivity under water-limiting conditions.

[0091] As used herein "increased water use efficiency" refers to the ability of plants to grow, develop, or yield faster or better than normal when subjected to the same amount of

available/applied water as under normal or standard conditions; ability of plants to grow, develop, or yield normally, or grow, develop, or yield faster or better when subjected to reduced amounts of available/applied water (water input) or under conditions of water stress or water deficit stress.

[0092] As used herein "increased drought tolerance" refers to the ability of plants to grow, develop, or yield normally, or grow, develop, or yield faster or better than normal when subjected to reduced amounts of available/applied water and/or under conditions of acute or chronic drought; ability of plants to grow, develop, or yield normally when subjected to reduced amounts of available/applied water (water input) or under conditions of water deficit stress or under conditions of acute or chronic drought.

[0093] As used herein "drought stress" refers to a period of dryness (acute or chronic/prolonged) that results in water deficit and subjects plants to stress and/or damage to plant tissues and/or negatively affects grain/crop yield; a period of dryness (acute or chronic/prolonged) that results in water deficit and/or higher temperatures and subjects plants to stress and/or damage to plant tissues and/or negatively affects grain/crop yield.

[0094] As used herein "water deficit" refers to the conditions or environments that provide less than optimal amounts of water needed for adequate/successful growth and development of plants.

[0095] As used herein "water stress" refers to the conditions or environments that provide improper (either less/insufficient or more/excessive) amounts of water than that needed for adequate/successful growth and development of plants/crops thereby subjecting the plants to stress and/or damage to plant tissues and/or negatively affecting grain/crop yield.

[0096] As used herein "water deficit stress" refers to the conditions or environments that provide less/insufficient amounts of water than that needed for adequate/successful growth and development of plants/crops thereby subjecting the plants to stress and/or damage to plant tissues and/or negatively affecting grain yield.

[0097] As used herein a "polynucleotide" is a nucleic acid molecule comprising a plurality of polymerized nucleotides. A polynucleotide may be referred to as a nucleic acid, a

oligonucleotide, or any fragment thereof. In many instances, a polynucleotide encodes a polypeptide (or protein) or a domain or a fragment thereof. Additionally, a polynucleotide can comprise a promoter, an intron, an enhancer region, a polyadenylation site, a translation initiation site, 5' or 3' untranslated regions, a reporter gene, a selectable marker, a scorable marker, or the like. A polynucleotide can be single-stranded or double-stranded DNA or RNA. A polynucleotide optionally comprises modified bases or a modified backbone. A polynucleotide can be, for example, genomic DNA or RNA, a transcript (such as an mRNA), a cDNA, a PCR product, a cloned DNA, a synthetic DNA or RNA, or the like. A polynucleotide can be combined with carbohydrate(s), lipid(s), protein(s), or other materials to perform a particular activity such as transformation or form a composition such as a peptide nucleic acid (PNA). A polynucleotide can comprise a sequence in either sense or antisense orientations. "Oligonucleotide" is substantially equivalent to the terms amplimer, primer, oligomer, element, target, and probe and is preferably single-stranded.

[0098] As used herein a "recombinant polynucleotide" or "recombinant DNA" is a polynucleotide that is not in its native state, for example, a polynucleotide comprises a series of nucleotides (represented as a nucleotide sequence) not found in nature, or a polynucleotide is in a context other than that in which it is naturally found; for example, separated from polynucleotides with which it typically is in proximity in nature, or adjacent (or contiguous with) polynucleotides with which it typically is not in proximity. The "recombinant polynucleotide" or "recombinant DNA" refers to polynucleotide or DNA which has been genetically engineered and constructed outside of a cell including DNA containing naturally occurring DNA or cDNA or synthetic DNA. For example, the polynucleotide at issue can be cloned into a vector, or otherwise recombined with one or more additional nucleic acids.

[0099] As used herein a "polypeptide" comprises a plurality of consecutive polymerized amino acid residues for example, at least about 15 consecutive polymerized amino acid residues. In many instances, a polypeptide comprises a series of polymerized amino acid residues that is a transcriptional regulator or a domain or portion or fragment thereof. Additionally, the polypeptide can comprise: (i) a localization domain; (ii) an activation domain; (iii) a repression domain; (iv) an oligomerization domain; (v) a protein-protein interaction domain; (vi) a DNA-binding domain; or the like. The polypeptide optionally comprises modified amino acid

residues, naturally occurring amino acid residues not encoded by a codon, non-naturally occurring amino acid residues.

[00100] As used herein "protein" refers to a series of amino acids, oligopeptide, peptide, polypeptide or portions thereof whether naturally occurring or synthetic.

[0101] As used herein a "recombinant polypeptide" is a polypeptide produced by translation of a recombinant polynucleotide.

[0102] A "synthetic polypeptide" is a polypeptide created by consecutive polymerization of isolated amino acid residues using methods known in the art.

[0103] An "isolated polypeptide", whether a naturally occurring or a recombinant polypeptide, is more enriched in (or out of) a cell than the polypeptide in its natural state in a wild-type cell, for example, more than about 5% enriched, more than about 10% enriched, or more than about 20%, or more than about 50%, or more, enriched, for example, alternatively denoted: 105%, 110%, 120%, 150% or more, enriched relative to wild type standardized at 100%. Such enrichment is not the result of a natural response of a wild-type plant. Alternatively, or additionally, the isolated polypeptide is separated from other cellular components, with which it is typically associated, for example, by any of the various protein purification methods.

[0104] As used herein, a "functional fragment" refers to a portion of a polypeptide provided herein which retains full or partial molecular, physiological or biochemical function of the full length polypeptide. A functional fragment often contains the domain(s), such as Pfam domains (see below), identified in the polypeptide provided in the sequence listing.

[0105] A "recombinant DNA construct" as used in the present disclosure comprises at least one expression cassette having a promoter operable in plant cells and a polynucleotide of the present disclosure. DNA constructs can be used as a means of delivering recombinant DNA constructs to a plant cell in order to effect stable integration of the recombinant molecule into the plant cell genome. In one embodiment, the polynucleotide can encode a protein or variant of a protein or fragment of a protein that is functionally defined to maintain activity in transgenic host cells including plant cells, plant parts, explants and whole plants. In another embodiment, the polynucleotide can encode a non-coding RNA that interferes with the functioning of endogenous

classes of small RNAs that regulate expression, including but not limited to taRNAs, siRNAs and miRNAs. Recombinant DNA constructs are assembled using methods known to persons of ordinary skill in the art and typically comprise a promoter operably linked to DNA, the expression of which provides the enhanced agronomic trait.

[0106] Other construct components can include additional regulatory elements, such as 5' leaders and introns for enhancing transcription, 3' untranslated regions (such as polyadenylation signals and sites), and DNA for transit or targeting or signal peptides.

[0107] Percent identity describes the extent to which polynucleotides or protein segments are invariant in an alignment of sequences, for example nucleotide sequences or amino acid sequences. An alignment of sequences is created by manually aligning two sequences, for example, a stated sequence, as provided herein, as a reference, and another sequence, to produce the highest number of matching elements, for example, individual nucleotides or amino acids, while allowing for the introduction of gaps into either sequence. An "identity fraction" for a sequence aligned with a reference sequence is the number of matching elements, divided by the full length of the reference sequence, not including gaps introduced by the alignment process into the reference sequence. "Percent identity" ("% identity") as used herein is the identity fraction times 100.

[0108] As used herein, a "homolog" or "homologues" means a protein in a group of proteins that perform the same biological function, for example, proteins that belong to the same Pfam protein family and that provide a common enhanced trait in transgenic plants of this disclosure. Homologs are expressed by homologous genes. With reference to homologous genes, homologs include orthologs, for example, genes expressed in different species that evolved from common ancestral genes by speciation and encode proteins retain the same function, but do not include paralogs, *i.e.,* genes that are related by duplication but have evolved to encode proteins with different functions. Homologous genes include naturally occurring alleles and artificially-created variants.

[0109] Degeneracy of the genetic code provides the possibility to substitute at least one base of the protein encoding sequence of a gene with a different base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. When optimally aligned,

homolog proteins, or their corresponding nucleotide sequences, have typically at least about 60% identity, in some instances at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or even at least about 99.5% identity over the full length of a protein or its corresponding nucleotide sequence identified as being associated with imparting an enhanced trait or altered phenotype when expressed in plant cells. In one aspect of the disclosure homolog proteins have at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5% identity to a consensus amino acid sequence of proteins and homologs that can be built from sequences disclosed herein.

[0110] Homologs are inferred from sequence similarity, by comparison of protein sequences, for example, manually or by use of a computer-based tool using known sequence comparison algorithms such as BLAST and FASTA. A sequence search and local alignment program, for example, BLAST, can be used to search query protein sequences of a base organism against a database of protein sequences of various organisms, to find similar sequences, and the summary Expectation value (E-value) can be used to measure the level of sequence similarity. Because a protein hit with the lowest E-value for a particular organism may not necessarily be an ortholog or be the only ortholog, a reciprocal query is used to filter hit sequences with significant E-values for ortholog identification. The reciprocal query entails search of the significant hits against a database of protein sequences of the base organism. A hit can be identified as an ortholog, when the reciprocal query's best hit is the query protein itself or a paralog of the query protein. With the reciprocal query process orthologs are further differentiated from paralogs among all the homologs, which allows for the inference of functional equivalence of genes. A further aspect of the homologs encoded by DNA useful in the transgenic plants of the invention are those proteins that differ from a disclosed protein as the result of deletion or insertion of one or more amino acids in a native sequence.

[0111] Other functional homolog proteins differ in one or more amino acids from those of a trait-improving protein disclosed herein as the result of one or more of known conservative amino acid substitutions, for example, valine is a conservative substitute for alanine and

threonine is a conservative substitute for serine. Conservative substitutions for an amino acid within the native sequence can be selected from other members of a class to which the naturally occurring amino acid belongs. Representative amino acids within these various classes include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; and (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. Conserved substitutes for an amino acid within a native protein or polypeptide can be selected from other members of the group to which the naturally occurring amino acid belongs. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side 30 chains is cysteine and methionine. Naturally conservative amino acids substitution groups are: valine-leucine, valine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alaninevaline, aspartic acid-glutamic acid, and asparagine-glutamine. A further aspect of the disclosure includes proteins that differ in one or more amino acids from those of a described protein sequence as the result of deletion or insertion of one or more amino acids in a native sequence.

[0112] In general, the term “variant” refers to molecules with some differences, generated synthetically or naturally, in their nucleotide or amino acid sequences as compared to a reference (native) polynucleotides or polypeptides, respectively. These differences include substitutions, insertions, deletions or any desired combinations of such changes in a native polynucleotide or amino acid sequence.

[0113] With regard to polynucleotide variants, differences between presently disclosed polynucleotides and polynucleotide variants are limited so that the nucleotide sequences of the former and the latter are similar overall and, in many regions, identical. Due to the degeneracy of the genetic code, differences between the former and the latter nucleotide sequences may be

silent (for example, the amino acids encoded by the polynucleotide are the same, and the variant polynucleotide sequence encodes the same amino acid sequence as the presently disclosed polynucleotide). Variant nucleotide sequences can encode different amino acid sequences, in which case such nucleotide differences will result in amino acid substitutions, additions, deletions, insertions, truncations or fusions with respect to the similarly disclosed polynucleotide sequences. These variations can result in polynucleotide variants encoding polypeptides that share at least one functional characteristic. The degeneracy of the genetic code also dictates that many different variant polynucleotides can encode identical and/or substantially similar polypeptides.

[0114] As used herein "gene" or "gene sequence" refers to the partial or complete coding sequence of a gene, its complement, and its 5' and/or 3' untranslated regions (UTRs) and their complements. A gene is also a functional unit of inheritance, and in physical terms is a particular segment or sequence of nucleotides along a molecule of DNA (or RNA, in the case of RNA viruses) involved in producing a polypeptide chain. The latter can be subjected to subsequent processing such as chemical modification or folding to obtain a functional protein or polypeptide. By way of example, a transcriptional regulator gene encodes a transcriptional regulator polypeptide, which can be functional or require processing to function as an initiator of transcription.

[0115] As used herein, the term "promoter" refers generally to a DNA molecule that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) to initiate transcription. A promoter can be initially isolated from the 5' untranslated region (5' UTR) of a genomic copy of a gene. Alternately, promoters can be synthetically produced or manipulated DNA molecules. Promoters can also be chimeric, that is a promoter produced through the fusion of two or more heterologous DNA molecules. Plant promoters include promoter DNA obtained from plants, plant viruses, fungi and bacteria such as *Agrobacterium* and *Bradyrhizobium* bacteria.

[0116] Promoters which initiate transcription in all or most tissues of the plant are referred to as "constitutive" promoters. Promoters which initiate transcription during certain periods or stages of development are referred to as "developmental" promoters. Promoters whose expression is

enhanced in certain tissues of the plant relative to other plant tissues are referred to as "tissue enhanced" or "tissue preferred" promoters. Promoters which express within a specific tissue of the plant, with little or no expression in other plant tissues are referred to as "tissue specific" promoters. A promoter that expresses in a certain cell type of the plant, for example a microspore mother cell, is referred to as a "cell type specific" promoter. An "inducible" promoter is a promoter in which transcription is initiated in response to an environmental stimulus such as cold, drought or light; or other stimuli such as wounding or chemical application. Many physiological and biochemical processes in plants exhibit endogenous rhythms with a period of about 24 hours. A "diurnal promoter" is a promoter which exhibits altered expression profiles under the control of a circadian oscillator. Diurnal regulation is subject to environmental inputs such as light and temperature and coordination by the circadian clock.

[0117] Sufficient expression in plant seed tissues is desired to affect improvements in seed composition. Exemplary promoters for use for seed composition modification include promoters from seed genes such as napin as disclosed in US Patent 5,420,034, maize L3 oleosin as disclosed in US Patent 6,433,252, zein Z27 as disclosed by Russell *et al.* (1997) *Transgenic Res.* 6(2):157-166, globulin 1 as disclosed by Belanger *et al* (1991) *Genetics* 129:863-872, glutelin 1 as disclosed by Russell (1997) *supra*, and peroxiredoxin antioxidant (Per1) as disclosed by Stacy *et al.* (1996) *Plant Mol Biol.* 31(6):1205-1216.

[0118] As used herein, the term "leader" refers to a DNA molecule isolated from the untranslated 5' region (5' UTR) of a genomic copy of a gene and is defined generally as a nucleotide segment between the transcription start site (TSS) and the protein coding sequence start site. Alternately, leaders can be synthetically produced or manipulated DNA elements. A leader can be used as a 5' regulatory element for modulating expression of an operably linked transcribable polynucleotide molecule. As used herein, the term "intron" refers to a DNA molecule that can be isolated or identified from the genomic copy of a gene and can be defined generally as a region spliced out during mRNA processing prior to translation. Alternately, an intron can be a synthetically produced or manipulated DNA element. An intron can contain enhancer elements that effect the transcription of operably linked genes. An intron can be used as a regulatory element for modulating expression of an operably linked transcribable

polynucleotide molecule. A DNA construct can comprise an intron, and the intron may or may not be with respect to the transcribable polynucleotide molecule.

[0119] As used herein, the term "enhancer" or "enhancer element" refers to a cis-acting transcriptional regulatory element, a.k.a. cis-element, which confers an aspect of the overall expression pattern, but is usually insufficient alone to drive transcription, of an operably linked polynucleotide. Unlike promoters, enhancer elements do not usually include a transcription start site (TSS) or TATA box or equivalent sequence. A promoter can naturally comprise one or more enhancer elements that affect the transcription of an operably linked polynucleotide. An isolated enhancer element can also be fused to a promoter to produce a chimeric promoter cis-element, which confers an aspect of the overall modulation of gene expression. A promoter or promoter fragment can comprise one or more enhancer elements that effect the transcription of operably linked genes. Many promoter enhancer elements are believed to bind DNA-binding proteins and/or affect DNA topology, producing local conformations that selectively allow or restrict access of RNA polymerase to the DNA template or that facilitate selective opening of the double helix at the site of transcriptional initiation. An enhancer element can function to bind transcription factors that regulate transcription. Some enhancer elements bind more than one transcription factor, and transcription factors can interact with different affinities with more than one enhancer domain.

[0120] Expression cassettes of this disclosure can include a "transit peptide" or "targeting peptide" or "signal peptide" molecule located either 5' or 3' to or within the gene(s). These terms generally refer to peptide molecules that when linked to a protein of interest directs the protein to a particular tissue, cell, subcellular location, or cell organelle. Examples include, but are not limited to, chloroplast transit peptides (CTPs), chloroplast targeting peptides, mitochondrial targeting peptides, nuclear targeting signals, nuclear exporting signals, vacuolar targeting peptides, and vacuolar sorting peptides. For description of the use of chloroplast transit peptides see US Patent No. 5,188,642 and US Patent No. 5,728,925. For description of the transit peptide region of an Arabidopsis EPSPS gene in the present disclosure, see Klee, H.J. *Et al* (*MGG* (1987) 210:437-442. Expression cassettes of this disclosure can also include an intron or introns. Expression cassettes of this disclosure can contain a DNA near the 3' end of the cassette that acts as a signal to terminate transcription from a heterologous nucleic acid and that

directs polyadenylation of the resultant mRNA. These are commonly referred to as "3'-untranslated regions" or "3'-non-coding sequences" or "3'-UTRs". The "3' non-translated sequences" means DNA sequences located downstream of a structural nucleotide sequence and include sequences encoding polyadenylation and other regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the mRNA precursor. The polyadenylation signal can be derived from a natural gene, from a variety of plant genes, or from T-DNA. An example of a polyadenylation sequence is the nopaline synthase 3' sequence (nos 3'; Fraley *et al., Proc. Natl. Acad. Sci. USA* 80: 4803-4807, 1983). The use of different 3' non-translated sequences is exemplified by Ingelbrecht *et al., Plant Cell* 1:671-680, 1989.

[0121] Expression cassettes of this disclosure can also contain one or more genes that encode selectable markers and confer resistance to a selective agent such as an antibiotic or an herbicide. A number of selectable marker genes are known in the art and can be used in the present disclosure: selectable marker genes conferring tolerance to antibiotics like kanamycin and paromomycin (*nptII*), hygromycin B (*aph IV*), spectinomycin (*aad*A), U.S. Patent Publication 2009/0138985A1 and gentamycin (*aac3* and *aac*C4) or tolerance to herbicides like glyphosate (for example, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), US Patent 5,627,061; US Patent 5,633,435; US Patent 6,040,497; US Patent 5,094,945), sulfonyl herbicides (for example, acetohydroxyacid synthase or acetolactate synthase conferring tolerance to acetolactate synthase inhibitors such as sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidyloxybenzoates and phthalide (US Patents 6,225,105; 5,767,366; 4,761,373; 5,633,437; 6,613,963; 5,013,659; 5,141,870; 5,378,824; 5,605,011)), bialaphos or phosphinothricin or derivatives (*e. g.*, phosphinothricin acetyltransferase (*bar*) tolerance to phosphinothricin or glufosinate (US Patents 5,646,024; 5,561,236; 5,276,268; 5,637,489; 5,273,894); dicamba (dicamba monooxygenase, Patent Application Publications US2003/0115626A1), or sethoxydim (modified acetyl-coenzyme A carboxylase for conferring tolerance to cyclohexanedione (sethoxydim)), and aryloxyphenoxypropionate (haloxyfop, US Patent 6,414,222).

[0122] Transformation vectors of this disclosure can contain one or more "expression cassettes", each comprising a native or non-native plant promoter operably linked to a polynucleotide sequence of interest, which is operably linked to a 3' UTR termination signal, for expression in

an appropriate host cell. It also typically comprises sequences required for proper translation of the polynucleotide or transgene. As used herein, the term “transgene” refers to a polynucleotide molecule artificially incorporated into a host cell’s genome. Such a transgene can be heterologous to the host cell. The term “transgenic plant” refers to a plant comprising such a transgene. The coding region usually codes for a protein of interest but can also code for a functional RNA of interest, for example an antisense RNA, a nontranslated RNA, in the sense or antisense direction, a miRNA, a noncoding RNA, or a synthetic RNA used in either suppression or over expression of target gene sequences. The expression cassette comprising the nucleotide sequence of interest can be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. As used herein the term “chimeric” refers to a DNA molecule that is created from two or more genetically diverse sources, for example a first molecule from one gene or organism and a second molecule from another gene or organism.

[0123] Recombinant DNA constructs in this disclosure generally include a 3’ element that typically contains a polyadenylation signal and site. Known 3’ elements include those from *Agrobacterium tumefaciens* genes such as *nos 3’*, *tml 3’*, *tmr 3’*, *tms 3’*, *ocs 3’*, *tr7 3’*, for example disclosed in US Patent 6,090,627; 3’ elements from plant genes such as wheat (*Triticum aesevitum*) heat shock protein 17 (*Hsp17 3’*), a wheat ubiquitin gene, a wheat fructose-1,6-biphosphatase gene, a rice glutelin gene, a rice lactate dehydrogenase gene and a rice beta-tubulin gene, all of which are disclosed in US Patent Application Publication 2002/0192813 A1; and the pea (*Pisum sativum)* ribulose biphosphate carboxylase gene (*rbs 3’)*, and 3’ elements from the genes within the host plant.

[0124] As used herein “operably linked” means the association of two or more DNA fragments in a recombinant DNA construct so that the function of one, for example, protein-encoding DNA, is controlled by the other, for example, a promoter.

[0125] Transgenic plants can comprise a stack of one or more polynucleotides disclosed herein resulting in the production of multiple polypeptide sequences. Transgenic plants comprising stacks of polynucleotides can be obtained by either or both of traditional breeding methods or through genetic engineering methods. These methods include, but are not limited to, crossing individual transgenic lines each comprising a polynucleotide of interest, transforming a

transgenic plant comprising a first gene disclosed herein with a second gene, and co-transformation of genes into a single plant cell. Co-transformation of genes can be carried out using single transformation vectors comprising multiple genes or genes carried separately on multiple vectors.

[0126] Transgenic plants comprising or derived from plant cells of this disclosure transformed with recombinant DNA can be further enhanced with stacked traits, for example, a crop plant having an enhanced trait resulting from expression of DNA disclosed herein in combination with herbicide and/or pest resistance traits. For example, genes of the current disclosure can be stacked with other traits of agronomic interest, such as a trait providing herbicide resistance, or insect resistance, such as using a gene from *Bacillus thuringensis* to provide resistance against lepidopteran, coliopteran, homopteran, hemiopteran, and other insects, or improved quality traits such as improved nutritional value. Herbicides for which transgenic plant tolerance has been demonstrated and the method of the present disclosure can be applied include, but are not limited to, glyphosate, dicamba, glufosinate, sulfonylurea, bromoxynil and norflurazon herbicides. Polynucleotide molecules encoding proteins involved in herbicide tolerance known in the art and include, but are not limited to, a polynucleotide molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) disclosed in US Patents 5,094,945; 5,627,061; 5,633,435 and 6,040,497 for imparting glyphosate tolerance; polynucleotide molecules encoding a glyphosate oxidoreductase (GOX) disclosed in US Patent 5,463,175 and a glyphosate-N-acetyl transferase (GAT) disclosed in US Patent Application Publication 2003/0083480 A1 also for imparting glyphosate tolerance; dicamba monooxygenase disclosed in US Patent Application Publication 2003/0135879 A1 for imparting dicamba tolerance; a polynucleotide molecule encoding bromoxynil nitrilase (*Bxn)* disclosed in US Patent 4,810,648 for imparting bromoxynil tolerance; a polynucleotide molecule encoding phytoene desaturase (*crtI*) described in Misawa et al, (1993) *Plant J.* 4:833-840 and in Misawa et al, (1994) *Plant J.* 6:481-489 for norflurazon tolerance; a polynucleotide molecule encoding acetohydroxyacid synthase (AHAS, *aka* ALS) described in Sathasiivan *et al.* (1990) *Nucl. Acids Res.* 18:2188-2193 for imparting tolerance to sulfonylurea herbicides; polynucleotide molecules known as *bar* genes disclosed in DeBlock, *et al.* (1987) *EMBO J.* 6:2513-2519 for imparting glufosinate and bialaphos tolerance; polynucleotide molecules disclosed in US Patent Application Publication 2003/010609 A1 for imparting N-amino methyl phosphonic acid tolerance; polynucleotide molecules disclosed in US Patent

6,107,549 for imparting pyridine herbicide resistance; molecules and methods for imparting tolerance to multiple herbicides such as glyphosate, atrazine, ALS inhibitors, isoxoflutole and glufosinate herbicides are disclosed in US Patent 6,376,754 and US Patent Application Publication 2002/0112260. Molecules and methods for imparting insect/nematode/virus resistance are disclosed in US Patents 5,250,515; 5,880,275; 6,506,599; 5,986,175 and US Patent Application Publication 2003/0150017 A1.

Plant Cell Transformation Methods

[0127] Numerous methods for transforming chromosomes in a plant cell with recombinant DNA are known in the art and are used in methods of producing a transgenic plant cell and plant. Two effective methods for such transformation are *Agrobacterium*-mediated transformation and microprojectile bombardment-mediated transformation. Microprojectile bombardment methods are illustrated in US Patents 5,015,580 (soybean); 5,550,318 (corn); 5,538,880 (corn); 5,914,451 (soybean); 6,160,208 (corn); 6,399,861 (corn); 6,153,812 (wheat) and 6,365,807 (rice). *Agrobacterium*-mediated transformation methods are described in US Patents 5,159,135 (cotton); 5,824,877 (soybean); 5,463,174 (canola); 5,591,616 (corn); 5,846,797 (cotton); 8,044,260 (cotton); 6,384,301 (soybean), 7,026,528 (wheat) and 6,329,571 (rice), US Patent Application Publication 2004/0087030 A1 (cotton), and US Patent Application Publication 2001/0042257 A1 (sugar beet), all of which are incorporated herein by reference in their entirety. Transformation of plant material is practiced in tissue culture on nutrient media, for example a mixture of nutrients that allow cells to grow *in vitro*. Recipient cell targets include, but are not limited to, meristem cells, shoot tips, hypocotyls, calli, immature or mature embryos, and gametic cells such as microspores, pollen, sperm and egg cells. Callus can be initiated from tissue sources including, but not limited to, immature or mature embryos, hypocotyls, seedling apical meristems, microspores and the like. Cells containing a transgenic nucleus are grown into transgenic plants.

[0128] In addition to direct transformation of a plant material with a recombinant DNA construct, a transgenic plant can be prepared by crossing a first plant comprising a recombinant DNA with a second plant lacking the recombinant DNA. For example, recombinant DNA can be introduced into a first plant line that is amenable to transformation, which can be crossed with

a second plant line to introgress the recombinant DNA into the second plant line. A transgenic plant with recombinant DNA providing an enhanced trait, for example, enhanced yield, can be crossed with a transgenic plant line having other recombinant DNA that confers another trait, for example herbicide resistance or pest resistance, to produce progeny plants having recombinant DNA that confers both traits. Typically, in such breeding for combining traits the transgenic plant donating the additional trait is a male line and the transgenic plant carrying the base traits is the female line. The progeny of this cross will segregate such that some of the plants will carry the DNA for both parental traits and some will carry DNA for one parental trait; such plants can be identified by markers associated with parental recombinant DNA, for example, marker identification by analysis for recombinant DNA or, in the case where a selectable marker is linked to the recombinant, by application of the selecting agent such as a herbicide for use with a herbicide tolerance marker, or by selection for the enhanced trait. Progeny plants carrying DNA for both parental traits can be crossed back into the female parent line multiple times, for example usually 6 to 8 generations, to produce a progeny plant with substantially the same genotype as the original transgenic parental line but for the recombinant DNA of the other transgenic parental line.

[0129] For transformation, DNA is typically introduced into only a small percentage of target plant cells in any one transformation experiment. Marker genes are used to provide an efficient system for identification of those cells that are stably transformed by receiving and integrating a recombinant DNA construct into their genomes. Preferred marker genes provide selective markers which confer resistance to a selective agent, such as an antibiotic or an herbicide. Any of the herbicides to which plants of this disclosure can be resistant is an agent for selective markers. Potentially transformed cells are exposed to the selective agent. In the population of surviving cells are those cells where, generally, the resistance-conferring gene is integrated and expressed at sufficient levels to permit cell survival. Cells can be tested further to confirm stable integration of the exogenous DNA. Commonly used selective marker genes include those conferring resistance to antibiotics such as kanamycin and paromomycin (*nptII*), hygromycin B (*aph IV*), spectinomycin (*aad*A) and gentamycin (*aac3* and *aac*C4) or resistance to herbicides such as glufosinate (*bar* or *pat*), dicamba (DMO) and glyphosate (*aroA* or EPSPS). Examples of such selectable markers are illustrated in US Patents 5,550,318; 5,633,435; 5,780,708; 6,118,047 and 8,030,544. Markers which provide an ability to visually screen transformants can

also be employed, for example, a gene expressing a colored or fluorescent protein such as a luciferase or green fluorescent protein (GFP) or a gene expressing a *beta*-glucuronidase or *uid*A gene (GUS) for which various chromogenic substrates are known.

[0130] Plant cells that survive exposure to a selective agent, or plant cells that have been scored positive in a screening assay, may be cultured in vitro to regenerate plantlets. Developing plantlets regenerated from transformed plant cells can be transferred to plant growth mix, and hardened off, for example, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO_2, and 25-250 microeinsteins m^{-2} s^{-1} of light, prior to transfer to a greenhouse or growth chamber for maturation. Plants are regenerated from about 6 weeks to 10 months after a transformant is identified, depending on the initial tissue, and plant species. Plants can be pollinated using conventional plant breeding methods known to those of skill in the art to produce seeds, for example self-pollination is commonly used with transgenic corn. The regenerated transformed plant or its progeny seed or plants can be tested for expression of the recombinant DNA and selected for the presence of an enhanced agronomic trait.

Transgenic Plants and Seeds

[0131] Transgenic plants derived from transgenic plant cells having a transgenic nucleus of this disclosure are grown to generate transgenic plants having an enhanced trait as compared to a control plant, and produce transgenic seed and haploid pollen of this disclosure. Such plants with enhanced traits are identified by selection of transformed plants or progeny seed for the enhanced trait. For efficiency a selection method is designed to evaluate multiple transgenic plants (events) comprising the recombinant DNA, for example multiple plants from 2 to 20 or more transgenic events. Transgenic plants grown from transgenic seeds provided herein demonstrate improved agronomic traits that contribute to increased yield or other traits that provide increased plant value, including, for example, improved seed quality. Of particular interest are plants having increased water use efficiency or drought tolerance, enhanced high temperature or cold tolerance, increased yield, and increased nitrogen use efficiency.

[0132] Table 1 provides a list of sequences of protein-encoding genes as recombinant DNA for production of transgenic plants with enhanced traits. The elements of Table 1 are described by reference to:

[0133] "NUC SEQ ID NO." which identifies a DNA sequence.

[0134] "PEP SEQ ID NO." which identifies an amino acid sequence.

[0135] "Gene ID" which refers to an arbitrary identifier.

[0136] "Gene Name and Description" which is a common name and functional description of the gene.

Table 1. Sequences for Protein-Coding Genes

NUC SEQ ID NO.	PEP SEQ ID NO.	Gene ID	Gene Name and Description
1	28	TRDX4-01	Arabidopsis mitochondrial import receptor subunit TOM5 homolog (TOM5)
2	29	TRDX4-02	Arabidopsis K+ independent Asparaginase
3	30	TRDX4-03	Arabidopsis plasma membrane (PM)-localized cyclic nucleotide gated channels (CNGCs)
4	31	TRDX4-04	Arabidopsis receptor like kinase
5	32	TRDX4-05	Rice gibberellin receptor gene GID1
6	33	TRDX4-06	Corn plastidial phosphoenolpyruvate (PEP) phosphate translocator (PPT)
7	34	TRDX4-07	Arabidopsis sulfolipid biosynthesis protein SQD1
8	35	TRDX4-08	Arabidopsis cytochrome P450 family protein
9	36	TRDX4-09	Pseudomonas syringae phosphoglycerate kinase
10	37	TRDX4-10	Corn phospholipase A (PLA1)
11	38	TRDX4-11	Arabidopsis plastidal glycolate/glycerate translocator 1 (PLGG1)
12	39	TRDX4-12	Corn coiled coil domain protein
13	40	TRDX4-13	Corn iron-phytosiderophore transporter protein yellow stripe 1 (YS1)
14	41	TRDX4-14	Arabidopsis ACT domain-containing protein 3 (ACR3)
15	42	TRDX4-15	E coli arginine-insensitive acetylglutamate kinase (NAGK)
16	43	TRDX4-16	Soybean NOS1 (mitochondrial constitutive NOS)
17	44	TRDX4-17	Corn thylakoid lumen protein CYP38
18	45	TRDX4-18	Arabidopsis glutaredoxin family protein
19	46	TRDX4-19	E coli aminobutyrate aminotransferase
20	47	TRDX4-20	Synechocystis sp. gene of unknown function
21	48	TRDX4-21	Corn putative forever young oxidoreductase
22	49	TRDX4-22	Corn MSH2 gene
23	50	TRDX4-23	Arabidopsis mitogen-activated protein kinase kinase kinase 19 (MAPKKK19)
24	51	TRDX4-24	Arabidopsis carbamoyl phosphate synthase EC 6.3.3.5 large subunit
25	52	TRDX4-25	Soybean gene improving Nitrogen Utilization Efficiency (NUE)
26	53	TRDX4-26	Arabidopsis casparian strip membrane protein 1 (CASP1)
27	54	TRDX4-27	E coli codon redesigned asparagine synthetase A (AsnA) gene

[0137] Table 2 provides a list of sequences for suppression of target protein-coding genes, as recombinant DNA for production of transgenic plants with enhanced traits. The elements of Table 2 are described by reference to:

[0138] "Target NUC SEQ ID NO." which identifies a nucleotide coding sequence of the suppression target gene.

[0139] "Target PEP SEQ ID NO." which identifies an amino acid sequence of the suppression target gene.

[0140] "Target Gene ID" which is an arbitrary identifier of the suppression target gene.

[0141] "Engineered miRNA precursor SEQ ID NO." which identifies a nucleotide sequence of the miRNA construct.

[0142] "miRNA recognition site SEQ ID NO." which identifies a nucleotide sequence of the miRNA recognition site.

[0143] "Target Gene Name and Description" which is a common name and functional description of the suppression target gene.

Table 2. Sequences for Gene Suppression

Target NUC SEQ ID NO.	Target PEP SEQ ID NO.	Target Gene ID	Engineered miRNA precursor SEQ ID NO.	miRNA recognition site SEQ ID NO.	Target Gene Name and Description
55	61	TRDX4-1T	67	73	corn homolog of NOX1 gene, Plastidial phosphoenolpyruvate (PEP) phosphate translocator (PPT)
56	62	TRDX4-3T	68	74	soybean SOUL gene
57	63	TRDX4-4T	69	75	soybean Elongated Hypocotyl 5 (Hy5)
58	64	TRDX4-5T	70	76	corn Proliferating cell nuclear antigen 2 (PCNA2)
59	65	TRDX4-6T	71	77	corn putative dolichyl-di-phosphooligosaccharide protein
60	66	TRDX4-7T	72	78	corn Peroxisomal_fatty_acid_beta-oxidation

Selection methods for transgenic plants with enhanced traits

[0144] Within a population of transgenic plants each regenerated from a plant cell with recombinant DNA many plants that survive to fertile transgenic plants that produce seeds and progeny plants will not exhibit an enhanced agronomic trait. Selection from the population is necessary to identify one or more transgenic plants with an enhanced trait. Transgenic plants having enhanced traits are selected from populations of plants regenerated or derived from plant cells transformed as described herein by evaluating the plants in a variety of assays to detect an enhanced trait, for example, increased water use efficiency or drought tolerance, enhanced high temperature or cold tolerance, increased yield, increased nitrogen use efficiency, enhanced seed composition such as enhanced seed protein and enhanced seed oil. These assays can take many

forms including, but not limited to, direct screening for the trait in a greenhouse or field trial or by screening for a surrogate trait. Such analyses can be directed to detecting changes in the chemical composition, biomass, physiological property, or morphology of the plant. Changes in chemical compositions such as nutritional composition of grain can be detected by analysis of the seed composition and content of protein, free amino acids, oil, free fatty acids, starch or tocopherols. Changes in chemical compositions can also be detected by analysis of contents in leaves, such as chlorophyll or carotenoid contents. Changes in biomass characteristics can be evaluated on greenhouse or field grown plants and can include plant height, stem diameter, root and shoot dry weights, canopy size; and, for corn plants, ear length and diameter. Changes in physiological properties can be identified by evaluating responses to stress conditions, for example assays using imposed stress conditions such as water deficit, nitrogen deficiency, cold growing conditions, pathogen or insect attack or light deficiency, or increased plant density. Changes in morphology can be measured by visual observation of tendency of a transformed plant to appear to be a normal plant as compared to changes toward bushy, taller, thicker, narrower leaves, striped leaves, knotted trait, chlorosis, albino, anthocyanin production, or altered tassels, ears or roots. Other selection properties include days to pollen shed, days to silking, leaf extension rate, chlorophyll content, leaf temperature, stand, seedling vigor, internode length, plant height, leaf number, leaf area, tillering, brace roots, stay green or delayed senescence, stalk lodging, root lodging, plant health, bareness/prolificacy, green snap, and pest resistance. In addition, phenotypic characteristics of harvested grain can be evaluated, including number of kernels per row on the ear, number of rows of kernels on the ear, kernel abortion, kernel weight, kernel size, kernel density and physical grain quality.

[0145] Assays for screening for a desired trait are readily designed by those practicing in the art. The following illustrates screening assays for corn traits using hybrid corn plants. The assays can be adapted for screening other plants such as canola, wheat, cotton and soybean either as hybrids or inbreds.

[0146] Transgenic corn plants having increased nitrogen use efficiency can be identified by screening transgenic plants in the field under the same and sufficient amount of nitrogen supply as compared to control plants, where such plants provide higher yield as compared to control plants. Transgenic corn plants having increased nitrogen use efficiency can also be identified by

screening transgenic plants in the field under reduced amount of nitrogen supply as compared to control plants, where such plants provide the same or similar yield as compared to control plants.

[0147] Transgenic corn plants having increased yield are identified by screening using progenies of the transgenic plants over multiple locations for several years with plants grown under optimal production management practices and maximum weed and pest control or standard agronomic practices (SAP). Selection methods can be applied in multiple and diverse geographic locations, for example up to 16 or more locations, over one or more planting seasons, for example at least two planting seasons, to statistically distinguish yield improvement from natural environmental effects.

[0148] Transgenic corn plants having increased water use efficiency or drought tolerance are identified by screening plants in an assay where water is withheld for a period to induce stress followed by watering to revive the plants. For example, a selection process imposes 3 drought/re-water cycles on plants over a total period of 15 days after an initial stress free growth period of 11 days. Each cycle consists of 5 days, with no water being applied for the first four days and a water quenching on the 5th day of the cycle. The primary phenotypes analyzed by the selection method are the changes in plant growth rate as determined by height and biomass during a vegetative drought treatment.

[0149] Although the plant cells and methods of this disclosure can be applied to any plant cell, plant, seed or pollen, for example, any fruit, vegetable, grass, tree or ornamental plant, the various aspects of the disclosure are applied to corn, soybean, cotton, canola, rice, barley, oat, wheat, turf grass, alfalfa, sugar beet, sunflower, quinoa and sugar cane plants.

Example 1. Corn Transformation

[0150] This example illustrates transformation methods in producing a transgenic corn plant cell, seed, and plant having altered phenotypes as shown in Tables 4-6, or an enhanced trait, for example, increased water use efficiency, increased nitrogen use efficiency, and increased yield as shown in Tables 7 and 9.

[0151] For *Agrobacterium*-mediated transformation of corn embryo cells corn plants were grown in the greenhouse and ears were harvested when the embryos were 1.5 to 2.0 mm in length. Ears were surface-sterilized by spraying or soaking the ears in 80% ethanol, followed by air drying. Immature embryos were isolated from individual kernels on surface-sterilized ears. Shortly after excision, immature maize embryos were inoculated with overnight grown *Agrobacterium* cells, and incubated at room temperature with *Agrobacterium* for 5-20 minutes. Inoculated immature embryos were then co-cultured with *Agrobacterium* for 1 to 3 days at 23°C in the dark. Co-cultured embryos were transferred to selection media and cultured for approximately two weeks to allow embryogenic callus to develop. Embryogenic calli were transferred to culture medium containing glyphosate and subcultured at about two week intervals. Transformed plant cells were recovered 6 to 8 weeks after initiation of selection.

[0152] For *Agrobacterium*-mediated transformation of maize callus immature embryos are cultured for approximately 8-21 days after excision to allow callus to develop. Callus is then incubated for about 30 minutes at room temperature with the *Agrobacterium* suspension, followed by removal of the liquid by aspiration. The callus and *Agrobacterium* are co-cultured without selection for 3-6 days followed by selection on paromomycin for approximately 6 weeks, with biweekly transfers to fresh media. Paromomycin resistant calli are identified about 6-8 weeks after initiation of selection.

[0153] To regenerate transgenic corn plants individual transgenic calli resulting from transformation and selection were placed on media to initiate shoot and root development into plantlets. Plantlets were transferred to potting soil for initial growth in a growth chamber at 26°C followed by a mist bench before transplanting to 5 inch pots where plants were grown to maturity. The regenerated plants were self-fertilized and seeds were harvested for use in one or more methods to select seeds, seedlings or progeny second generation transgenic plants (R2 plants) or hybrids, for example, by selecting transgenic plants exhibiting an enhanced trait as compared to a control plant.

[0154] The above process can be repeated to produce multiple events of transgenic corn plants from cells that were transformed with recombinant DNA from the genes identified in Table 1 or with recombinant DNA from Table 2 that is transcribed into a non-coding miRNA. Progeny

transgenic plants and seeds of the transformed plants were screened for the presence and single copy of the inserted gene, and for increased water use efficiency, increased yield, increased nitrogen use efficiency, and altered phenotypes as shown in Tables 4-6. From each group of multiple events of transgenic plants with a specific recombinant DNA from Table 1 or Table 2, the event(s) that showed increased yield, increased water use efficiency, increased nitrogen use efficiency, and altered phenotypes was (were) identified.

Example 2. Soybean transformation

[0155] This example illustrates plant transformation in producing a transgenic soybean plant cell, seed, and plant having altered phenotypes, or an enhanced trait, for example, increased water use efficiency or drought tolerance and increased yield as shown in Tables 7 and 9.

[0156] For *Agrobacterium* mediated transformation, soybean seeds were imbibed overnight and the meristem explants excised. Soybean explants were mixed with induced *Agrobacterium* cells containing plasmid DNA with the gene of interest cassette and a plant selectable marker cassette no later than 14 hours from the time of initiation of seed imbibition, and wounded using sonication. Following wounding, explants were placed in co-culture for 2-5 days at which point they were transferred to selection media to allow selection and growth of transgenic shoots. Resistant shoots were harvested in approximately 6-8 weeks and placed into selective rooting media for 2-3 weeks. Shoots producing roots were transferred to the greenhouse and potted in soil. Shoots that remained healthy on selection, but did not produce roots were transferred to non-selective rooting media for an additional two weeks. Roots from any shoots that produced roots off selection were tested for expression of the plant selectable marker before they were transferred to the greenhouse and potted in soil.

[0157] The above process can be repeated to produce multiple events of transgenic soybean plants from cells that were transformed with recombinant DNA from the genes identified in Table 1 or recombinant DNA transcribed into a miRNA identified in Table 2. Progeny transgenic plants and seed of the transformed plant cells were screened for the presence and single copy of the inserted gene, and for increased water use efficiency and increased yield as shown in Tables 7 and 9.

Example 3. Identification of altered phenotypes in automated greenhouse

[0158] This example illustrates screening and identification of transgenic plants for altered phenotypes in an automated greenhouse (AGH). The apparatus and the methods for automated phenotypic screening of plants are disclosed in US Patent publication No. US20110135161 (filed on Nov. 10, 2010), which is incorporated by reference herein in its entirety.

Screening and identification of transgenic corn plants for altered phenotypes.

[0159] Corn plants were tested in 3 screens in AGH under different conditions including non-stress, nitrogen deficit and water deficit stress conditions. All screens began with a non-stress condition during day 0-5 germination phase, after which the plants were grown for 22 days under screen specific conditions as shown in Table 3.

Table 3. Description of the 3 AGH screens for corn plants

Screen	Description	Germination phase (5 days)	Screen specific phase (22 days)
Non-stress	well watered	55% VWC	55% VWC
	sufficient nitrogen	water	8 mM nitrogen
Water deficit	limited watered	55% VWC	30% VWC
	sufficient nitrogen	water	8 mM nitrogen
Nitrogen deficit	well watered	55% VWC	55% VWC
	low nitrogen	water	2 mM nitrogen

[0160] Water deficit is defined as a specific Volumetric Water Content (VWC) that is lower than the VWC of non-stress plant. For example, a non-stressed plant might be maintained at 55% VWC and the VWC for a water-deficit assay might be defined around 30% VWC as shown in Table 3. Data were collected using visible light and hyperspectral imaging as well as direct measurement of pot weight and amount of water and nutrient applied to individual plants on a daily basis.

[0161] Nitrogen deficit is defined in part as a specific mM concentration of nitrogen that is lower than the nitrogen concentration of non-stress plants. For example, a non-stress plant might be

maintained at 8mM nitrogen while the nitrogen concentration applied in a nitrogen-deficit assay might be maintained at a concentration of 2mM.

[0162] Up to ten parameters were measured for each screen. The visible light color imaging based measurements are: biomass, canopy area and plant height. Biomass (Bmass) is defined as estimated shoot fresh weight (g) of the plant obtained from images acquired from multiple angles of view. Canopy Area (Cnop) is defined as area of leaf as seen in top-down image (mm^2). Plant Height (PlntH) refers to the distance from the top of the pot to the highest point of the plant derived from side image (mm). Anthocyanin score and area, chlorophyll score and concentration, and water content score are hyperspectral imaging based parameters. Anthocyanin Score (AntS) is an estimate of anthocyanin in the leaf canopy obtained from a top-down hyperspectral image. Anthocyanin Area (AntA) is an estimate of anthocyanin in the stem obtained from a side-view hyperspectral image. Chlorophyll Score (ClrpS) and Cholrophyll Concentration (ClrpC) are both measurements of chlorophyll in the leaf canopy obtained from a top-down hyperspectral image, where Chlorophyll Score measures in relative units and is done for soybean plants, and Chlorophyll Concentration measures in ppm units and is done for corn plants. Water Content Score (WtrCt) is a measurement of water in the leaf canopy obtained from a top-down hyperspectral image. Water Use Efficiency (WUE) is derived from the grams of plant biomass per liter of water added. Water Applied (WtrAp) is a direct measurement of water added to a pot (pot with no hole) during the course of an experiment.

[0163] These physiological screen runs were set up so that tested transgenic lines were compared to a control line. The collected data were analyzed against the control using % delta and certain p-value cutoff. Tables 4-6 are summaries of transgenic corn plants comprising the disclosed recombinant DNA constructs with altered phenotypes under non stress, nitrogen deficit, and water deficit conditions, respectively.

[0164] The test results are represented by three numbers: the first number before letter "p" denotes number of events with an increase in the tested parameter at $p \leq 0.1$; the second number before letter "n" denotes number of events with an decrease in the tested parameter at $p \leq 0.1$; the third number before letter "t" denotes total number of transgenic events tested for a given parameter in a specific screen. The increase or decrease is measured in comparison to non-

transgenic control plants. A "-" means that it has not been tested. For example, 2p1n5t indicates that 5 transgenic plant events were screened, of which 2 events showed increase and 1 showed decrease of the measured parameter. Note that two constructs of gene TRDX4-19 were tested, and the results are listed as TRDX4-19 and TRDX4-19x.

Table 4. Summary of transgenic corn plants with altered phenotypes in AGH non-stress screens

Gene_ID	AntA	AntS	Bmass	ClrpC	ClrpS	Cnop	PlntH	WUE	WtrAp	WtrCt
TRDX4-01	0p0n5t	1p0n5t	0p2n5t	0p1n5t	-	0p3n5t	0p0n5t	0p1n5t	0p3n5t	-
TRDX4-02	0p1n5t	0p1n5t	0p2n5t	0p2n5t	-	0p3n5t	0p2n5t	0p2n5t	0p3n5t	-
TRDX4-03	-	0p2n5t	0p1n5t	1p0n5t	-	0p2n5t	0p1n5t	0p2n5t	0p1n5t	-
TRDX4-04	1p0n5t	0p0n5t	0p2n5t	0p0n5t	-	0p1n5t	0p3n5t	0p1n5t	0p1n5t	-
TRDX4-05	1p0n5t	0p0n5t	0p0n5t	0p0n5t	-	0p0n5t	0p1n5t	0p0n5t	0p1n5t	-
TRDX4-07	-	0p0n5t	1p0n5t	-	1p0n5t	1p0n5t	0p1n5t	0p0n5t	3p0n5t	1p0n5t
TRDX4-09	0p0n5t	0p0n5t	0p0n5t	0p0n5t	-	0p0n5t	1p0n5t	0p2n5t	1p0n5t	-
TRDX4-11	0p1n5t	0p0n5t	0p1n5t	0p0n5t	-	0p0n5t	0p1n5t	0p1n5t	1p1n5t	-
TRDX4-12	0p2n5t	0p2n5t	0p0n5t	1p0n5t	-	0p0n5t	0p0n5t	0p0n5t	2p0n5t	-
TRDX4-13	1p0n5t	0p0n5t	0p0n5t	0p1n5t	-	0p1n5t	0p1n5t	0p0n5t	0p0n5t	-
TRDX4-14	0p0n5t	1p0n5t	0p2n5t	1p0n5t	-	0p2n5t	0p4n5t	0p2n5t	0p2n5t	-
TRDX4-16	0p0n5t	0p0n5t	0p0n5t	0p0n5t	-	0p1n5t	0p1n5t	0p0n5t	0p0n5t	-
TRDX4-17	0p1n5t	0p0n5t	1p0n5t	1p0n5t	-	2p0n5t	0p0n5t	0p0n5t	4p0n5t	-
TRDX4-18	0p2n5t	0p0n5t	2p1n5t	0p0n5t	-	1p1n5t	0p1n5t	1p1n5t	3p1n5t	-
TRDX4-19	0p1n4t	0p0n4t	0p1n4t	0p0n4t	-	0p1n4t	0p1n4t	0p1n4t	0p1n4t	-
TRDX4-19x	1p0n5t	1p0n5t	0p0n5t	0p0n5t	-	0p3n5t	0p1n5t	0p0n5t	0p1n5t	-
TRDX4-1T	0p0n5t	0p0n5t	0p1n5t	3p0n5t	-	0p1n5t	0p1n5t	0p0n5t	0p1n5t	-
TRDX4-20	0p0n3t	0p1n3t	0p1n3t	0p1n3t	-	0p1n3t	0p1n3t	0p1n3t	0p1n3t	-

TRDX4-21	1p1n5t	0p0n5t	0p0n5t	0p0n5t	-	1p0n5t	0p0n5t	0p0n5t	1p0n5t	-
TRDX4-22	0p0n3t	1p0n3t	0p0n3t	0p0n3t	-	0p1n3t	0p0n3t	0p0n3t	0p1n3t	-
TRDX4-23	0p0n5t	1p0n5t	0p0n5t	1p0n5t	-	0p1n5t	0p0n5t	0p0n5t	0p1n5t	-
TRDX4-25	1p0n5t	0p0n5t	0p0n5t	2p0n5t	-	0p1n5t	0p0n5t	0p0n5t	0p0n5t	-
TRDX4-26	0p0n5t	0p0n5t	0p2n5t	0p1n5t	-	0p2n5t	0p1n5t	0p3n5t	0p0n5t	-
TRDX4-27	0p0n7t	0p0n7t	0p1n7t	0p0n7t	-	0p1n7t	0p1n7t	0p0n7t	0p0n7t	-
TRDX4-5T	0p0n3t	0p0n3t	0p1n3t	0p0n3t	-	0p1n3t	0p0n3t	0p1n3t	0p1n3t	-
TRDX4-6T	-	0p1n5t	0p4n5t	0p0n5t	-	0p3n5t	0p1n5t	0p4n5t	0p2n5t	-
TRDX4-7T	1p0n2t	0p0n2t	0p0n2t	0p0n2t	-	0p0n2t	0p1n2t	0p0n2t	0p0n2t	-

Table 5. Summary of transgenic corn plants with altered phenotypes in AGH nitrogen-deficit screens

Gene_ID	AntA	AntS	Bmass	ClrpC	ClrpS	Cnop	PlntH	WUE	WtrAp	WtrCt
TRDX4-01	3p0n5t	0p1n5t	0p1n5t	0p0n5t	-	0p3n5t	0p2n5t	0p2n5t	1p2n5t	-
TRDX4-02	0p1n5t	0p1n5t	5p0n5t	3p0n5t	-	4p0n5t	3p1n5t	5p0n5t	5p0n5t	-
TRDX4-03	-	0p0n5t	0p3n5t	0p0n5t	-	0p4n5t	0p2n5t	0p2n5t	0p3n5t	-
TRDX4-04	0p0n5t	0p0n5t	0p0n5t	1p0n5t	-	0p0n5t	0p2n5t	0p0n5t	1p0n5t	-
TRDX4-05	5p0n5t	0p1n5t	0p2n5t	0p0n5t	-	0p1n5t	0p0n5t	0p3n5t	0p4n5t	-
TRDX4-07	-	0p0n5t	1p0n5t	-	0p1n5t	0p0n5t	0p1n5t	1p0n5t	1p0n5t	0p0n5t
TRDX4-09	0p0n5t	0p0n5t	0p0n5t	0p0n5t	-	0p0n5t	0p0n5t	0p0n5t	0p1n5t	-
TRDX4-11	0p0n5t	0p0n5t	0p2n5t	0p1n5t	-	0p0n5t	0p0n5t	0p0n5t	0p3n5t	-
TRDX4-12	0p4n5t	0p0n5t	1p0n5t	1p0n5t	-	2p0n5t	0p0n5t	1p0n5t	0p0n5t	-
TRDX4-13	0p2n5t	0p0n5t	4p0n5t	0p1n5t	-	1p0n5t	3p0n5t	3p0n5t	3p0n5t	-
TRDX4-14	3p0n5t	0p0n5t	0p2n5t	0p0n5t	-	0p1n5t	0p4n5t	0p1n5t	0p3n5t	-
TRDX4-16	1p0n5t	0p0n5t	0p2n5t	0p0n5t	-	0p1n5t	1p2n5t	0p2n5t	0p1n5t	-
TRDX4-17	0p2n5t	1p0n5t	0p0n5t	1p1n5t	-	2p0n5t	0p1n5t	0p0n5t	0p4n5t	-

TRDX4-18	0p3n5t	0p1n5t	2p0n5t	0p0n5t	-	1p0n5t	0p0n5t	3p0n5t	0p1n5t	-
TRDX4-19	0p1n3t	0p0n3t	1p0n3t	0p0n3t	-	2p0n3t	1p0n3t	0p0n3t	2p0n3t	-
TRDX4-19x	0p0n5t	0p2n5t	0p0n5t	0p0n5t	-	0p1n5t	0p0n5t	1p0n5t	0p1n5t	-
TRDX4-1T	1p0n5t	0p1n5t	1p1n5t	0p0n5t	-	0p1n5t	0p2n5t	1p1n5t	1p0n5t	-
TRDX4-20	0p2n3t	0p2n3t	2p0n3t	0p0n3t	-	1p0n3t	1p0n3t	2p0n3t	2p0n3t	-
TRDX4-21	0p2n5t	0p0n5t	1p1n5t	2p0n5t	-	1p1n5t	0p1n5t	2p0n5t	0p3n5t	-
TRDX4-22	0p2n3t	0p0n3t	2p0n3t	0p0n3t	-	1p0n3t	2p0n3t	2p0n3t	3p0n3t	-
TRDX4-23	0p0n5t	0p0n5t	1p0n5t	0p0n5t	-	1p0n5t	1p0n5t	1p0n5t	1p0n5t	-
TRDX4-25	0p0n5t	0p0n5t	0p0n5t	1p0n5t	-	0p1n5t	0p2n5t	1p0n5t	0p2n5t	-
TRDX4-26	0p0n5t	0p2n5t	0p0n5t	4p0n5t	-	0p1n5t	0p5n5t	0p0n5t	0p5n5t	-
TRDX4-27	1p1n7t	0p1n7t	0p0n7t	1p0n7t	-	0p1n7t	0p1n7t	0p0n7t	1p1n7t	-
TRDX4-5T	0p0n3t	0p0n3t	0p1n3t	0p0n3t	-	0p2n3t	0p1n3t	0p2n3t	0p3n3t	-
TRDX4-6T	-	0p2n5t	5p0n5t	5p0n5t	-	3p0n5t	4p0n5t	5p0n5t	5p0n5t	-
TRDX4-7T	0p1n3t	0p1n3t	3p0n3t	0p0n3t	-	3p0n3t	0p0n3t	3p0n3t	2p0n3t	-

Table 6. Summary of transgenic corn plants with altered phenotypes in AGH water-deficit screens

Gene_ID	AntA	AntS	Bmass	ClrpC	ClrpS	Cnop	PlntH	WUE	WtrAp	WtrCt
TRDX4-01	0p0n5t	1p1n5t	1p0n5t	1p0n5t	-	1p1n5t	1p1n5t	0p0n5t	1p0n5t	-
TRDX4-02	0p0n5t	3p0n5t	2p0n5t	0p0n5t	-	0p0n5t	1p1n5t	1p1n5t	0p3n5t	-
TRDX4-03	-	0p1n5t	0p0n5t	1p0n5t	-	1p0n5t	0p0n5t	0p1n5t	1p0n5t	-
TRDX4-04	0p2n5t	0p0n5t	1p0n5t	0p0n5t	-	1p0n5t	0p2n5t	1p0n5t	0p0n5t	-
TRDX4-05	1p0n5t	0p0n5t	0p1n5t	0p0n5t	-	0p1n5t	1p0n5t	0p1n5t	1p2n5t	-
TRDX4-07	-	4p0n5t	0p1n5t	-	3p1n5t	0p2n5t	0p2n5t	0p1n5t	0p4n5t	4p1n5t
TRDX4-09	1p0n5t	0p0n5t	0p0n5t	0p1n5t	-	0p1n5t	0p1n5t	0p0n5t	1p4n5t	-

TRDX4-11	2p0n5t	0p0n5t	0p0n5t	2p0n5t	-	0p0n5t	0p0n5t	0p0n5t	1p2n5t	-
TRDX4-12	1p1n5t	0p1n5t	1p1n5t	0p0n5t	-	2p1n5t	2p0n5t	1p1n5t	2p0n5t	-
TRDX4-13	4p0n5t	0p1n5t	0p5n5t	0p0n5t	-	0p5n5t	0p5n5t	0p3n5t	0p5n5t	-
TRDX4-14	0p0n5t	0p0n5t	0p1n5t	0p1n5t	-	0p1n5t	1p1n5t	0p0n5t	0p2n5t	-
TRDX4-16	0p0n5t	0p0n5t	0p0n5t	0p0n5t	-	0p0n5t	1p0n5t	0p0n5t	0p0n5t	-
TRDX4-17	0p1n5t	0p0n5t	4p0n5t	2p0n5t	-	2p0n5t	4p0n5t	1p0n5t	4p0n5t	-
TRDX4-18	0p2n5t	0p0n5t	3p0n5t	1p0n5t	-	1p0n5t	1p2n5t	2p1n5t	4p0n5t	-
TRDX4-19	0p1n4t	1p0n4t	0p2n4t	0p0n4t	-	1p1n4t	0p2n4t	0p1n4t	0p1n4t	-
TRDX4-19x	0p1n5t	0p0n5t	0p0n5t	0p0n5t	-	0p0n5t	0p0n5t	0p0n5t	1p2n5t	-
TRDX4-1T	0p1n2t	0p0n2t	1p0n2t	0p0n2t	-	0p0n2t	0p0n2t	1p0n2t	0p0n2t	-
TRDX4-20	0p1n3t	0p0n3t	0p0n3t	0p0n3t	-	0p0n3t	0p1n3t	0p0n3t	0p0n3t	-
TRDX4-21	0p1n5t	0p0n5t	3p1n5t	2p0n5t	-	3p0n5t	2p0n5t	1p0n5t	4p1n5t	-
TRDX4-22	0p0n1t	0p0n1t	1p0n1t	0p1n1t	-	0p0n1t	0p0n1t	0p0n1t	1p0n1t	-
TRDX4-23	0p3n5t	0p1n5t	4p0n5t	1p0n5t	-	4p0n5t	0p0n5t	1p0n5t	4p0n5t	-
TRDX4-25	1p0n5t	1p0n5t	1p0n5t	1p0n5t	-	1p0n5t	0p0n5t	0p0n5t	0p1n5t	-
TRDX4-26	1p0n5t	3p0n5t	5p0n5t	0p0n5t	-	5p0n5t	2p0n5t	3p0n5t	1p0n5t	-
TRDX4-27	4p0n7t	0p0n7t	0p2n7t	1p1n7t	-	1p0n7t	0p2n7t	0p1n7t	0p1n7t	-
TRDX4-5T	2p0n3t	0p0n3t	0p3n3t	0p0n3t	-	0p3n3t	0p3n3t	0p3n3t	0p3n3t	-
TRDX4-6T	-	0p1n5t	0p0n5t	0p0n5t	-	0p0n5t	1p0n5t	0p0n5t	0p0n5t	-
TRDX4-7T	0p0n3t	0p0n3t	0p0n3t	0p1n3t	-	3p0n3t	0p0n3t	1p0n3t	0p0n3t	-

Example 4. Phenotypic evaluation of transgenic plants for increased nitrogen use efficiency, increased water use efficiency and increased yield

[0165] Corn field trials were conducted to identify genes that can improve nitrogen use efficiency (NUE) under nitrogen limiting conditions leading to increased yield performance as compared to non transgenic controls. For the Nitrogen field trial results shown in Tables 7 and 9, each field was planted under nitrogen limiting condition (60 lbs/acre) and corn ear weight or yield was compared to non transgenic control plants.

[0166] Corn field trials were conducted to identify genes that can improve water use efficiency (WUE) under water limiting conditions leading to increased yield performance as compared to non transgenic controls. The water use efficiency trials for results shown in Tables 7 and 9 were conducted under managed water limiting conditions, and the corn ear weight or yield was compared to non transgenic control plants.

[0167] Corn and soybean field trials were conducted to identify genes that can improve broad-acre yield (BAY) under standard agronomic practice. The broad-acre yield trials for results shown in Tables 7 and 9 were conducted under standard agronomic practice, and the corn or soybean yield was compared to non transgenic control plants.

[0168] Table 7 provides a list of genes for producing transgenic plants with increased nitrogen use efficiency (NUE), increased water use efficiency (WUE), and increased broad-acre yield (BAY) as compared to a control plant. Polynucleotide sequences in constructs with at least one event showing significant yield or ear weight increase across multiple locations at $p \leq 0.2$ are included. The genes were expressed with constitutive promoters unless noted otherwise under "Specific Expression Pattern". Promoter of specific expression pattern was chosen over constitutive promoter, based on the understanding of the gene function, or based on the observed lack of significant yield increase when the gene was expressed with constitutive promoter. The elements of Table 7 are described by reference to:

[0169] "Crop" which refers to the crop in trial, which is either corn or soybean;

[0170] "Condition" which refers to the type of field trial, which is BAY for broad acre yield trial under standard agronomic practice (SAP), WUE for water use efficiency trial, and NUE for nitrogen use efficiency trial;

[0171] "Specific Expression Pattern" which refers to the expected expression pattern or promoter type, instead of constitutive;

[0172] "Gene ID" which refers to the gene identifier as defined in Table 1;

[0173] "Yield results" which refers to the recombinant DNA in a construct with at least one event showing significant yield increase at p ≤0.2 across locations. The first number refers to the number of events with significant yield or ear weight increase, whereas the second number refers to the total number of events tested for each recombinant DNA in the construct.

Table 7. Recombinant DNA with protein-coding genes for increased nitrogen use efficiency, increased water use efficiency and increased yield

Crop	Condition	Specific Expression Pattern	Gene ID	Yield Results
corn	BAY	leaf preferred	TRDX4-01	2/15
corn	BAY	seed preferred	TRDX4-02	1/13
corn	BAY		TRDX4-03	2/8
corn	BAY		TRDX4-04	1/23
corn	BAY		TRDX4-05	1/23
corn	BAY		TRDX4-06	1/7
corn	WUE		TRDX4-07	2/4
corn	BAY	cold inducible	TRDX4-08	4/26
corn	BAY		TRDX4-09	1/25
corn	NUE		TRDX4-10	1/12
corn	BAY		TRDX4-11	1/20
corn	BAY		TRDX4-12	8/20
corn	BAY		TRDX4-13	2/32
corn	BAY		TRDX4-14	2/20
soybean	BAY		TRDX4-15	4/13
corn	BAY		TRDX4-16	1/20
corn	BAY	leaf preferred	TRDX4-17	2/13
corn	BAY		TRDX4-18	2/19
corn	WUE	leaf preferred	TRDX4-19	1/5
corn	BAY		TRDX4-19	2/22
corn	BAY	leaf preferred	TRDX4-20	1/20

corn	BAY		TRDX4-21	4/19
corn	NUE		TRDX4-22	2/10
corn	BAY	leaf preferred	TRDX4-23	1/19
soybean	BAY		TRDX4-24	3/15
corn	BAY		TRDX4-25	2/18
corn	BAY	root preferred	TRDX4-26	3/21
corn	BAY		TRDX4-27	3/24

[0174] Table 8 provides a list of polynucleotide sequences of promoters with specific expression patterns. To convey the specific expression patterns, choices of promoters are not limited to those listed in Table 8.

Table 8. Promoter sequences and expression patterns

Nucleotide SEQ ID NO.	Promoter Expression Pattern
97	Cold inducible
98	Seed preferred
99	Leaf preferred
100	Leaf preferred

[0175] Table 9 provides a list of suppression target genes and miRNA construct elements provided as recombinant DNA for production of transgenic corn or soybean plants with increased nitrogen use efficiency, increased water use efficiency and increased yield. The elements of Table 9 are described by reference to:

[0176] "Crop" which refers to the crop in trial, which is either corn or soy;

[0177] "Condition" which refers to the type of field trial, which is BAY for broad acre yield trial under standard agronomic practice, WUE for water use efficiency trial, and NUE for nitrogen use efficiency trial;

[0178] "Target Gene ID" which refers to the suppression target gene identifier as defined in Table 2;

[0179] "Engineered miRNA precursor SEQ ID NO." which identifies a nucleotide sequence of the miRNA construct;

[0180] "Yield results" which refers to the recombinant DNA in a construct with at least one event showing significant yield increase at p ≤0.2 across locations. The first number refers to the number of events with significant yield or ear weight increase, whereas the second number refers to the total number of events tested for each sequence in the construct.

Table 9. miRNA Recombinant DNA constructs suppressing targeted genes for increased nitrogen use efficiency, increased water use efficiency and increased yield

Crop	Condition	Target Gene ID	Engineered miRNA precursor SEQ ID NO.	Yield Results
corn	BAY	TRDX4-1T	67	1/13
soybean	BAY	TRDX4-3T	68	4/15
soybean	BAY	TRDX4-4T	69	3/14
corn	BAY	TRDX4-5T	70	2/20
corn	NUE	TRDX4-6T	71	1/6
corn	WUE	TRDX4-6T	71	1/6
corn	BAY	TRDX4-6T	71	2/20
corn	WUE	TRDX4-7T	72	1/5

Example 5. Homolog Identification

[0181] This example illustrates the identification of homologs of proteins encoded by the DNA identified in Table 1 which were used to provide transgenic seed and plants having enhanced agronomic traits. From the sequences of the homolog proteins, corresponding homologous DNA sequences can be identified for preparing additional transgenic seeds and plants with enhanced agronomic traits.

[0182] An "All Protein Database" was constructed of known protein sequences using a proprietary sequence database and the National Center for Biotechnology Information (NCBI)

non-redundant amino acid database (nr.aa). For each organism from which a polynucleotide sequence provided herein was obtained, an “Organism Protein Database” was constructed of known protein sequences of the organism; it is a subset of the All Protein Database based on the NCBI taxonomy ID for the organism.

[0183] The All Protein Database was queried using amino acid sequences provided in Table 1 using NCBI “blastp” program with E-value cutoff of 1e-8. Up to 1000 top hits were kept, and separated by organism names. For each organism other than that of the query sequence, a list was kept for hits from the query organism itself with a more significant E-value than the best hit of the organism. The list contains likely duplicated genes of the polynucleotides provided herein, and is referred to as the Core List. Another list was kept for all the hits from each organism, sorted by E-value, and referred to as the Hit List.

[0184] The Organism Protein Database was queried using polypeptide sequences provided in Table 1 using NCBI “blastp” program with E-value cutoff of 1e-4. Up to 1000 top hits were kept. A BLAST searchable database was constructed based on these hits, and is referred to as “SubDB”. SubDB is queried with each sequence in the Hit List using NCBI “blastp” program with E-value cutoff of 1e-8. The hit with the best E-value was compared with the Core List from the corresponding organism. The hit is deemed a likely ortholog if it belongs to the Core List, otherwise it is deemed not a likely ortholog and there is no further search of sequences in the Hit List for the same organism. Homologs with at least 95% identity over 95% of the length of the polypeptide sequences provided in Table 1 are reported below in Tables 10 and 11.

[0185] Table 10 provides a list of homolog genes, of which the elements are described by reference to:

[0186] “PEP SEQ ID NO.” which identifies an amino acid sequence.

[0187] “Homolog ID” which refers to an alphanumeric identifier, the numeric part of which is the NCBI Genbank GI number.

[0188] “Gene Name and Description” which is a common name and functional description of the gene.

Table 10. Homolog genes information

PEP SEQ ID NO.	Homolog ID	Gene Name and Description
79	gi_735918	gi|735918|emb|CAA84367.1| asparaginase [Arabidopsis thaliana]
80	gi_110742427	gi|110742427|dbj|BAE99132.1| cyclic nucleotide-gated cation channel [Arabidopsis thaliana]
81	gi_215261267	gi|215261267|pdb|3EBL|A Chain A, Crystal Structure Of Rice Gid1 Complexed With Ga4 [Oryza sativa Japonica group]
82	gi_193211383	gi|193211383|ref|NP_001105952.1| plastid phosphate/phosphoenolpyruvate translocator1 [Zea mays]
83	gi_3164136	gi|3164136|dbj|BAA28535.1| cytochrome P450 monooxygenase [Arabidopsis thaliana]
84	gi_28867617	gi|28867617|ref|NP_790236.1| phosphoglycerate kinase [Pseudomonas syringae pv. tomato str. DC3000]
85	gi_71734219	gi|71734219|ref|YP_276916.1| phosphoglycerate kinase [Pseudomonas syringae pv. phaseolicola 1448A]
86	gi_66048014	gi|66048014|ref|YP_237855.1| phosphoglycerate kinase [Pseudomonas syringae pv. syringae B728a]
87	gi_242053823	gi|242053823|ref|XP_002456057.1| hypothetical protein SORBIDRAFT_03g029630 [Sorghum bicolor]
88	gi_21593232	gi|21593232|gb|AAM65181.1| unknown [Arabidopsis thaliana]
89	gi_226510490	gi|226510490|ref|NP_001148910.1| LOC100282530 [Zea mays] gi|195623174|gb|ACG33417.1| pre-mRNA-splicing factor ISY1 [Zea mays]
90	gi_242065688	gi|242065688|ref|XP_002454133.1| hypothetical protein SORBIDRAFT_04g025200 [Sorghum bicolor]
91	gi_21593552	gi|21593552|gb|AAM65519.1| unknown [Arabidopsis thaliana]
92	gi_21593833	gi|21593833|gb|AAM65800.1| glutaredoxin-like protein [Arabidopsis thaliana]
93	gi_226506654	gi|226506654|ref|NP_001146301.1| DNA mismatch repair protein MSH2 [Zea mays]
94	gi_242050756	gi|242050756|ref|XP_002463122.1| hypothetical protein SORBIDRAFT_02g038230 [Sorghum bicolor]
95	gi_255639875	gi|255639875|gb|ACU20230.1| unknown [Glycine max]
96	gi_195623972	gi|195623972|gb|ACG33816.1| triose phosphate/phosphate translocator, non-green plastid, chloroplast precursor [Zea mays]

[0189] Table 11 describes the correspondence between the protein-coding genes in Table 1, suppression target genes in Table 2, and their homologs, and the level of protein sequence alignment between the gene and its homolog. Note that homologs can be from Table 1, 2 or 10.

Table 11. Correspondence of Genes and Homologs

Gene ID	Homolog ID	Percent Gene Coverage	Percent Homolog Coverage	Percent Identity
TRDX4-02	gi_735918	100	100	99
TRDX4-03	gi_110742427	100	100	99
TRDX4-05	gi_215261267	100	97	100
TRDX4-06	TRDX4-1T	100	100	99
TRDX4-06	gi_193211383	100	100	99
TRDX4-08	gi_3164136	100	100	99
TRDX4-09	gi_28867617	99	100	100
TRDX4-09	gi_71734219	99	100	96
TRDX4-09	gi_66048014	99	100	96
TRDX4-10	gi_242053823	100	100	95
TRDX4-11	gi_21593232	100	100	99
TRDX4-12	gi_226510490	100	100	99
TRDX4-12	gi_242065688	100	100	98
TRDX4-14	gi_21593552	100	100	99
TRDX4-18	gi_21593833	99	100	100
TRDX4-22	gi_226506654	100	100	99
TRDX4-22	gi_242050756	100	100	95
TRDX4-25	gi_255639875	100	100	99
TRDX4-1T	TRDX4-06	100	100	99
TRDX4-1T	gi_195623972	100	100	99
TRDX4-1T	gi_193211383	100	100	99

Example 6. Use of suppression methods to suppress expression of target genes

[0190] This example illustrates monocot and dicot plant transformation with recombinant DNA constructs that are useful for stable integration into plant chromosomes in the nuclei of plant cells to provide transgenic plants having enhanced traits by suppression of the expression of target genes.

[0191] Various recombinant DNA constructs for use in suppressing the expression of a target gene in transgenic plants are constructed based on the nucleotide sequence of the gene encoding the protein that has an amino acid sequence selected from the group consisting of SEQ ID NOs: 61-66, where the DNA constructs are designed to express (a) a miRNA that targets the gene for suppression, (b) an RNA that is a messenger RNA for a target protein and has a synthetic miRNA recognition site that results in down modulation of the target protein, (c) an RNA that forms a dsRNA and that is processed into siRNAs that effect down regulation of the target protein, (d) a ssRNA that forms a transacting siRNA which results in the production of siRNAs that effect down regulation of the target protein.

[0192] Each of the various types of recombinant DNA constructs is used in transformation of a corn cell using the vector and method of Examples 1 and 2 to produce multiple events of transgenic corn cell. Such events are regenerated into transgenic corn plants and are screened to confirm the presence of the recombinant DNA and its expression of RNA for suppression of the target protein. The population of transgenic plants from multiple transgenic events are also screened to identify the transgenic plants that exhibit altered phenotype or enhanced trait.

We claim:

1. A recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:
    a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;
    b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;
    c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-95 and 96; or
    d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.
2. The recombinant DNA construct of claim 1, wherein said RNA is a double-stranded RNA, an antisense RNA, a miRNA precursor, or a ta-siRNA.
3. The recombinant DNA construct of claim 1, wherein said RNA is a miRNA precursor that produces a mature miRNA, and wherein said mature miRNA has a nucleic acid sequence with at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity or complementarity to a fragment of at least 19, 20, 21, 22, 23, 24, 25, 26 or 27 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60.
4. The recombinant DNA construct of claim 1, wherein said RNA is a miRNA precursor that produces a mature miRNA having a nucleic acid sequence with 100% identity or 100%

complementarity to a fragment of 21 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60.

5. The recombinant DNA construct of claim 1, wherein said DNA comprises a sequence selected from the group consisting of SEQ ID NOs: 67-72.
6. A plant comprising a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:
    a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;
    b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;
    c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79- 96; or
    d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.
7. The plant of claim 6, wherein said plant has an altered phenotype or an enhanced trait as compared to a control plant.
8. The plant of claim 6, wherein said plant is a progeny, a propagule, or a field crop.
9. The plant of claim 6, wherein said plant is a field crop selected from the group consisting of corn, soybean, cotton, canola, rice, barley, oat, wheat, turf grass, alfalfa, sugar beet, sunflower, quinoa and sugar cane.

10. The plant of claim 6, wherein said plant is a propagule selected from the group consisting of cell, pollen, ovule, flower, embryo, leaf, root, stem, shoot, meristem, grain and seed.
11. The plant of claim 7, wherein said enhanced trait is selected from the group consisting of increased yield, increased nitrogen use efficiency, and increased water use efficiency as compared to a control plant.
12. The plant of claim 7, wherein said phenotype is selected from the group consisting of anthocyanin content, biomass, canopy area, chlorophyll content, plant height, water applied, water content and water use efficiency.
13. A method for increasing yield, increasing nitrogen use efficiency, or increasing water use efficiency in a plant comprising producing a plant comprising a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to:
    a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55;
    b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60;
    c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-96; or
    d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.

14. The method of claim 13 wherein said plant is produced by transforming a plant cell or tissue with said recombinant DNA construct and regenerating a plant from said cell or tissue containing said recombinant DNA construct.
15. The method of claim 13 comprising producing said plant by crossing said plant through breeding with:
    a) itself;
    b) a second plant from the same plant line;
    c) a wild type plant; or
    d) a second plant from a different line of plants

to produce a seed, growing said seed to produce a plurality of progeny plants; and selecting a progeny plant with increased yield, increased nitrogen use efficiency, or increased water use efficiency as compared to a control plant.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01H 5/00; C07H 21/04 (2016.01)
CPC - C12N 15/8271, 15/8273, 15/8261
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)- A01H 5/00; C07H 21/04 (2016.01)
CPC- C12N 15/8271, 15/8273, 15/8261, 9/0008; C07K 14/415, C12Y 102/01016

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); The Lens; Google/Google Scholar; NCBI/BLAST/PubMed; EBSCO;
Search terms used: transgenic, plant, trait, characteristic, construct, vector, plasmid, recombinant, sequence, amino acid, promoter, operably link, TOM5, microRNA, miRNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0090998 A1 (ABAD, M et al.) April 17, 2008; paragraphs [0005]-[0006], [0010], [0020], [0052], [0054]-[0055], [0080]; table 2; SEQ ID NOs: 136, 405	1-2, 6-10, 12-15
Y	WO 95/02060 (ZENECA LIMITED) January 19, 1995; paragraphs [0009], [0014]	1-2, 6-10, 12-15
A	WO 2014/102773 A1 (EVOGENE LTD) July 03, 2014; page 12 lines 10-21, page 49 lines 28-31; SEQ ID NO: 2347	1-2, 6-10, 12-15
A	US 2012/0005773 A1 (AASEN, ED et al.) January 05, 2012; SEQ ID NO: 106	1-2, 6-10, 12-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2016 (04.04.2016)	14 APR 2016

Name and mailing address of the ISA/
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/63306

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

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

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

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+: Claims 1-2, 6-10, 12-15; SEQ ID NOs: 1 (Arabidopsis thaliana DNA sequence), 28 (Atabidopsis thaliana amino acid sequence)

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

-***-Continued from Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-15 are directed toward a recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to a polynucleotide that comprises a nucleotide sequence that encodes a polypeptide having an amino acid sequence or a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence, for suppressing the expression of a target protein having an amino acid sequence; as well as a plant comprising the DNA and a method for increasing yield, increasing nitrogen use efficiency, or increasing water use efficiency in a plant comprising producing a plant comprising the recombinant DNA construct.

The construct, plant and method will be searched to the extent that they encompass a nucleic acid encompassing SEQ ID NO: 1 (Arabidopsis thaliana DNA sequence)) encoding an amino acid sequence of SEQ ID NO: 28 (Arabidopsis thaliana amino acid sequence). It is believed that Claims 1 (in-part), 2 (in-partt), 6 (in-part), 7 (in-part), 8 (in-part), 9 (in-part), 10 (in-part), 12 (in-part), 13 (in-part), 14 (in-part) and 15 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 1 (Arabidopsis thaliana DNA sequence) and 28 (Arabidopsis thaliana amino acid sequence). Applicant is invited to elect additional nucleotide sequence(s) with specified SEQ ID NO: for each, and corresponding amino acid sequence(s) with specified SEQ ID NO: for each, to be searched. Additional nucleic acid and corresponding amino acid sequences will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected nucleic acid sequence(s) and/or corresponding amino acid sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NOs: 6 (Zea mays DNA sequence), SEQ ID NO: 3 (Zea mays amino acid sequence).

No technical features are shared between the sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a recombinant DNA construct; a plant comprising a recombinant DNA construct; and a method for increasing yield, increasing nitrogen use efficiency, or increasing water use efficiency in a plant comprising producing a plant comprising a recombinant DNA construct; the recombinant DNA construct comprising a heterologous promoter functional in a plant cell and operably linked to: a) a polynucleotide that comprises a nucleotide sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 1-27 and 55; b) a DNA encoding RNA for suppressing the expression of a target mRNA transcribed from a polynucleotide having a nucleic acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 6 and 55-60; c) a polynucleotide that encodes a polypeptide having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 28-54, 61, 79-95 and 96; or d) a DNA encoding RNA for suppressing the expression of a target protein having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence selected from the group consisting of SEQ ID NOs: 33, 61-66, 82, and 96.

However, these shared technical features are previously disclosed by EP 2,314,606 A1 (BASF Plant Science GmbH) (hereinafter 'BASF') in view of the publication entitled 'Glucose-6-Phosphate/Phosphate-Translocator Precursor Homolog - Maize: PIR: T04096' by Fischer, et al. (hereinafter 'Fischer').

BASF discloses a recombinant DNA construct (a vector comprising a sequence (a recombinant DNA construct); paragraphs [0026], [0030]); a plant comprising a recombinant DNA construct (a transgenic plant comprising a vector comprising a sequence; paragraphs [0014], [0026], [0030]); and a method for increasing yield in a plant (a method for enhancing (increasing) yield in a plant; paragraph [0061]) comprising producing a plant comprising a recombinant DNA construct (comprising producing a plant comprising a recombinant vector (DNA construct); paragraphs [0014], [0026], [0030]); the recombinant DNA construct comprising a heterologous promoter functional in a plant cell (the recombinant vector (DNA construct) comprising a heterologous promoter functional in a plant cell; paragraphs [0026], [0030]) and operably linked to a polynucleotide that comprises a nucleotide sequence (operably-linked to a polynucleotide which comprises a nucleotide sequence; paragraphs [0026], [0030]) that encodes a polypeptide having an amino acid sequence (abstract; paragraph [0012]); wherein the polypeptide has phosphate/phosphoenolpyruvate translocator activity (abstract).

BASF does not disclose a polypeptide having an amino acid sequence with at least 99% identity to SEQ ID NO: 33.

Fischer discloses a phosphate/phosphoenolpyruvate translocator (page 1) polypeptide having an amino acid sequence with at least 99% identity to SEQ ID NO: 33 (page 1, wherein the disclosed sequence is 99.5% identical to SEQ ID NO: 33 of the instant PCT application).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the previous disclosure of BASF, for including the use of known phosphate/phosphoenolpyruvate translocator sequences, such as those from common crop plants, including maize, as disclosed by Fischer, for providing a phosphate/phosphoenolpyruvate translocator, which would have been well-expressed and functional in a transgenic recipient plant, as disclosed by BASF, and which would have expanded the range of source organisms for the translocators, for providing a wider range of useful recipient plants.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the BASF and Fischer references, unity of invention is lacking.