The NR enzymes described herein were discovered in the red algae of Porphyra perforata (PpNR) and Porphyra yezoensis (PyNR). The present invention provides methods and compositions relating to altering NR activity, nitrogen utilization and/or uptake in plants. The invention relates to a method for the production of plants with maintained or increased yield under low nitrogen fertility. The invention provides isolated nitrate reductase (NR) nucleic acids and their encoded proteins. The invention further provides recombinant expression cassettes, host cells, and transgenic plants. Plants transformed with nucleotide sequences encoding the NR enzyme show improved properties, for example, increased yield and growth.
Published:
— with international search report
— before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments
— with sequence listing part of description published sepa-
rately in electronic form and available upon request from
the International Bureau
NITRATE REDUCTASES FROM PORPHYRA, COMPOSITIONS AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

The domestication of many plants has correlated with dramatic increases in yield. The identification of specific genes responsible for the dramatic differences in yield, in domesticated plants, has become an important focus of agricultural research.

One group of genes effecting yield are the nitrate reductase genes. These genes have utility for improving the use of nitrogen in crop plants, especially maize. The genes can be used to alter the genetic composition of the plants rendering them more productive with current fertilizer application standards, or maintaining their productive rates with significantly reduced fertilizer input.

Increased nitrogen use efficiency can result from enhanced uptake and assimilation of nitrogen fertilizer and/or the subsequent remobilization and reutilization of accumulated nitrogen reserves. Plants containing these genes can therefore be used for the enhancement of yield. Improving the nitrogen use efficiency in corn would increase corn harvestable yield per unit of input nitrogen fertilizer, both in developing nations where access to nitrogen fertilizer is limited and in developed nations were the level of nitrogen use remains high. Nitrogen utilization improvement also allows decreases in on-farm input costs, decreased use and dependence on the non-renewable energy sources required for nitrogen fertilizer production, and decreases the environmental impact of nitrogen fertilizer manufacturing and agricultural use.

Many efforts have recently been made to improve nitrogen efficiency of crop plants through overexpression of nitrate reductase (NR) in plants. One group has applied expression of the Nicotiana plumbaginifolia & Arabidopsis thaliana NR cDNA or gene under control of different promoters such as 35S CaMV (Nicotiana plumbaginifolia) Ferraho, et al., (1995) "Effects of constitutive expression of nitrate reductase in transgenic Nicotiana plumbaginifolia L. in response to varying nitrogen supply" Planta 196:288-294; and Lhcb1-3::Nia1-2 (Arabidopsis thaliana) Nejidat, et al., (1997) "Increased protein content in transgenic Arabidopsis Thalina over-expressing nitrate reductase activity" Plant Science 130:41-49. (references).
Although increases in NR expression levels up to 2-5-fold were detected with the 35S CaMV::Nia-2 gene in Nicotiana plumbaginifolia plants (Foyer, et al., (1994) "Adaptations of photosynthetic electron transport, carbon assimilation and carbon partitioning in transgenic Nicotiana plumbaginifolia plants to changes in nitrate reductase activity” Plant Physiol. 104:171-178), no improved nitrogen efficiency was observed. All these attempts to over express this enzyme have not resulted in improved growth under lower nitrogen fertility.

Therefore, despite several attempts to improve NR efficiency, no satisfactory composition or method has been provided that leads to an improvement of growth, productivity, and/or yield for agricultural crop plants. For these and other reasons, there is a need for the present invention.

**BRIEF SUMMARY OF THE INVENTION**

The present invention provides polynucleotides, related polypeptides and all conservatively modified variants of the present NR sequences. The invention provides sequences for the NR genes.

The present invention presents methods to alter the genetic composition of crop plants, especially maize, so that such crops can be more productive with current fertilizer applications and/or as productive with significantly reduced fertilizer input. The utility of this class of invention is then both yield enhancement and reduced fertilizer costs with corresponding reduced impact to the environment.

Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising an isolated polynucleotide sequence encoding an NR gene. One embodiment of the invention is an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence comprising SEQ ID NO: 1, 2, 3, 4, 5 or 6; and (c) the nucleotide sequence comprising at least 70% sequence identity to SEQ ID NO: 1, 2, 3, 4, 5 or 6 wherein said polynucleotide encodes a polypeptide having increased NR activity.

Compositions of the invention include an isolated polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence comprising SEQ ID NO: 7, 8, 9, 10, 11 or 12 and (b) the amino acid
sequence comprising at least 70% sequence identity to SEQ ID NO: 7, 8, 9, 10, 11 or 12 wherein said polypeptide has increased NR activity.

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

In yet another embodiment, the present invention is directed to a transgenic plant or plant cells, containing the nucleic acids of the present invention. Preferred plants containing the polynucleotides of the present invention include but are not limited to maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, tomato, and millet. In another embodiment, the transgenic plant is a maize plant or plant cells. Another embodiment is the transgenic seeds from the transgenic NR polypeptide of the invention operably linked to a promoter that drives expression in the plant. The plants of the invention can have altered NR as compared to a control plant. In some plants, the NR is altered in a vegetative tissue, a reproductive tissue, or a vegetative tissue and a reproductive tissue. Plants of the invention can have at least one of the following phenotypes including but not limited to: increased root mass, increased root length, increased leaf size, increased ear size, increased seed size, increased endosperm size, and increased biomass.

Another embodiment of the invention would be plants that have been genetically modified at a genomic locus, wherein the genomic locus encodes a NR polypeptide of the invention.

Methods for increasing the activity of a NR polypeptide in a plant are provided. The method can comprise introducing into the plant an NR polynucleotide of the invention.

Methods for reducing or eliminating the level of NR polypeptide in the plant are provided. The level or activity of the polypeptide could also be reduced or eliminated in specific tissues, causing alteration in plant growth, growth rate or
nitrogen utilization efficiency (NUE). Reducing the level and/or activity of the NR polypeptide may lead to smaller stature or slower growth of plants.

BRIEF DESCRIPTION OF THE DRAWINGS

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

Figure 1. Peptide sequence comparison between Porphyra perforata (PpNR - SEQ ID NO: 5) and P. yezoensis (PyNR - SEQ ID NO: 10). PpNR and PyNR are approximately 94% identical. The identical amino acid residues are in bold and similar ones are underlined.

Figure 2. Yeast nitrate transporter YNT1 was cloned from Pichia angusta based on the published sequence (GenBank Accession Number Z69783). YNT1 driven by a constitutive promoter glyceraldehyde-3-phosphate dehydrogenase (GAP) from P. pastoris with histidine auxotroph selection marker (p3.5GAP-YNT1) was integrated into His4 locus of P. pastoris strain KM71 (Invitrogen) using Pichia EasyComp transformation kit (Invitrogen). The recombinant strains were confirmed to carry the YNT1 expression cassette by PCR. The KM71-containing YNT1 line was re-transformed with nitrate reductase gene PPNR from Porphyra perforata driven by GAP promoter with Zeocin selection marker (pAOXGAP-PPNR) which integrated into AOX1 locus of P. pastoris genome. Nitrate reductase enzyme activity was assayed in vivo. Four transformants and KM71 wild type were cultured in rich media (YPD) at 30°C for overnight. Yeast cells were collected and washed with water twice then re-suspended in 20 mM MOPS, pH6.5 and 1% glucose containing 5 mM NaNO₃. After 1 hour incubation at 30°C, the supernatant was collected for nitrite assay with 1% Sulfanilamide, 0.01 % N-(1-Naphthyl) ethylene-diamine dihydrochloride and 15% (v/v) H₃PO₄. Strong NR activity was detected in transformants carrying PPNR comparing to KM71 wild type strain.

Figure 3. The KM71-containing YNT1 line (see Fig.2) was re-transformed with nitrate reductase gene PYNR from Porphyra yezoensis driven by GAP promoter with Zeocin selection marker (pAOXGAP-PYNR) which integrated into AOX1 locus of P. pastoris genome. In this case, the nitrate reductase enzyme activity from two transformants and KM71 wild type was assayed in vivo as before.
The NR activity was detected from the transformants carrying PYNR comparing to KM71 wild type strain. However, the PYNR activity is much weaker than PPNR in P. pastoris.

Figure 4. Yeast nitrate reductase YNR1 was cloned from Pichia angusta based on the published sequence (GenBank Accession Number Z49110). The KM71-containing YNT1 line was re-transformed with nitrate reductase gene YNR1 driven by GAP promoter with Zeocin selection marker (pAOXGAP-YNR1) which integrated into AOX1 locus of P. pastoris genome. The transformant with the best Vmax was used for kinetic study.

Maize nitrate reductase ZmNR was cloned from maize B73 (assembled from two truncated ESTs) based on the published genomic sequence (GenBank Accession Number AF153448). The cloned ZmNR has three different amino acid residues compared to AF153448. The KM71-containing YNT1 line was re-transformed with nitrate reductase gene ZMNR driven by GAP promoter with Zeocin selection marker (pAOXGAP-ZMNR) which integrated into AOX1 locus of P. pastoris genome. The transformant with the best Vmax was used for kinetic study.

The transformants carrying YNT1/YNR1, YNT1/ZMNR, or YNT1/PPNR, and KM71 wild type were cultured in rich media (YPD) at 30°C for overnight. Yeast cells were collected and washed with water twice then re-suspended in 20 μM MOPS, pH6.5, or 20μM MES, pH5.5, or 20 μM Tris, pH7.5 with 1% glucose containing 24 different concentrations of NaNO3 from 0 up to 30 mM (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 2, 4, 8, 10, 15, 20, 25 and 30 mM). The reduced nitrite was assayed as mentioned above. The Km was estimated as the substrate concentration at ½ Vmax. The Km and preferred pH (with the best Vmax) of YNR1, ZMNR, and PPNR was summarized in the table. Please note that although the diagram shows YNR1 as the lowest line on the chart, only one tenth the volume of YNR1 material was used in the experiment, therefore the YNR1 had the best Vmax in the experiment.

Figure 5. The constructs of PPNR and PYNR driven by UBI promoter and/or maize nitrate reductase ZMNR promoter were made. The resulting constructs, PHP27800, PHP27801, PHP28335, and PHP28334 were used for maize transformation. Transgenic maize segregating 1:1 for UBI.Porphyra perforata NR was grown to anthesis in hydroponics medium maintained at 1mM
KNO₃ as the sole nitrogen source. Plants containing the transgene were identified during growth. All plants were harvested shortly after anthesis. The ear and the remaining plant were separated, dried in a forced air oven at 70°C for 72 hr and weighed. Mean ear dry weight of each event transgenic plants were compared to the ear dry weight of the corresponding nulls. Similar comparisons were made between transgenic mean plant dry weight and the corresponding event nulls. Comparisons marked with * are statistically significant (p > t = 0.1). This figure is a graphical representation of the data described in Example 10.

BRIEF DESCRIPTION OF THE SEQUENCES

The application provides details of NR sequences as shown in Table 1 below.
### Table 1

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Polynucleotide (pnt) or polypeptide (ppt)</th>
<th>Length</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pnt</td>
<td>2865</td>
<td>PpNR.</td>
</tr>
<tr>
<td>2</td>
<td>pnt</td>
<td>2865</td>
<td>Codon optimized polynucleotide sequence encoding PpNR S561Ala</td>
</tr>
<tr>
<td>3</td>
<td>pnt</td>
<td>2865</td>
<td>Codon optimized polynucleotide sequence encoding PpNR S561Asp</td>
</tr>
<tr>
<td>4</td>
<td>pnt</td>
<td>2871</td>
<td>PyNR</td>
</tr>
<tr>
<td>5</td>
<td>pnt</td>
<td>2871</td>
<td>Codon optimized polynucleotide sequence encoding PyNR S563Ala</td>
</tr>
<tr>
<td>6</td>
<td>pnt</td>
<td>2871</td>
<td>Codon optimized polynucleotide sequence encoding PyNR S563Asp</td>
</tr>
<tr>
<td>7</td>
<td>ppt</td>
<td>954</td>
<td>PpNR.</td>
</tr>
<tr>
<td>8</td>
<td>ppt</td>
<td>954</td>
<td>PpNR S561Ala - encoded by codon optimized polynucleotide</td>
</tr>
<tr>
<td>9</td>
<td>ppt</td>
<td>954</td>
<td>PpNR S561Asp – encoded by codon optimized polynucleotide</td>
</tr>
<tr>
<td>10</td>
<td>ppt</td>
<td>954</td>
<td>PyNR</td>
</tr>
<tr>
<td>11</td>
<td>ppt</td>
<td>956</td>
<td>PyNR S563Ala - encoded by codon optimized polynucleotide</td>
</tr>
<tr>
<td>12</td>
<td>ppt</td>
<td>956</td>
<td>PyNR S563Asp - encoded by codon optimized polynucleotide</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Polynucleotide (pnt) or polypeptide (ppt)</th>
<th>Length</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>pnt</td>
<td>26</td>
<td>PYNR sense primer</td>
</tr>
<tr>
<td>14</td>
<td>pnt</td>
<td>20</td>
<td>PYNR anti-sense primer</td>
</tr>
<tr>
<td>15</td>
<td>pnt</td>
<td>20</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>16</td>
<td>pnt</td>
<td>20</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>17</td>
<td>pnt</td>
<td>20</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>18</td>
<td>pnt</td>
<td>25</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>19</td>
<td>pnt</td>
<td>24</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>20</td>
<td>pnt</td>
<td>25</td>
<td>PpNR walking primer</td>
</tr>
<tr>
<td>21</td>
<td>pnt</td>
<td>21</td>
<td>ORF PpNR sense primer</td>
</tr>
<tr>
<td>22</td>
<td>pnt</td>
<td>21</td>
<td>ORF PpNR anti-sense primer</td>
</tr>
<tr>
<td>23</td>
<td>pnt</td>
<td>19</td>
<td>ORF PyNR sense primer</td>
</tr>
<tr>
<td>24</td>
<td>pnt</td>
<td>22</td>
<td>ORF PyNR anti-sense primer</td>
</tr>
</tbody>
</table>
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

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

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

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

OVERVIEW

Nitrate is the most important source of nitrogen for higher plants. Nitrate is absorbed by the roots, transported to various tissues of the plant and then reduced to aqueous ammonia in two steps. The first step requires the enzyme nitrate reductase (NR), which catalyzes the reduction of nitrate to nitrite in the cytoplasm. In a second step, the nitrite is then reduced in the chloroplast by nitrite reductase. The reduction of nitrate is considered to be limiting step in nitrate metabolism in plants.

Plant nitrate reductases are regulated at both transcriptional and post-translational levels. Several environmental factors such as light or nitrate regulate
NR gene expression at transcriptional level. NR activity is also regulated at post-translational level by light. NR becomes inactive/active in response to dark/light by phosphorylation/dephosphorylation at putative Ser residues. The inactive form of NR binds to 14-3-3 protein in the dark. The putative regulation sites include N-terminal region (Plant Cell (1995) 7:61 1-621), hinge 1 (Plant J. (2003) 35:566-573) and other regions.

The present invention relates to the discovery of novel NRs from red algae (division of Rhodophyta) of Porphyra perforate (PpNR) and Porphyra yezoensis (PyNR). As described herein, the inventors have identified two novel NR cDNAs in red algae that share approximately 94% amino acid consensus with respect to one another but only 52% amino acid identity to a maize homolog of NR from B73 (Genbank Ace NoAFI 53448). The algal NR polynucleotides of this invention are 2865 bp (PpNR) and 2871 bp (PyNR), nucleotides in length encoding polypeptides with calculated molecular weight of 104.9 KDa (PpNR) and 105.2 KDa (PyNR). Also contemplated are variants of these sequences. For example, mutating the serine residue in a putative phosphorylation motif R/K-S/T-X-pS-X-P (J of Experimental Botany (2004) 55:1 275-1 282) at position 561 to an alanine or aspartic acid of the PpNR or an alanine or aspartic acid at position 563 of the PyNR sequence is believed to increase and maintain high level of active form of nitrate reductase in transgenic plants to facilitate nitrate assimilation.

Red algae (division of Rhodophyta) of Porphyra perforate (PpNR) and Porphyra yezoensis (PyNR) grow in concentrations of nitrate that are approximately one hundred times lower than the nitrate concentrations in which plants are capable of growing. These red algae have more efficient nitrate reductase enzymes that saturate at lower substrate concentrations than higher plant nitrate reductase. In particular, Porphyra perforate and Porphyra yezoensis have been reported in the literature to have a NR enzyme with a low Km for nitrate (30-65 µM). This is in contrast to a maize NR from B73 which has a Km for nitrate reductase of 300 µM. In vivo kinetic measurements made of PpNR and PyNR expressed in Pichia pastoris show this enzyme has a Km for nitrate of 30-50 µM compared to maize nitrate reductase Km of 200 µM. Without wishing to be bound by this theory, it is believed that expressing more efficient NRs that saturate at lower concentrations of substrate than the higher plant NR enzymes will be more efficient in nitrogen reduction. Modulation of the NRs of the present invention
would provide a mechanism for manipulating a plant's nitrogen utilization efficiency (NUE). Accordingly, the present invention provides methods, polynucleotides, and polypeptides for the production of plants with improved or maintained yield under limited nitrogen supply. In one aspect, the methods include introducing into a plant cell, plant tissue or plant one or more polynucleotides encoding NR polypeptides having the enzymatic activity of nitrate reductase. This may be accomplished by introducing the nitrate reductase polynucleotides driven by a constitutive promoter or a mesophyll cell preferred promoter into the plant nuclear genome.

Advantageously, plants expressing a NR of the present invention will provide the customer increased revenue by lowering input costs and/or increasing yields with a significant reduction in applied nitrogen fertilizer. Furthermore, yields may be maintained or increased in plants expressing a NR of the present invention even under non-favorable growth conditions, for example, where nitrogen is in limited supply.


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

5 Definitions.

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

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

By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., Diagnostic Molecular Microbiology: Principles and Applications, Persing, et al, eds., American Society for Microbiology, Washington, DC (1993). The product of amplification is termed an amplicon.

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids that encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; one exception is Micrococcus
rubens, for which GTG is the methionine codon (Ishizuka, et al., (1993) J. Gen. Microbiol. 139:425-32) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid, which encodes a polypeptide of the present invention, is implicit in each described polypeptide sequence and incorporated herein by reference.

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

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

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).


As used herein, "consisting essentially of" means the inclusion of additional sequences to an object polynucleotide where the additional sequences do not selectively hybridize, under stringent hybridization conditions, to the same cDNA as the polynucleotide and where the hybridization conditions include a wash step in 0.1 X SSC and 0.1 % sodium dodecyl sulfate at 65°C.
By "encoding" or "encoded," with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium Mycoplasma capricolum (Yamao, et al., 1985) Proc. Natl. Acad. Sci. USA 82:2306-9), or the ciliate Macronucleus, may be used when the nucleic acid is expressed using these organisms.

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

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

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

The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

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

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

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules, which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, (1987) *Guide To Molecular Cloning Techniques*, from the series *Methods in Enzymology*, vol. 152, Academic Press, Inc., San Diego, CA; Sambrook, *et al.*, (1989) *Molecular Cloning: A

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

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants, which can be used in the methods of the invention, is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants including species from the genera: Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciahorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesis, Pelargonium, Panieum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Pisum, Phaseolus, Lolium, Oryza, Avena, Hordeum, Secale, Allium, and Triticum. A particularly preferred plant is Zea mays.

As used herein, "yield" may include reference to bushels per acre of a grain crop at harvest, as adjusted for grain moisture (15% typically for maize, for example), and the volume of biomass generated (for forage crops such as alfalfa, and plant root size for multiple crops). Grain moisture is measured in the grain at harvest. The adjusted test weight of grain is determined to be the weight in pounds per bushel, adjusted for grain moisture level at harvest. Biomass is measured as the weight of harvestable plant material generated.

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

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

As used herein "promoter" includes reference to a region of DNA upstream
from the start of transcription and involved in recognition and binding of RNA
polymerase and other proteins to initiate transcription. A "plant promoter" is a
promoter capable of initiating transcription in plant cells. Exemplary plant
promoters include, but are not limited to, those that are obtained from plants, plant
viruses, and bacteria which comprise genes expressed in plant cells such
Agrobacterium or Rhizobium. Examples are promoters that preferentially initiate
transcription in certain tissues, such as leaves, roots, seeds, fibres, xylem vessels,
tracheids, or sclerenchyma. Such promoters are referred to as "tissue preferred."
A "cell type" specific promoter primarily drives expression in certain cell types in
one or more organs, for example, vascular cells in roots or leaves. An "inducible"
or "regulatable" promoter is a promoter, which is under environmental control.
Examples of environmental conditions that may effect transcription by inducible
promoters include anaerobic conditions or the presence of light. Another type of promoter is a developmentally regulated promoter, for example, a promoter that drives expression during pollen development. Tissue preferred, cell type specific, developmentally regulated, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter, which is active under most environmental conditions, for example, the ubiquitin gene promoter UBI (GenBank Accession Number S94464).

As used herein, the term nitrate reductase (NR) includes but is not limited to the sequences disclosed herein, such as NR, their conservatively modified variants, regardless of source and any other variants which retain the biological properties of the NR, for example, NR activity as disclosed herein. The term "NR polypeptide" refers to one or more amino acid sequences. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof. A "NR protein" comprises a NR polypeptide. Unless otherwise stated, the term "NR nucleic acid" means a nucleic acid comprising a polynucleotide ("NR polynucleotide") encoding a NR polypeptide.

As used interchangeably herein, a "NR activity", "biological activity of NR" or "functional activity of NR", refers to an activity exerted by a NR protein, polypeptide or portion thereof as determined in vivo, or in vitro, according to standard techniques. In one aspect, a NR activity is the reduction of nitrate to nitrite. In one aspect, NR activity includes but is not limited to increased nitrate reduction rate and/or specificity for nitrate, for example, decreased $K_m$ for nitrate and NADH, increased velocity ($V_{max}$) for nitrate reduction and the like as compared to NR activity of an endogenous NR of a crop plant of interest. In another aspect, NR activity includes but is not limited to increasing NUE and/or plant productivity/yield as compared to a control plant. NUE may be inferred from amount and/or rate of nitrogen uptake from the soil or medium as described herein in Example 11.

The expression level of the NR polypeptide may be measured directly, for example, by measuring the level of the NR polypeptide by Western in the plant, or indirectly, for example, by measuring the NR activity of the NR polypeptide in the plant. Methods for determining the NR activity may be determined using standard techniques such as Hageman, et al., Methods Enzymol. (1971) 23:491-503, Tucker,
et al., (2004). "Control of nitrate reductase by circadium and diurnal rhythms in tomato" *Planta* 219:277-285. and Scheible, et al., (1997) "Accumulation of nitrate in the shoot acts as a signal to regulate shootroot allocation in tobacco" *Plant J.* 11:671-691, including the evaluation of activity in various expression systems, for example of Xenopus oocytes (see, Miller, et al., (2000) "Xenopus Oocytes as an Expression System for Plant Transporters", *Biochimica et Biophysica Acta* 1465:343-358.) or yeast such as *Pichia pastoris* (US Provisional Patent Application Serial Number 60/944,223 filed June 15, 2007), NR activity may also include evaluation of phenotypic changes, such as increased or maintained yield or NUE in a plant grown under nitrate limiting conditions such as lower nitrogen fertility. Examples of phenotypic changes include but are not limited to increased ear size in maize, increased ear growth rate, increased biomass, higher grain yields, synchronous flowering so that pollen is shed at approximately the same time as silking, enhanced root growth, increased seed size, increased seed weight, seed with increased embryo size, increased leaf size, increased seedling vigor, enhanced silk emergence, and greater chlorophyll content (greener).

Maintained or increased yield may be achieved through NRs of the present invention. Thus, modulation of NR activity of the NRs of the present invention in a plant cell provides a novel strategy for maintaining or increasing yield or NUE of a plant grown under limited nitrogen supply or lower nitrogen fertility.

Accordingly, the present invention further provides plants having increased yield or a maintained yield when grown under limited nitrogen fertility. In some embodiments, the plants having an increased or maintained yield when grown under limited nitrogen fertility have a modulated level/activity of a NR polypeptide of the invention.

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

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

As used herein "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention; or may have reduced or eliminated expression of a native gene. The term "recombinant" as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

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

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

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

The terms "stringent conditions" or "stringent hybridization conditions" include reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which can be up to 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Optimally, the probe is approximately 500 nucleotides in length, but can vary greatly in length from less than 500 nucleotides to equal to the entire length of the target sequence.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide or Denhardt's. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the $T_m$ can be approximated from the equation of Meinkoth and Wahl,
(1984) Anal. Biochem. 138:267-84: \( T_m = 81.5^\circ C + 16.6 \log M + 0.41 \%GC - 0.61 \% \text{ form} - 500/L; \) where \( M \) is the molarity of monovalent cations, \( \%GC \) is the percentage of guanosine and cytosine nucleotides in the DNA, \( \% \text{ form} \) is the percentage of formamide in the hybridization solution, and \( L \) is the length of the hybrid in base pairs. The \( T_m \) is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. \( T_m \) is reduced by about 1°C for each 1% of mismatching; thus, \( T_m \), hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the \( T_m \) can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (\( T_m \)) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3 or 4°C lower than the thermal melting point (\( T_m \)); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9 or 10°C lower than the thermal melting point (\( T_m \)). Low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15 or 20°C lower than the thermal melting point (\( T_m \)). Using the equation, hybridization and wash compositions, and desired \( T_m \), those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a \( T_m \) of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology - Hybridization with Nucleic Acid Probes, part I, chapter 2, Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, New York (1993); and Current Protocols in Molecular Biology, chapter 2, Ausubel, et al, eds, Greene Publishing and Wiley-Interscience, New York (1995). Unless otherwise stated, in the present application high stringency is defined as hybridization in 4X SSC, 5X Denhardt's (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin in 500ml of water), 0.1 mg/ml boiled salmon sperm DNA, and 25 mM Na phosphate at 65°C, and a wash in 0.1 X SSC, 0.1% SDS at 65°C.
As used herein, "transgenic plant" includes reference to a plant, which comprises within its genome a heterologous polynucleotid. Generally, the heterologous polynucleotid is stably integrated within the genome such that the polynucleotid is passed on to successive generations. The heterologous polynucleotid may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

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

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides or polypeptides: (a) "reference sequence," (b) "comparison window," (c) "sequence identity," (d) "percentage of sequence identity," and (e) "substantial identity."

As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

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

Methods of alignment of nucleotide and amino acid sequences for
comparison are well known in the art. The local homology algorithm (BESTFIT) of
alignment of sequences for comparison; by the homology alignment algorithm
(GAP) of Needleman and Wunsch, (1970) J. Mol. Biol. 48:443-53; by the search
Acad. Sci. USA 85:2444; by computerized implementations of these algorithms,
including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics,
Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the
Wisconsin Genetics Software Package, Version 8 (available from Genetics
Computer Group (GCG® programs (Accelrys, Inc., San Diego, CA)). The
CLUSTAL program is well described by Higgins and Sharp, (1988) Gene
The preferred program to use for optimal global alignment of multiple sequences is
PileUp (Feng and Doolittle, (1987) J. Mol. Evol, 25:351-60 which is similar to the
method described by Higgins and Sharp, (1989) CABIOS 5:1 51-53 and hereby
incorporated by reference). The BLAST family of programs which can be used for
database similarity searches includes: BLASTN for nucleotide query sequences
against nucleotide database sequences; BLASTX for nucleotide query sequences
against protein database sequences; BLASTP for protein query sequences
against protein database sequences; TBLASTN for protein query sequences
against nucleotide database sequences; and TBLASTX for nucleotide query
sequences against nucleotide database sequences. See, Current Protocols in
Molecular Biology, Chapter 19, Ausubel et al., eds., Greene Publishing and Wiley-

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

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see, Henikoff and Henikoff, (1989) Proc. Natl. Acad. Sci. USA 89:1 0915).


As those of ordinary skill in the art will understand, BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences, which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, (1993) Comput.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences, which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences, which differ by such conservative substitutions, are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, (1988) Computer Applic. Biol. Sci. 4:1 1-17, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.
The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has between 50-100% sequence identity, preferably at least 50% sequence identity, preferably at least 60% sequence identity, preferably at least 70%, more preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of between 55-100%, preferably at least 55%, preferably at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

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

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

Nucleic Acids

The present invention provides, *inter alia*, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising a NR polynucleotide.

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

The NR nucleic acids of the present invention comprise isolated NR polynucleotides which are inclusive of:

- (a) a polynucleotide encoding a NR polypeptide and conservatively modified and polymorphic variants thereof;
- (b) a polynucleotide having at least 70% sequence identity with polynucleotides of (a);
- (c) complementary sequences of polynucleotides of (a) or (b).

Construction of Nucleic Acids

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

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the isolation of the translated polynucleotide of the
present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention. The nucleic acid of the present invention - excluding the polynucleotide sequence - is optionally a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art. Exemplary nucleic acids include such vectors as: M13, lambda ZAP Express, lambda ZAP II, lambda gt10, lambda gt11, pBK-CMV, pBK-RSV, pBluescript II, lambda DASH II, lambda EMBL 3, lambda EMBL 4, pWE15, SuperCos 1, SurfZap, Uni-ZAP, pBC, pBS+/-, pSG5, pBK, pCR-Schpt, pET, pSPUTK, p3'SS, pGEM, pSK+/-, pGEX, pSPORTI and II, pOPRSVI CAT, pOPI3 CAT, pXT1, pSG5, pPbac, pMbac, pMCl neo, pOG44, pOG45, pFRT βGAL, pNEO βGAL, pRS403, pRS404, pRS405, pRS406, pRS413, pRS414, pRS415, pRS416, lambda MOSSlox, and lambda MOSElox. Optional vectors for the present invention, include but are not limited to, lambda ZAP II, and pGEX. For a description of various nucleic acids see, e.g., Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA); and, Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, IL).

**Synthetic Methods for Constructing Nucleic Acids**

oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

**UTRs and Codon Preference**


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

**Sequence Shuffling**


Generally, sequence shuffling provides a means for generating libraries of polynucleotides having a desired characteristic, which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides, which comprise sequence regions, which have substantial sequence identity and can be homologously recombined in vitro or in vivo. The population of sequence-recombined polynucleotides comprises a subpopulation of polynucleotides which possess desired or advantageous characteristics and which can be selected by a suitable selection or screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be an altered $K_m$ and/or $K_{ca}$ over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled wild-type polynucleotide. In yet other embodiments, a protein or polynucleotide generated from sequence shuffling will have an altered pH optimum as compared to the non-shuffled wild-type polynucleotide. The increase in such properties can be at least 110%, 120%, 130%, 140% or greater than 150% of the wild-type value.
Recombinant Expression Cassettes

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

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

A number of promoters can be used in the practice of the invention, including the native promoter of the endogenous NR polynucleotide sequence of the crop plant of interest. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, inducible, or other promoters for expression in plants.

A plant promoter or promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the 1’- or 2’- promoter derived from T-DNA of Agrobacterium tumefaciens, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US Patent Number 5,683,439), the Nos promoter, the rubisco promoter, the GRP1-8 promoter, the 35S promoter from cauliflower mosaic virus (CaMV), as described in Odell, et al., (1985) Nature 313:810-2; rice actin (McElroy, et al., (1990) Plant Cell 163-171);


A mesophyllic cell preferred promoter includes but is not limited to promoters such as known phosphoenopyruvate decarboxylase (PEPC) promoters or putative PEPC promoters from any number of species, for example, Zea mays, Oryza sativa, Arabidopsis thaliana, Glycine max or Sorghum bicolor. Examples include Zea mays PEPC of GenBank Accession Number gi:16268332_HTG AC190686, (SEQ ID NO: 25) and gCAT GSS composite sequence (SEQ ID NO: 30); Oryza sativa PEPC of GenBank Accession Number gi|20804452|dbj|AP003052.3| (SEQ ID NO: 26); Arabidopsis thaliana PEPC of GenBank Accession Number gi|5541653|dbj|AP000370.1|AP000370 (SEQ ID NO: 27); gi:7769847 (SEQ ID NO: 28); or gi|20198070|gb|AC007087.7 (SEQ ID NO: 29); Glycine max (GSS contigs) (SEQ ID NOS: 31-32); or Sorghum bicolor


Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire, et al., (1992) Plant Mol. Biol. 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991 ) Plant Cell 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger, et al., (1990) Plant Mol. Biol. 15(3):533-553 (root-specific promoter of the mannopine synthase (MAS) gene of Agrobacterium tumefaciens); and Miao, et al., (1991 ) Plant Cell 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also, Bogusz, et al., (1990) Plant Cell 2(7):633-651 , where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume Parasponia andersonii and the related non-nitrogen-fixing nonlegume Trema tomentosa are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume Nicotiana tabacum and the legume Lotus corniculatus, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991 ) describe their analysis of the promoters of the highly expressed rol C and rol D root-inducing genes of Agrobacterium rhizogenes (see, Plant Science (Limerick) 79(1):69-76). They
concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri, et al., (1989) used gene fusion to lacZ to show that the Agrobacterium T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see, EMBO J. 8(2):353-350). The TRV gene, fused to nptII (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VIENOD-GRP3 gene promoter (Kuster, et al., (1995) Plant Mol. Biol. 29(5):759-772); rolB promoter (Capana, et al., (1995) Plant Mol. Biol. 25(5):681-691; and the CRWAQ81 root-preferred promoter with the ADH first intron (US Provisional Application Number 60/509,878, filed October 9, 2003, herein incorporated by reference). See also, US Patent Numbers 5,837,876; 5,750,386; 5,633,363; 5,559,252; 5,501,836; 5,110,732; and 5,023,179.

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

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

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from a variety of plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene. Examples of such

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


The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene, which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes including genes coding for resistance to
the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resistance to herbicides which act to inhibit the action of acetylacetate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetylacetate synthase (ALS) gene containing mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The bar gene encodes resistance to the herbicide basta, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

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

Expression of Proteins in Host Cells

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

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.
In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter, such as ubiquitin, to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Constitutive promoters are classified as providing for a range of constitutive expression. Thus, some are weak constitutive promoters, and others are strong constitutive promoters. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended at levels of about 1/1,000 transcripts to about 1/10,000 transcripts to about 1/500,000 transcripts. Conversely, a "strong promoter" drives expression of a coding sequence at a "high level," or about 1/1,000 transcripts to about 1/1,000,000 transcripts.

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

**Expression in Prokaryotes**

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly

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

**Expression in Eukaryotes**

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

Synthesis of heterologous proteins in yeast is well known. Sherman, et al., (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.
A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates or the pellets. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

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

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

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Other useful terminators for practicing this invention include, but are not limited to, pinll (see, An, et al., (1989) Plant Cell 1(1):1 15-1 22), glb1 (see, Genbank Accession Number L22345), gz (see, gzw64a terminator, Genbank Accession Number S78780), and the nos terminator from Agrobacterium.
Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, et al., (1983) J. Virol. 45:773-81). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors (Saveria-Campo, "Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector," in DNA Cloning: A Practical Approach, vol. II, Glover, ed., IRL Press, Arlington, VA, pp. 213-38 (1985)).

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

**Plant Transformation Methods**

Numerous methods for introducing foreign genes into plants are known and can be used to insert an NR polynucleotide into a plant host, including biological and physical plant transformation protocols. See, e.g., Miki, et al., (1993) "Procedure for Introducing Foreign DNA into Plants," in Methods in Plant Molecular Biology and Biotechnology, Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pp. 67-88. The methods chosen vary with the host plant, and include chemical transfection methods such as calcium phosphate, microorganism-mediated gene transfer such as *Agrobacterium* (Horsch, et al., (1985) Science 227:1229-31), electroporation, micro-injection, and biolistic bombardment.

Expression cassettes and vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are known and available. See, e.g., Gruber, et al., "Vectors for Plant Transformation," in Methods in Plant Molecular Biology and Biotechnology, supra, pp. 89-19.

The isolated polynucleotides or polypeptides may be introduced into the plant by one or more techniques typically used for direct delivery into cells. Such protocols may vary depending on the type of organism, cell, plant or plant cell, i.e. monocot or dicot, targeted for gene modification. Suitable methods of transforming plant cells include microinjection (Crossway, et al., (1986)

**Agrobacterium-me iate Transformation**


Similarly, the gene can be inserted into the T-DNA region of a Ti or Ri plasmid derived from A. *tumefaciens* or A. *rhizogenes*, respectively. Thus, expression cassettes can be constructed as above, using these plasmids. Many control sequences are known which when coupled to a heterologous coding sequence and transformed into a host organism show fidelity in gene expression with respect to tissue/organ specificity of the original coding sequence. See, e.g., Benfey and Chua, (1989) *Science* 244:174-81. Particularly suitable control sequences for use in these plasmids are promotors for constitutive leaf-specific expression of the gene in the various target plants. Other useful control sequences include a promoter and terminator from the nopaline synthase gene (NOS). The NOS promoter and terminator are present in the plasmid pARC2, available from the American Type Culture Collection and designated ATCC 67238. If such a system is used, the virulence (*vir*) gene from either the Ti or Ri plasmid must also be present, either along with the T-DNA portion, or via a binary system where the *vir* gene is present on a separate vector. Such systems, vectors for use therein, and methods of transforming plant cells are described in US Patent Number 4,658,082; US Patent Application Number 913,914, filed Oct. 1, 1986, as referenced in US Patent Number 5,262,306, issued November 16, 1993; and Simpson, *et al.*, (1986) *Plant Mol. Biol.* 6:403-15 (also referenced in the '306 patent); all incorporated by reference in their entirety.
Once constructed, these plasmids can be placed into \textit{A. rhizogenes} or \textit{A. tumefaciens} and these vectors used to transform cells of plant species, which are ordinarily susceptible to \textit{Fusarium} or \textit{Alternaria} infection. Several other transgenic plants are also contemplated by the present invention including but not limited to soybean, corn, sorghum, alfalfa, rice, clover, cabbage, banana, coffee, celery, tobacco, cowpea, cotton, melon and pepper. The selection of either \textit{A. tumefaciens} or \textit{A. rhizogenes} will depend on the plant being transformed thereby. In general \textit{A. tumefaciens} is the preferred organism for transformation. Most dicotyledonous plants, some gymnosperms, and a few monocotyledonous plants (e.g., certain members of the \textit{Liliales} and \textit{Arales}) are susceptible to infection with \textit{A. tumefaciens}. \textit{A. rhizogenes} also has a wide host range, embracing most dicots and some gymnosperms, which includes members of the \textit{Leguminosae}, \textit{Compositae}, and \textit{Chenopodiaceae}. Monocot plants can now be transformed with some success. European Patent Application Number 604 662 A1 discloses a method for transforming monocots using \textit{Agrobacterium}. European Application Number 672 752 A1 discloses a method for transforming monocots with \textit{Agrobacterium} using the scutellum of immature embryos. Ishida, \textit{et al.}, discuss a method for transforming maize by exposing immature embryos to \textit{A. tumefaciens} (\textit{Nature Biotechnology} 14:745-50 (1996)).

Once transformed, these cells can be used to regenerate transgenic plants. For example, whole plants can be infected with these vectors by wounding the plant and then introducing the vector into the wound site. Any part of the plant can be wounded, including leaves, stems and roots. Alternatively, plant tissue, in the form of an explant, such as cotyledonary tissue or leaf disks, can be inoculated with these vectors, and cultured under conditions, which promote plant regeneration. Roots or shoots transformed by inoculation of plant tissue with \textit{A. rhizogenes} or \textit{A. tumefaciens}, containing the gene coding for the fumonisin degradation enzyme, can be used as a source of plant tissue to regenerate fumonisin-resistant transgenic plants, either via somatic embryogenesis or organogenesis. Examples of such methods for regenerating plant tissue are disclosed in Shahin, (1985) \textit{Theor. Appl. Genet.} 69:235-40; US Patent Number 4,658,082; Simpson, \textit{et al.}, \textit{supra}; and US Patent Application Numbers 913,913 and 913,914, both filed Oct. 1, 1986, as referenced in US Patent Number...

**Direct Gene Transfer**

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

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


Increasing the Activity and/or Level of a NR Polypeptide

Methods are provided to increase the activity and/or level of the NR polypeptide of the invention. An increase in the level and/or activity of the NR polypeptide of the invention can be achieved by providing to the plant a NR polypeptide. The NR polypeptide can be provided by introducing the amino acid sequence encoding the NR polypeptide into the plant, introducing into the plant a nucleotide sequence encoding a NR polypeptide or alternatively by modifying a genomic locus encoding the NR polypeptide of the invention.

As discussed elsewhere herein, many methods are known the art for providing a polypeptide to a plant including, but not limited to, direct introduction of the polypeptide into the plant, introducing into the plant (transiently or stably) a polynucleotide construct encoding a polypeptide having enhanced nitrogen utilization activity. It is also recognized that the methods of the invention may employ a polynucleotide that is not capable of directing, in the transformed plant, the expression of a protein or an RNA. Thus, the level and/or activity of a NR polypeptide may be increased by altering the gene encoding the NR polypeptide or its promoter. See, e.g., Kmiec, US Patent Number 5,565,350; Zarling, et al., PCT/US93/03868. Therefore mutagenized plants that carry mutations in NR genes, where the mutations increase expression of the NR gene or increase the NR activity of the encoded NR polypeptide are provided.

Reducing the Activity and/or Level of a NR Polypeptide

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

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

In other embodiments of the invention, the activity of the NR polypeptides is reduced or eliminated by transforming a plant cell with an expression cassette comprising a polynucleotide encoding a polypeptide that inhibits the activity of a NR polypeptide. The enhanced nitrogen utilization activity of a NR polypeptide is inhibited according to the present invention if the NR activity of the NR polypeptide is less than 70% of the NR activity of the same NR polypeptide in a plant that has not been modified to inhibit the NR activity of that NR polypeptide. In particular embodiments of the invention, the NR activity of the NR polypeptide in a modified plant according to the invention is less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% of the NR activity of the same NR polypeptide in a plant that has not been modified to inhibit the expression of that NR polypeptide. The NR activity of a NR polypeptide is "eliminated" according to the invention when it is not detectable by the assay methods described elsewhere herein. Methods of determining the alteration of nitrogen utilization activity of a NR polypeptide are described elsewhere herein.

In other embodiments, the activity of a NR polypeptide may be reduced or eliminated by disrupting the gene encoding the NR polypeptide. The invention encompasses mutagenized plants that carry mutations in NR genes, where the mutations reduce expression of the NR gene or inhibit the nitrogen utilization activity of the encoded NR polypeptide.

Thus, many methods may be used to reduce or eliminate the activity of a NR polypeptide. In addition, more than one method may be used to reduce the activity of a single NR polypeptide.
1. **Polynucleotide-Based Methods:**

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

Examples of polynucleotides that inhibit the expression of a NR polypeptide are given below.

/. **Sense Suppression/Cosuppression**

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

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


*ii. Antisense Suppression*

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

The polynucleotide for use in antisense suppression may correspond to all or part of the complement of the sequence encoding the NR polypeptide, all or
part of the complement of the 5’ and/or 3’ untranslated region of the NR transcript, or all or part of the complement of both the coding sequence and the untranslated regions of a transcript encoding the NR polypeptide. In addition, the antisense polynucleotide may be fully complementary (i.e., 100% identical to the complement of the target sequence) or partially complementary (i.e., less than 100% identical to the complement of the target sequence) to the target sequence. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, US Patent Number 5,942,657. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, 300, 400, 450, 500, 550 or greater may be used. Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu, et al., (2002) Plant Physiol. 129:1732-1743 and US Patent Numbers 5,759,829 and 5,942,657, each of which is herein incorporated by reference. Efficiency of antisense suppression may be increased by including a poly-dT region in the expression cassette at a position 3’ to the antisense sequence and 5’ of the polyadenylation signal. See, US Patent Publication Number 20020048814, herein incorporated by reference.

Hi. Double-Stranded RNA Interference

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

Expression of the sense and antisense molecules can be accomplished by designing the expression cassette to comprise both a sense sequence and an antisense sequence. Alternatively, separate expression cassettes may be used for the sense and antisense sequences. Multiple plant lines transformed with the dsRNA interference expression cassette or expression cassettes are then screened to identify plant lines that show the greatest inhibition of NR polypeptide expression. Methods for using dsRNA interference to inhibit the expression of

iv. Hairpin RNA Interference and Intron-Containing Hairpin RNA Interference

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

For hpRNA interference, the expression cassette is designed to express an RNA molecule that hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem. The base-paired stem region comprises a sense sequence corresponding to all or part of the endogenous messenger RNA encoding the gene whose expression is to be inhibited, and an antisense sequence that is fully or partially complementary to the sense sequence. Alternatively, the base-paired stem region may correspond to a portion of a promoter sequence controlling expression of the gene to be inhibited. Thus, the base-paired stem region of the molecule generally determines the specificity of the RNA interference. hpRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants. See, for example, Chuang and Meyerowitz, (2000) Proc. Natl. Acad. Sci. USA 97:4985-4990; Stoutjesdijk, et al., (2002) Plant Physiol. 129:1723-1731; and Waterhouse and Helliwell, (2003) Nat. Rev. Genet. 4:29-38. Methods for using hpRNA interference to inhibit or silence the expression of genes are described, for example, in Chuang and Meyerowitz, (2000) Proc. Natl. Acad. Sci. USA 97:4985-4990; Stoutjesdijk, et al., (2002) Plant Physiol. 129:1723-1731; Waterhouse and Helliwell, (2003) Nat. Rev. Genet. 4:29-38; Pandolfini et al., BMC Biotechnology 3:7, and US Patent Publication Number 2003/0175965; each of which is herein incorporated by reference. A transient assay for the efficiency of hpRNA constructs to silence


v. Amplicon-Mediated Interference

Amplicon expression cassettes comprise a plant virus-derived sequence that contains all or part of the target gene but generally not all of the genes of the native virus. The viral sequences present in the transcription product of the expression cassette allow the transcription product to direct its own replication. The transcripts produced by the amplicon may be either sense or antisense relative to the target sequence (i.e., the messenger RNA for the NR polypeptide).

vi. Ribozymes

In some embodiments, the polynucleotide expressed by the expression cassette of the invention is catalytic RNA or has hbozyme activity specific for the messenger RNA of the NR polypeptide. Thus, the polynucleotide causes the degradation of the endogenous messenger RNA, resulting in reduced expression of the NR polypeptide. This method is described, for example, in US Patent Number 4,987,071, herein incorporated by reference.

vii. Small Interfering RNA or Micro RNA

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

For miRNA interference, the expression cassette is designed to express an RNA molecule that is modeled on an endogenous miRNA gene. The miRNA gene encodes an RNA that forms a hairpin structure containing a 22-nucleotide sequence that is complementary to another endogenous gene (target sequence).

For suppression of NR expression, the 22-nucleotide sequence is selected from a NR transcript sequence and contains 22 nucleotides of said NR sequence in sense orientation and 21 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence. miRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants.

2. Polypeptide-Based Inhibition of Gene Expression

In one embodiment, the polynucleotide encodes a zinc finger protein that binds to a gene encoding a NR polypeptide, resulting in reduced expression of the
gene. In particular embodiments, the zinc finger protein binds to a regulatory region of a NR gene. In other embodiments, the zinc finger protein binds to a messenger RNA encoding a NR polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described, for example, in US Patent Number 6,453,242, and methods for using zinc finger proteins to inhibit the expression of genes in plants are described, for example, in US Patent Application Publication Number 2003/0037355; each of which is herein incorporated by reference.

3. **Polypeptide-Based Inhibition of Protein Activity**

In some embodiments of the invention, the polynucleotide encodes an antibody that binds to at least one NR polypeptide, and reduces the enhanced nitrogen utilization activity of the NR polypeptide. In another embodiment, the binding of the antibody results in increased turnover of the antibody-NR complex by cellular quality control mechanisms. The expression of antibodies in plant cells and the inhibition of molecular pathways by expression and binding of antibodies to proteins in plant cells are well known in the art. See, for example, Conrad and Sonnewald, (2003) *Nature Biotech.* 21:35-36, incorporated herein by reference.

4. **Gene Disruption**

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

/. **Transposon Tagging**

In one embodiment of the invention, transposon tagging is used to reduce or eliminate the NR activity of one or more NR polypeptide. Transposon tagging comprises inserting a transposon within an endogenous NR gene to reduce or eliminate expression of the NR polypeptide. "NR gene" is intended to mean the gene that encodes a NR polypeptide according to the invention.
In this embodiment, the expression of one or more NR polypeptide is reduced or eliminated by inserting a transposon within a regulatory region or coding region of the gene encoding the NR polypeptide. A transposon that is within an exon, intron, 5' or 3' untranslated sequence, a promoter, or any other regulatory sequence of a NR gene may be used to reduce or eliminate the expression and/or activity of the encoded NR polypeptide.


ii. Mutant Plants with Reduced Activity

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

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

In another embodiment of this invention, dominant mutants can be used to trigger RNA silencing due to gene inversion and recombination of a duplicated gene locus. See, for example, Kusaba, et al., (2003) *Plant Cell* 15:1455-1467.

The invention encompasses additional methods for reducing or eliminating the activity of one or more NR polypeptide. Examples of other methods for altering or mutating a genomic nucleotide sequence in a plant are known in the art and include, but are not limited to, the use of RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides, and recombinogenic oligonucleobases. Such vectors and methods of use are known in the art. See, for example, US Patent Numbers 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; each of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham, et al., (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; each of which is herein incorporated by reference.

**Hi. Modulating nitrogen utilization activity**

In specific methods, the level and/or activity of a NR regulator in a plant is decreased by increasing the level or activity of the NR polypeptide in the plant. The increased expression of a negative regulatory molecule may decrease the level of expression of downstream one or more genes responsible for an improved NR phenotype.

Methods for increasing the level and/or activity of NR polypeptides in a plant are discussed elsewhere herein. Briefly, such methods comprise providing a NR polypeptide of the invention to a plant and thereby increasing the level and/or activity of the NR polypeptide. In other embodiments, a NR nucleotide sequence encoding a NR polypeptide can be provided by introducing into the plant a
polynucleotide comprising a NR nucleotide sequence of the invention, expressing the NR sequence, increasing the activity of the NR polypeptide, and thereby decreasing the number of tissue cells in the plant or plant part. In other embodiments, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

In other methods, the growth of a plant tissue is increased by decreasing the level and/or activity of the NR polypeptide in the plant. Such methods are disclosed in detail elsewhere herein. In one such method, a NR nucleotide sequence is introduced into the plant and expression of said NR nucleotide sequence decreases the activity of the NR polypeptide, and thereby increasing the tissue growth in the plant or plant part. In other embodiments, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

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

In other embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising a NR nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

iv. **Modulating Root Development**

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

Methods for modulating root development in a plant are provided. The methods comprise modulating the level and/or activity of the NR polypeptide in the plant. In one method, a NR sequence of the invention is provided to the plant. In another method, the NR nucleotide sequence is provided by introducing into the plant a polynucleotide comprising a NR nucleotide sequence of the invention, expressing the NR sequence, and thereby modifying root development. In still
other methods, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant. A change in NR activity can result in at least one or more of the following alterations to root development, including but not limited to alterations in root biomass and length.

As used herein, "root growth" encompasses all aspects of growth of the plant, both adventitious and adventitious roots, etc. including the primary root, lateral roots, adventitious roots, etc. The term "root biomass" refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.

Exemplary root-preferred promoters have been disclosed elsewhere herein. Exemplary root-preferred promoters have been disclosed elsewhere herein. The term "resistance to lodging" in the plant refers to the ability of a plant to fix itself to the soil. For plants with erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse environmental conditions. This trait relates to the size, shape and morphology of the root system. In addition, stimulating root growth and increasing root mass by decreasing the activity of the NR polypeptide also use in promoting the level of the NR polypeptide also used in promoting the activity of the NR polypeptide in the plant. Exemplary promoters for this embodiment include constitutive promoters and root-preferred promoters.
Accordingly, the present invention further provides plants having modulated root development when compared to the root development of a control plant. In some embodiments, the plant of the invention has an increased level/activity of the NR polypeptide of the invention and has enhanced root growth and/or root biomass. In other embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising a NR nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.

v. **Modulating Shoot and Leaf Development**

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

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

In specific embodiments, shoot or leaf development is modulated by altering the level and/or activity of the NR polypeptide in the plant. A change in NR activity can result in at least one or more of the following alterations in shoot and/or leaf development, including, but not limited to, changes in leaf number,
altered leaf surface, altered vasculature, internodes and plant growth, and alterations in leaf senescence, when compared to a control plant.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate shoot and leaf development of the plant. Exemplary promoters for this embodiment include constitutive promoters, shoot-preferred promoters, shoot mehstem-preferred promoters, and leaf-preferred promoters. Exemplary promoters have been disclosed elsewhere herein.

Increasing NR activity and/or level in a plant results in altered internodes and growth. Thus, the methods of the invention find use in producing modified plants. In addition, as discussed above, NR activity in the plant modulates both root and shoot growth. Thus, the present invention further provides methods for altering the root/shoot ratio. Shoot or leaf development can further be modulated by altering the level and/or activity of the NR polypeptide in the plant.

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

vi. Modulating Reproductive Tissue Development

Methods for modulating reproductive tissue development are provided. In one embodiment, methods are provided to modulate floral development in a plant. By "modulating floral development" is intended any alteration in a structure of a plant's reproductive tissue as compared to a control plant in which the activity or level of the NR polypeptide has not been modulated. "Modulating floral development" further includes any alteration in the timing of the development of a plant's reproductive tissue (i.e., a delayed or a accelerated timing of floral development) when compared to a control plant in which the activity or level of the NR polypeptide has not been modulated. Macroscopic alterations may include changes in size, shape, number, or location of reproductive organs, the developmental time period that these structures form, or the ability to maintain or proceed through the flowering process in times of environmental stress. Microscopic alterations may include changes to the types or shapes of cells that make up the reproductive organs.
The method for modulating floral development in a plant comprises modulating NR activity in a plant. In one method, a NR sequence of the invention is provided. A NR nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising a NR nucleotide sequence of the invention, expressing the NR sequence, and thereby modifying floral development. In other embodiments, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

In specific methods, floral development is modulated by increasing the level or activity of the NR polypeptide in the plant. A change in NR activity can result in at least one or more of the following alterations in floral development, including, but not limited to, altered flowering, changed number of flowers, modified male sterility, and altered seed set, when compared to a control plant. Inducing delayed flowering or inhibiting flowering can be used to enhance yield in forage crops such as alfalfa. Methods for measuring such developmental alterations in floral development are known in the art. See, for example, Mouradov, et al., (2002) The Plant Cell 14 S111-S130, herein incorporated by reference.

As discussed above, one of skill will recognize the appropriate promoter to use to modulate floral development of the plant. Exemplary promoters for this embodiment include constitutive promoters, inducible promoters, shoot-preferred promoters, and inflorescence-preferred promoters.

In other methods, floral development is modulated by altering the level and/or activity of the NR sequence of the invention. Such methods can comprise introducing a NR nucleotide sequence into the plant and changing the activity of the NR polypeptide. In other methods, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant. Altering expression of the NR sequence of the invention can modulate floral development during periods of stress. Such methods are described elsewhere herein. Accordingly, the present invention further provides plants having modulated floral development when compared to the floral development of a control plant. Compositions include plants having a altered level/activity of the NR polypeptide of the invention and having an altered floral development. Compositions also include plants having a modified level/activity of the NR polypeptide of the invention wherein the plant maintains or proceeds through the flowering process in times of stress.
Methods are also provided for the use of the NR sequences of the invention to increase seed size and/or weight. The method comprises increasing the activity of the NR sequences in a plant or plant part, such as the seed. An increase in seed size and/or weight comprises an increased size or weight of the seed and/or an increase in the size or weight of one or more seed part including, for example, the embryo, endosperm, seed coat, aleurone, or cotyledon.

As discussed above, one of skill will recognize the appropriate promoter to use to increase seed size and/or seed weight. Exemplary promoters of this embodiment include constitutive promoters, inducible promoters, seed-preferred promoters, embryo-preferred promoters, and endosperm-preferred promoters.

The method for altering seed size and/or seed weight in a plant comprises increasing NR activity in the plant. In one embodiment, the NR nucleotide sequence can be provided by introducing into the plant a polynucleotide comprising a NR nucleotide sequence of the invention, expressing the NR sequence, and thereby decreasing seed weight and/or size. In other embodiments, the NR nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

It is further recognized that increasing seed size and/or weight can also be accompanied by an increase in the speed of growth of seedlings or an increase in early vigor. As used herein, the term "early vigor" refers to the ability of a plant to grow rapidly during early development, and relates to the successful establishment, after germination, of a well-developed root system and a well-developed photosynthetic apparatus. In addition, an increase in seed size and/or weight can also result in an increase in plant yield when compared to a control.

Accordingly, the present invention further provides plants having an increased seed weight and/or seed size when compared to a control plant. In other embodiments, plants having an increased vigor and plant yield are also provided. In some embodiments, the plant of the invention has a modified level/activity of the NR polypeptide of the invention and has an increased seed weight and/or seed size. In other embodiments, such plants have stably incorporated into their genome a nucleic acid molecule comprising a NR nucleotide sequence of the invention operably linked to a promoter that drives expression in the plant cell.
vii. Method of Use for NR polynucleotide, expression cassettes, and additional polynucleotides

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

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

The polynucleotides of the present invention may be stacked with any gene or combination of genes to produce plants with a variety of desired trait combinations, including but not limited to traits desirable for animal feed such as high oil genes (e.g., US Patent Number 6,232,529); balanced amino acids (e.g., hordothionins (US Patent Numbers 5,990,389; 5,885,801; 5,885,802; and 5,703,409); barley high lysine (Williamson, et al., (1987) Eur. J. Biochem. 165:99-106; and WO 98/20122); and high methionine proteins (Pedersen, et al., (1986) J. Biol. Chem. 261:6279; Kirihara, et al., (1988) Gene 71:359; and Musumura, et al.,
increased digestibility (e.g., modified storage proteins (US Patent Application Serial Number 10/053,410, filed November 7, 2001); and thioredoxins (US Patent Application Serial Number 10/005,429, filed December 3, 2001)), the disclosures of which are herein incorporated by reference. The polynucleotides of the present invention can also be stacked with traits desirable for insect, disease or herbicide resistance (e.g., *Bacillus thuringiensis* toxic proteins (US Patent Numbers 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; Geiser, *et al.*, (1986) *Gene* 48:109); lectins (Van Damme, *et al.*, (1994) *Plant Mol. Biol.* 24:825); fumonisin detoxification genes (US Patent Number 5,792,931); avirulence and disease resistance genes (Jones, *et al.*, (1994) *Science* 266:789; Martin, *et al.*, (1993) *Science* 262:1432; Mindrinos, *et al.*, (1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene); and traits desirable for processing or process products such as high oil (e.g., US Patent Number 6,232,529); modified oils (e.g., fatty acid desaturase genes (US Patent Number 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., US Patent Number 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert, *et al.*, (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)), the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides affecting agronomic traits such as male sterility (e.g., see US Patent Number 5,583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (e.g., WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated by reference.

In one embodiment, sequences of interest improve plant growth and/or crop yields. For example, sequences of interest include agronomically important genes that result in improved primary or lateral root systems. Such genes include, but are not limited to, nutrient/water transporters and growth induces. Examples of such genes, include but are not limited to, maize plasma membrane H+-ATPase

Additional, agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch. Hordeothionin protein modifications are described in US Patent Numbers 5,703,049, 5,885,801, 5,885,802, and 5,990,389, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in US Patent Number 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson, et al., (1987) Eur. J. Biochem. 165:99-106, the disclosures of which are herein incorporated by reference.

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, US Patent Application Serial Number 08/740,682, filed November 1, 1996, and WO 98/20133, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley, et al., (1989) Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen, et al., (1986) J. Biol. Chem. 261:6279; Kirihara, et al., (1988) Gene 71:359; both of which are herein incorporated by reference); and rice (Musumura, et al., (1989) Plant Mol. Biol. 12:1 23, herein incorporated by reference). Other agronomically
important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (US Patent Numbers 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser, *et al./, (1986) Gene 48:1 09); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (US Patent Number 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones, *et al./, (1994) Science 266:789; Martin, *et al./, (1993) Science 262:1432; and Mindrinos, *et al./, (1994) Cell 78:1 089); and the like.

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

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in US Patent Number 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. In corn, modified hordothionin proteins are described in US Patent Numbers 5,703,049, 5,885,801, 5,885,802, and 5,990,389.

Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the
production of polymers and bioplastics such as described in US Patent Number 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxyburyrate synthase), and acetoacetyl-CoA reductase (see, Schubert, et al., (1988) J. Bacteriol. 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs).

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

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

**EXAMPLES**

**Example 1. Isolation of NR sequences**

20,778 Porphyra yezoensis ESTs were identified in Genbank then downloaded. The dataset was converted to fasta, formatted for BLAST algorithm searching, and searched with maize nitrate reductase as the query.

Multiple ESTs likely encoding fragments of nitrate reductase genes were identified, and EST contigs were built, supplemented by 5'- and 3'-directional EST walking and final contig assembly of the gene transcript region. Prior to this contig assembly the gene and its coding region existed in the public databases as short EST sequences representing merely fragments of the gene. The resulting *Porphyra yezoensis* nitrate reductase transcript contig represented a 5'UTR, the N-terminus, and all but the last (C-terminal) 167 codons of the peptide.

Assuming the two strains of *Porphyra* were closely related organisms, oligonucleotide primers were designed from the assembled *Porphyra yezoensis* nitrate reductase gene with sense: attgtgggtcacaacaaggtgtatga (SEQ ID NO: 13) and anti-sense primers: tcggtttcagatgtctctgt (SEQ ID NO: 14). These primers were used to amplify a 448 bp internal region of the gene using Porphyra perforata genomic DNA. A Genome Walking kit from Bio S&T (Montreal, Quebec, Canada) was then used to determine both ends of the gene, walking out from the internal
fragment. (Standard conditions for nested PCR, ligation and digestion as per the provided SOP from Bio S&T were used). For the Porphyra perforata gene discovery, the following walking primers were designed - cgtgtacctccgcaactct (SEQ ID NO: 15), gctgaccttgtgcgcaacc (SEQ ID NO: 16), gcatggagccggcgaacaa (SEQ ID NO: 17), gcttgccgctggctcactttgcag (SEQ ID NO: 18), ccgtgagctcctgatgcgtgc (SEQ ID NO: 19) and ctacagccacgctgctgacgca (SEQ ID NO: 20).

Again assuming the Porphyra were closely related organisms, Porphyra perforata primers for full length nitrate reductase were used to amplify the gene from the closely related Porphyra yezoensis red algae, but with a lower annealing temperature of 52°C in the initial PCR set-up for 40 cycles. Regions were sequence verified as correct, then using the same Genome Walking kit from Bio S&T ambiguous regions and the remaining ends of the Porphyra yezoensis along with associated untranslated regions were determined.

The entire ORF of the nitrate reductase gene from both species was cloned as a large PCR product using a high-fidelity Taq polymerase and a minimal of at least three independent clones was used to verify the correct internal sequence. The ORF of the Porphyra perforata nitrate reductase gene was amplified with sense: catggagctgttctgctgc (SEQ ID NO: 21) and anti-sense primers: tcaacagctgtgcgtgcggtgca (SEQ ID NO: 22). The ORF of the Porphyra yezoensis nitrate reductase gene was amplified with sense: cccatcaccgcacagccgca (SEQ ID NO: 23) and anti-sense primers: agagagccgcctgctgcgtgt (SEQ ID NO: 24). For most regions of the gene, more clones were used to validate the full-length sequence. Protein and nucleotide alignments confirmed that both genes were closely associated and had many conserved protein residues when compared to other nitrate reductase enzymes. There are no introns for either Nitrate Reductase coding DNA sequence.

**Example 2. Pichia pastoris expression vectors and yeast transformation:**

Pichia pastoris is a non-nitrate assimilating yeast and requires both functional nitrate transporter and nitrate reductase to uptake nitrate (Unkles, *et al.*, (2004) J. Biol. Chem. 279:28182-28186). P. pastoris has been used as a model system for identification and characterization of nitrate reductases and/or plant nitrate transporters. The nitrate reductase activity can be determined *in vitro or in*
vivo when a nitrate transporter gene was co-expressed. The wild type nitrate reductase genes from *Porphyra performa* (PpNR) and *Porphyra yezoensis* (PyNR) driven by a yeast constitutive promoter (GAP promoter, glyceraldehydes-3-phosphate dehydrogenase) was integrated into AOX1 locus of *P. pastoris* stain KM71 carrying a yeast nitrate transporter gene (YNT1).

Details: YNT1 (nitrate transporter from *Pichia angusta*) driven by GAP promoter (vector: p3.5GAP-YNT1) was integrated into *P. pastoris* strain KM71 genome at His4 locus to generate YNT1-containing lines. After the YNT1-containing lines were confirmed by PCR, PpNR or PyNR driven by GAP promoter was integrated into the genome at AOX1 locus (vectors: pAOXGAP-PPNR and PAOXGAP-PYNR). (*Pichia* transformation kit is from Invitrogen. Both expression vector backbones were modified based on the versions from Invitrogen.)

**Functional expression in *P. pastoris***: Recombinant KM71 transformed with p3.5GAP-YNT1 and/or pAOXGAP-PPNR, pAOXGAP-PYNR were screened for NR activity. Transformants were cultured in rich media (YPD) at 30°C for overnight. Yeast cells were collected and washed with water twice then re-suspended in 20 uM MOPS, pH6.5 and 1% glucose containing 1 mM NaNO3. After 2 hours incubation at 30°C, the supernatant was collected for nitrite assay with 2% and 2%. The Vmax of PpNR is 70-80X higher than Py-NR. The transformants containing PpNR showed much stronger nitrate reductase activity than PyNR in *P. pastoris in vivo*.

**Kinetics in *P. pastoris***: The nitrite concentration of *P. pastoris* KM71-containing YNT1 and PpNR line incubated with different nitrate concentration from 0-30 mM at three pH, pH5.5, 6.5, and 7.5 was assayed in vivo. PpNR has better Vmax at pH6.5. The Km is about 30-40 µM. This data match the predicted results (published PyNR with Km 64 µM). At the same time, maize NR, ZM-NR, and yeast NR from *P. angusta*, YNR1, were assayed at similar conditions. The Km of ZM-NR is about 300 uM and YNR1 has Km about 200-400 µM. They also have the better Vmax at pH6.5. The PPNR is an efficient NR with Km 10X lower than others.

To maintain high level of active form of PPNR in transgenic plants, the serine residue in a putative phosphorylation motif R/K-S/T-X-pS-X-P (J. of Experimental Botany (2004) 55:1275-1282) at position 561 will be mutated to either alanine (S561A mutant, sequence provided) or aspartic acid (S561D mutant). The mutated PPNRs will be generated in P. pastoris and tested in YNT1-carrying line for NR activity as described before. The functional S561A or S561D will be expressed in transgenic plants driven by a constitutive promoter or a mesophyll preferred promoter. The NR activity will be tested during light/dark transitions. Other putative post-translational regions such as N-terminal region of PPNR may be modified or deleted to de-regulate by light at post-translational level.

Example 4. Transformation and Regeneration of Transgenic Plants

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the NR sequence operably linked to the drought-inducible promoter RAB17 promoter (Vilardell, et ai, (1990) Plant Mol Biol 14:423-432) and the selectable marker gene PAT, which confers resistance to the herbicide Bialaphos. (Miller, et ai, (2000) Biochimica et Biophysica Acta 1465:343-358). Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue:

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

Preparation of DNA:

A plasmid vector comprising the NR sequence operably linked to an ubiquitin promoter is made. This plasmid DNA plus plasmid DNA containing a
PAT selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a CaCl₂ precipitation procedure as follows:

100 µl prepared tungsten particles in water
10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)
100 µl 2.5 M CaCl₂
10 µl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multtube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet.

For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

**Particle Gun Treatment:**

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

**Subsequent Treatment:**

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

**Bombardment and Culture Media:**

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-151 1), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H2O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H2O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-151 1), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H2O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H2O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H2O) (Murashige and Skoog, (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H2O after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H2O); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I
H$_2$O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-l H$_2$O after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-l H$_2$O), sterilized and cooled to 60°C.

Example 5. Agrobacterium-mediated Transformation

For Agrobacterium-mediated transformation of maize with an antisense sequence of the NR sequence of the present invention, preferably the method of Zhao is employed (US Patent Number 5,981,840, and PCT Patent Application Publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of Agrobacterium tumefaciens, where the bacteria are capable of transferring the antisense NR sequences to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an Agrobacterium tumefaciens suspension for the initiation of inoculation. The embryos are co-cultured for a time with the Agrobacterium (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of Agrobacterium without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of Agrobacterium and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants. Plants are monitored and scored for a modulation in meristem development (for instance, alterations of size and appearance of the shoot and floral meristems and/or increased yields of leaves, flowers, and/or fruits).
Example 6. Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing an antisense NR sequences operably linked to an ubiquitin promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.


A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell, et al., (1985) Nature 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from E. coli; Gritz, et al., (1983) Gene 25:179-188), and the 3’ region of the nopaline synthase gene from the T-DNA of the Ti plasmid of Agrobacterium tumefaciens. The expression cassette comprising an antisense NR sequence operably linked to the ubiquitin promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

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

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

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

Example 7. Sunflower Meristem Tissue Transformation

Sunflower meristem tissues are transformed with an expression cassette containing an antisense NR sequences operably linked to a ubiquitin promoter as follows (see also, European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg, et al., (1994) Plant Science 103:1 99-207). Mature sunflower seed (Helianthus annuus L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled water.

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

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

Disarmed Agrobacterium tumefaciens strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the NR gene operably linked to the ubiquitin promoter is introduced into Agrobacterium strain EHA105 via freeze-thawing as described by Holsters, et al., (1978) Mol. Gen. Genet. 163:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e., nptII). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD600 of about 0.4 to 0.8. The Agrobacterium cells are pelleted and resuspended at a final OD600 of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l NH₄Cl, and 0.3 gm/l MgSO₄.

Freshly bombarded explants are placed in an Agrobacterium suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days.
After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on selection and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assaying for a modulation in mehstems development (i.e., an alteration of size and appearance of shoot and floral mehstems).

NPTII-positive shoots are grafted to Pioneer® hybrid 6440 in wrosgrown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of in vitro culture. Grafts in soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T₀ plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by NR activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T₀ plants are identified by NR activity analysis of small portions of dry seed cotyledon.

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

Approximately 18.8 mg of 1.8 μm tungsten particles are resuspended in 150 μl absolute ethanol. After sonication, 8 μl of it is dropped on the center of the surface of macrorrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

The plasmid of interest is introduced into Agrobacterium tumefaciens strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C in a liquid YEP medium (10 g/l yeast extract, 10 g/l Bactopeptone, and 5 g/l NaCl, pH 7.0) in the presence of 50 μg/l kanamycin is resuspended in an inoculation medium (12.5 mM 2-mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH₄Cl and 0.3 g/l MgSO₄ at pH 5.7) to reach a final concentration of 4.0 at OD₆₀₀. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no BAP, IAA, GA3 and supplemented with 250 μg/ml cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C incubation conditions.

Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for a modulation in meristem development (i.e., an alteration of size and appearance of shoot and floral meristems). After positive explants are identified, those shoots that fail to exhibit modified NR activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have
been enriched in the transgenic sector detected in the initial assay prior to nodal
culture.

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

Example 8. Rice Tissue Transformation

Genetic Confirmation of the NR gene

One method for transforming DNA into cells of higher plants that is
available to those skilled in the art is high-velocity ballistic bombardment using
metal particles coated with the nucleic acid constructs of interest (see, Klein, et al.,
Biolistic PDS-1000/He (BioRAD Laboratories, Hercules, CA) is used for these
complementation experiments. The particle bombardment technique is used to
transform the NR mutants and wild type rice with DNA fragments

The bacterial hygromycin B phosphotransferase (Hpt II) gene from
Streptomyces hygroscopicus that confers resistance to the antibiotic is used as
the selectable marker for rice transformation. In the vector, pML18, the Hpt II
gene was engineered with the 35S promoter from Cauliflower Mosaic Virus and
the termination and polyadenylation signals from the octopine synthase gene of
Agrobacterium tumefaciens. pML18 was described in WO 97/47731, which was
published on December 18, 1997, the disclosure of which is hereby incorporated
by reference.
Embryogenic callus cultures derived from the scutellum of germinating rice seeds serve as source material for transformation experiments. This material is generated by germinating sterile rice seeds on a callus initiation media (MS salts, Nitsch and Nitsch vitamins, 1.0 mg/l 2,4-D and 10 µM AgNO₃s) in the dark at 27-28°C. Embryogenic callus proliferating from the scutellum of the embryos is the transferred to CM media (N6 salts, Nitsch and Nitsch vitamins, 1 mg/l 2,4-D, Chu, et al., 1985, Sci. Sinica 18: 659-668). Callus cultures are maintained on CM by routine sub-culture at two week intervals and used for transformation within 10 weeks of initiation.

Callus is prepared for transformation by subculturing 0.5-1.0 mm pieces approximately 1 mm apart, arranged in a circular area of about 4 cm in diameter, in the center of a circle of Whatman #541 paper placed on CM media. The plates with callus are incubated in the dark at 27-28°C for 3-5 days. Prior to bombardment, the filters with callus are transferred to CM supplemented with 0.25 M mannitol and 0.25 M sorbitol for 3 hr in the dark. The petri dish lids are then left ajar for 20-45 minutes in a sterile hood to allow moisture on tissue to dissipate.

Each genomic DNA fragment is co-precipitated with pML18 containing the selectable marker for rice transformation onto the surface of gold particles. To accomplish this, a total of 10 µg of DNA at a 2:1 ratio of trait: selectable marker DNAs are added to 50 µl aliquot of gold particles that have been resuspended at a concentration of 60 mg ml⁻¹. Calcium chloride (50 µl of a 2.5 M solution) and spermidine (20 µl of a 0.1 M solution) are then added to the gold-DNA suspension as the tube is vortexing for 3 min. The gold particles are centrifuged in a microfuge for 1 sec and the supernatant removed. The gold particles are then washed twice with 1 ml of absolute ethanol and then resuspended in 50 µl of absolute ethanol and sonicated (bath sonicator) for one second to disperse the gold particles. The gold suspension is incubated at -70°C for five minutes and sonicated (bath sonicator) if needed to disperse the particles. Six µl of the DNA-coated gold particles are then loaded onto mylar macrocarrier disks and the ethanol is allowed to evaporate.

At the end of the drying period, a petri dish containing the tissue is placed in the chamber of the PDS-1000/He. The air in the chamber is then evacuated to a vacuum of 28-29 inches Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock
tube reaches 1080-1100 psi. The tissue is placed approximately 8 cm from the stopping screen and the callus is bombarded two times. Two to four plates of tissue are bombarded in this way with the DNA-coated gold particles. Following bombardment, the callus tissue is transferred to CM media without supplemental sorbitol or mannitol.

Within 3-5 days after bombardment the callus tissue is transferred to SM media (CM medium containing 50 mg/l hygromycin). To accomplish this, callus tissue is transferred from plates to sterile 50 ml conical tubes and weighed. Molten top-agar at 40°C is added using 2.5 ml of top agar/100 mg of callus. Callus clumps are broken into fragments of less than 2 mm diameter by repeated dispensing through a 10 ml pipet. Three ml aliquots of the callus suspension are plated onto fresh SM media and the plates are incubated in the dark for 4 weeks at 27-28°C. After 4 weeks, transgenic callus events are identified, transferred to fresh SM plates and grown for an additional 2 weeks in the dark at 27-28°C.

Growing callus is transferred to RM1 media (MS salts, Nitsch and Nitsch vitamins, 2% sucrose, 3% sorbitol, 0.4% gelrite +50 ppm hyg B) for 2 weeks in the dark at 25°C. After 2 weeks the callus is transferred to RM2 media (MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 0.4% gelrite + 50 ppm hyg B) and placed under cool white light (-40 µEm²s⁻¹) with a 12 hr photoperiod at 25°C and 30-40% humidity. After 2-4 weeks in the light, callus begin to organize, and form shoots. Shoots are removed from surrounding callus/media and gently transferred to RM3 media (1/2 x MS salts, Nitsch and Nitsch vitamins, 1% sucrose + 50 ppm hygromycin B) in phytatrails (Sigma Chemical Co., St. Louis, MO) and incubation is contiNRd using the same conditions as described in the previous step.

Plants are transferred from RM3 to 4" pots containing Metro mix 350 after 2-3 weeks, when sufficient root and shoot growth have occurred. The seed obtained from the transgenic plants is examined for genetic complementation of the NR mutation with the wild-type genomic DNA containing the NR gene.

**Example 9. Variants of NR Sequences**

A. **Variant Nucleotide Sequences of NR That Do Not Alter the Encoded Amino Acid Sequence**

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

B. Variant Amino Acid Sequences of NR Polypeptides

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

C. Additional Variant Amino Acid Sequences of NR Polypeptides

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

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

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

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Strongly Similar and Optimal Substitution</th>
<th>Rank of Order to Change</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>L, V</td>
<td>1</td>
<td>50:50 substitution</td>
</tr>
<tr>
<td>L</td>
<td>I, V</td>
<td>2</td>
<td>50:50 substitution</td>
</tr>
<tr>
<td>V</td>
<td>I, L</td>
<td>3</td>
<td>50:50 substitution</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>E</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>D</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>Y</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>W</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>T</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>S</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>R</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>K</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Q</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>N</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Y</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>L</td>
<td>17</td>
<td>First methionine cannot change</td>
</tr>
<tr>
<td>H</td>
<td>Na</td>
<td>No good substitutes</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Na</td>
<td>No good substitutes</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>Na</td>
<td>No good substitutes</td>
<td></td>
</tr>
</tbody>
</table>

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

The variant amino acid sequences are written as output. Perl script is used to calculate the percent identities. Using this procedure, variants of the NR polypeptides are generating having about 80%, 85%, 90% and 95% amino acid identity to the starting ORF nucleotide sequence of SEQ ID NO: 1, 2, 3, 4, 5 or 6.

**Example 10. Transgenic Maize Plants**

T₀ transgenic maize plants containing the NR construct under the control of a promoter were generated. To improve nitrate assimilation, PPNR and PYNR driven by constitutive promoter (ZM-UBI) and tissue-specific promoter (ZM-NR) (4 constructs) were transformed in transgenic maize plants via Agrobacteria.

These plants were grown in greenhouse conditions, under the FASTCORN system, as detailed in US Patent Application Publication Number 2003/0221212, US Patent Application Number 10/367,417.

Each of the plants was analyzed for measurable alteration in one or more of the following characteristics in the following manner:

Ti progeny derived from cross fertilization each T₀ plant containing a single copy of each NR construct that were found to segregate 1:1 for the transgenic event were analyzed for improved growth rate in low (1 mM) KNO₃. Growth was monitored up to anthesis when cumulative plant growth, growth rate and ear weight were determined for transgene positive, transgene null, and non-transformed controls events. The distribution of the phenotype of individual plants was compared to the distribution of a control set and to the distribution of all the remaining treatments. Transgenic means were compared to the grand mean, the block mean in which the transgene resides and the corresponding transgenic null mean and tested for statistical significance using a Students’ t-test. Variances for each set were calculated and used as the denominator of the Student's t-test after
adjustment for the number of observations in each mean compared to: Student's t
\[ t = \frac{(\text{transgene mean} - \text{transgenic null mean})}{\text{Sqr(variance} \times (1/\text{number of observations of transgene mean} + 1/\text{number of observations of transgenic null mean}))}. \]

The probability of obtaining the calculated Student's t-test at random was calculated based on the magnitude of the Student's t-test and the number of degrees of freedom associated with the variance. A probability of 0.1 (1 chance in 10 of obtaining the result at random) or lower was used to indicate a significant response to KNO3 fertility.

Example 11. Transgenic event analysis from field plots

Transgenic events are evaluated in field plots where yield is limited by reducing fertilizer application by 30% or more. Transgenic maize will be grown hydroponically. Improvements in yield, yield components, or other agronomic traits between transgenic and non-transgenic plants in these reduced nitrogen fertility plots are used to assess improvements in nitrogen utilization contributed by expression of transgenic events.

The rate of nitrate removal from hydroponics medium, the nitrate, and total nitrogen level in the plant may be determined using routine techniques. Nitrate levels in the growth medium will be monitored and compared to the nitrate levels in the growth medium of transgenic nulls. The rate of nitrate loss from the medium is an indication of nitrate utilization efficiency. Plant samples will be dried, ground and nitrate extracted and quantified. Total N will be determined in the tissue by micro-Kjeldahl. (Yasuhura and Nokihara (2001) "High-Throughput Analysis of Total Nitrogen Content that Replaces Classic Kjeldahl Method" J Agric Food Chem 49:4581-4583). Comparisons are made in plots supplemented with recommended nitrogen fertility rates. Effective transgenic events are those that achieve similar yields in the nitrogen-limited and normal nitrogen experiments.

Example 12. Maize backcross analysis

Segregating $T_4$ backcrosses to Gaspe-3 were grown in nutrient solutions containing 1 or 2mM KNO$_3$ as the sole nitrogen source till anthesis. Leaf color (SPAD), stem diameter, vegetative and ear dry weight were determined and compared to the corresponding segregating null plants. There were 9 replicates of all treatments. Results for the plants containing the PpNR transgene showed
statistically significant (at 0.001 level) improvement in ear dry weight (at both 1 and 2 mM KNO₃ concentrations) in comparison to non-transgenic control plants. At 2mM KNO3, there were also statistically significant improvements noted in SPAD, ear dry weight and total dry weight, as compared to non-transgenic control plants. This demonstrates the gene is stable after 5 cycles of backcrossing.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

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

1. An isolated nitrate reductase (NR) polynucleotide comprising a member
selected from the group consisting of:

   (a) a polynucleotide that encodes the polypeptide of SEQ ID NO: 7, 8, 9,
       10, 11 or 12;

   (b) a polynucleotide comprising the sequence set forth in SEQ ID NO: 1,
       2, 3, 4, 5 or 6; and

   (c) a polynucleotide comprising at least 30 nucleotides in length which
       hybridizes under stringent conditions to a polynucleotide of (a) or (b),
       wherein the conditions include hybridization in 40 to 45% formamide,
       1 M NaCl, 1% SDS at 37°C and a wash in 0.5 X to 1 X SSC at 55 to
       60°C; and

   (d) a polynucleotide having at least 70% sequence identity to SEQ ID
       NO: 1, 2, 3, 4, 5 or 6, wherein the % sequence identity is based on
       the entire encoding region and is determined by BLAST 2.0 under
       default parameters wherein the polynucleotide encodes a
       polypeptide having nitrate reductase (NR) activity;; and

   (e) an isolated polynucleotide degenerate from any of (a) to (e) as a
       result of the genetic code;

   f) a polynucleotide complimentary to a polynucleotide of any one of (a)
       to (e).

2. An isolated polynucleotide according to claim 1 that encodes a NR
polypeptide that confers increased yield or nitrogen utilization efficiency
under lower fertility.

3. A vector comprising at least one polynucleotide of claim 1.

4. An expression cassette comprising at least one polynucleotide of claim 1
operably linked to a promoter, wherein the polynucleotide is in sense
orientation.

5. A host cell into which is introduced at least one expression cassette of
claim 4.

6. The host cell of claim 5 that is a plant cell.

7. A transgenic plant comprising at least one expression cassette of claim 4.
8. The transgenic plant of claim 7, wherein the plant is selected from the
group consisting of: corn, soybean, sunflower, sorghum, canola, wheat,
alalfa, cotton, rice, barley and millet.

9. A seed from the transgenic plant of claim 7.

10. The seed of claim 9, wherein the seed is corn, soybean, sunflower,
sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet.

11. An isolated polypeptide selected from the group consisting of:
   a) an isolated polypeptide comprising any one of SEQ ID NOS: 7, 8, 9,
      10, 11 or 12 said polypeptide having NR activity;
   b) a polypeptide that is at least 70% identical to the amino acid
      sequence of any of SEQ ID NOS: 7, 8, 9, 10, 11 or 12 said
      polypeptide having NR activity;
   c) a polypeptide that is encoded by a nucleic acid molecule comprising
      a nucleotide sequence that is at least 70% identical to any one of
      SEQ ID NOS: 1, 2, 3, 4, 5 or 6 or a complement thereof, said
      polypeptide having NR activity;
   d) a polypeptide that is encoded by a nucleic acid molecule that
      hybridizes with a nucleic acid probe consisting of the nucleotide
      sequence of any of SEQ ID NOS: 1, 2 or 3, or a complement thereof
      following at least one wash in 0.2X SSC at 55°C for 20 minutes, said
      polypeptide having NR activity;
   e) a fragment comprising at least 200 consecutive amino acids of any
      of SEQ ID NOS: 7, 8, 9, 10, 11, or 12, said polypeptide having NR
      activity.

12. A recombinant expression cassette comprising a polynucleotide operably
    linked to a promoter, wherein the polynucleotide encodes the polypeptide of
    claim 11.

13. A transformed host cell comprising the isolated polypeptide of claim 11.

14. The host cell of claim 13, wherein the host cell is a transformed plant cell.

15. The plant cell of claim 14, wherein the plant cell is selected from the group
    consisting of sorghum, maize, rice, wheat, soybean, sunflower, canola,
alalfa, barley and millet.

16. A transformed plant regenerated from the plant cell of claim 14.
17. The plant of claim 16, wherein the plant is sorghum, maize, rice, wheat, soybean, sunflower, canola, alfalfa, barley or millet.

18. A transformed seed of the plant of claim 16.

19. An isolated polypeptide encoded by the polynucleotide of SEQ ID NO: 1, 2, 3, 4, 5 or 6.

20. The isolated polypeptide of claim 11 wherein the expression of NR in a plant results in an increased yield in the plant as compared to a control plant, wherein the control plant that does not contain the polynucleotide encoding the NR.

21. A method of modulating the level of nitrate reductase (NR) protein in a plant cell, comprising:
   (a) transforming a plant cell with a NR polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense orientation; and
   (b) expressing the polynucleotide for a time sufficient to modulate the NR protein in the plant cell.

22. The method of claim 21, wherein the plant is corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet.

23. The method of claim 21, wherein NR protein is increased.

24. The method of claim 21, wherein NR protein is decreased.

25. A method of modulating the level of nitrate reductase (NR) protein in a plant, comprising:
   (a) stably transforming a plant cell with a NR polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense orientation; and
   (b) regenerating the transformed plant cell into a transformed plant that expresses the NR polynucleotide in an amount sufficient to modulate the level of NR protein in the plant.

26. The method of claim 25, wherein the plant is corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet.

27. The method of claim 25, wherein NR protein is increased as compared to a control plant, wherein the control plant that does not contain the polynucleotide encoding the NR.
28. The method of claim 25, wherein NR protein is decreased as compared to a control plant, wherein the control plant that does not contain the polynucleotide encoding the NR.

29. A method for increasing yield in a plant, said method comprising the steps of:

(a) introducing into plant cells a construct comprising a polynucleotide encoding a nitrate reductase (NR), wherein said NR polynucleotide is operably linked to a promoter functional in plant cells to yield transformed plant cells, and wherein the NR encoding the NR protein is selected from the group consisting of:

(1) a polynucleotide that encodes the polypeptide of SEQ ID NO: 7, 8, 9, 10, 11 or 12;

(2) a polynucleotide comprising the sequence set forth in the encoding region of SEQ ID NO: 7, 8, 9, 10, 11 or 12; and

(3) a polynucleotide comprising at least 30 nucleotides in length which hybridizes under moderate stringency conditions to a polynucleotide of (a) or (b), wherein the conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C. and a wash in 0.5 X to 1 X SSC at 55 to 60°C; and

(4) a polynucleotide having at least 70% sequence identity to SEQ ID NO: 1, 2, 3, 4, 5 or 6, wherein the % sequence identity is based on the entire encoding region and is determined by BLAST 2.0 under default parameters; and

(5) an isolated polynucleotide degenerate from any of (1) to (4) as a result of the genetic code;

(6) a polynucleotide complimentary to a polynucleotide of any one of (1) to (5);

(b) regenerating a transgenic plant from said transformed plant cells, wherein said NR is expressed in the cells of said transgenic plant at levels sufficient to maintain or increase yield in said transgenic plant.

30. The method of claim 29, wherein increased yield comprises enhanced root growth, increased seed size, increased seed weight, the plant has seed with increased embryo size, increased leaf size, increased seedling vigor, enhanced silk emergence, increased ear size or chlorophyll content.
31. The method of claim 29, wherein the plant is grown under limited nitrogen fertility.

32. The method of claim 31, wherein the nitrogen utilization efficiency of the plant is increased.

33. The method of claim 31, wherein NR activity of the NR polynucleotide is increased compared to the activity of a NR endogenous to the plant.

34. The method of claim 29, wherein the expression of the NR polynucleotide is driven by a phosphoenopyruvate decarboxylase (PEPC) promoter.

35. The method of claim 29, wherein said polynucleotide encoding the NR is constitutively expressed.

36. The method of claim 29, wherein said polynucleotide encoding the NR is inducibly expressed.

37. The method of claim 29, wherein said polynucleotide encoding the NR is expressed in a cell-specific, tissue-specific, or organ-specific manner.

38. The method of claim 29, wherein the plant is a dicotyledonous plant.

39. The method of claim 29, wherein the plant is a monocotyledonous plant.

40. The method of claim 29, wherein the yield of the plant is compared to a control plant, wherein the control plant does not contain the polynucleotide encoding the NR.
1
Pp-NR (1) MEAAGSLERLEKGVQWPDVKVPGRSSLKTPIATPESGLRGSGLYT
Py-NR (1) MEAAGSLERLEKGVQWPDVKVPGRSSLKTPIATPESGLRGSGLYT
Consensus (1) MEAAGSLERLEKGVQWPDVKVPGRSSLKTPIATPESGLRGSGLYT
51
Pp-NR (51) ARSQH--AAGANDYMAANGVSSSTASGSLFAPSGSGSGSRRGVTELNL
Py-NR (51) TAAAGGGAGAAAGGMAANGVSSSTASGSLFAPSGSGSGSRRGVTELNL
Consensus (51) RA AAGANGVSSSTASGSLFAPSGSGSGSRRGVTELNL
101
Pp-NR (99) DALNKLALKSMILKDKVHAEVDDDRDVKTDPNWRHPALRILRTQKHF
Py-NR (101) DALNKLALKSMILKDKVHAEVDDDRDVKTDPNWRHPALRILRTQKHF
Consensus (101) DALNKLALKSMILKDKVHAEVDDDRDVKTDPNWRHPALRILRTQKHF
151
Pp-NR (149) NEAPLSMLDGQLFITPSLHFVNRHGAAPQLSDFDRLEVTGLVDPLTL
Py-NR (151) NEAPLSMLDGQLFITPSLHFVNRHGAAPQLSDFDRLEVTGLVDPLTL
Consensus (151) NEAPLSMLDGQLFITPSLHFVNRHGAAPQLSDFDRLEVTGLVDPLTL
201
Pp-NR (199) LSNMADILAMPSVTPVTCLGNNRKEQNMRTQTVGFSWAGASTNCFW
Py-NR (201) LSNMADILAMPSVTPVTCLGNNRKEQNMRTQTVGFSWAGASTNCFW
Consensus (201) LSNMADILAMPSVTPVTCLGNNRKEQNMRTQTVGFSWAGASTNCFW
251
Pp-NR (249) GVRVLDVLKQAGMDKARHCVFVGCDNLPGGKYTSQLDATAMQDFGEV
Py-NR (251) GVRVLDVLKQAGMDKARHCVFVGCDNLPGGKYTSQLDATAMQDFGEV
Consensus (251) GVRVLDVLKQAGMDKARHCVFVGCDNLPGGKYTSQLDATAMQDFGEV
301
Pp-NR (299) MLAYEQNGIRLTDFGHAPLRVLPWGIQRVVRMVWPARGTLSVSTESESEQEHYH
Py-NR (301) MLAYEQNGIRLTDFGHAPLRVLPWGIQRVVRMVWPARGTLSVSTESESEQEHYH
Consensus (301) MLAYEQNGIRLTDFGHAPLRVLPWGIQRVVRMVWPARGTLSVSTESESEQEHYH
351
Pp-NR (349) FFDNRLIPHPVDMLAEKSEGWWYKRELYLQNBQLNISAISSPANGREMSLS
Py-NR (351) FFDNRLIPHPVDMLAEKSEGWWYKRELYLQNBQLNISAISSPANGREMSLS
Consensus (351) FFDNRLIPHPVDMLAEKSEGWWYKRELYLQNBQLNISAISSPANGREMSLS
401
Pp-NR (399) GAGYVTILGQAYGSGGRKVRTRVESVDGKTWLALTDHPEEDRHSHAPSY
Py-NR (401) GAGYVTILGQAYGSGGRKVRTRVESVDGKTWLALTDHPEEDRHSHAPSY
Consensus (401) GAGYVTILGQAYGSGGRKVRTRVESVDGKTWLALTDHPEEDRHSHAPSY
451
Pp-NR (449) GRYWCWFWEYTDIFKALNAATSSGELLVRADEGNNTQPAKTIWNLMLG
Py-NR (451) GRYWCWFWEYTDIFKALNAATSSGELLVRADEGNNTQPAKTIWNLMLG
Consensus (451) GRYWCWFWEYTDIFKALNAATSSGELLVRADEGNNTQPAKTIWNLMLG
501
Pp-NR (499) MGNCNFYVRTVPAPKQSSGFLELFHPTVGAPEAGGWMPPQESVVAAA
Py-NR (501) MGNCNFYVRTVPAPKQSSGFLELFHPTVGAPEAGGWMPPQESVVAAA
Consensus (501) MGNCNFYVRTVPAPKQSSGFLELFHPTVGAPEAGGWMPPQESVVAAA
551
Pp-NR (549) AAVSFLITRKASAPQINMKDQDSKITEEMEVAKHTEDSVWVHNK
Py-NR (551) AAVSFLITRKASAPQINMKDQDSKITEEMEVAKHTEDSVWVHNK
Consensus (551) AAVSFLITRKASAPQINMKDQDSKITEEMEVAKHTEDSVWVHNK
601
Pp-NR (599) YDCTPFKHKDPQGGAIVMNAGACTEEDFDAOIHTSRKMLDDYYIGELA
Py-NR (601) YDCTPFKHKDPQGGAIVMNAGACTEEDFDAOIHTSRKMLDDYYIGELA
Consensus (601) YDCTPFKHKDPQGGAIVMNAGACTEEDFDAOIHTSRKMLDDYYIGELA

FIGURE 1A
FIGURE 1B
<table>
<thead>
<tr>
<th>NR</th>
<th>Species</th>
<th>Form</th>
<th>Km</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPNR</td>
<td><em>Porphyra perforata</em></td>
<td>NADH-specific</td>
<td>30-40 uM</td>
<td>6.5&gt;7.5&gt;5.5</td>
</tr>
<tr>
<td>YNR1</td>
<td><em>Pichia angusta</em></td>
<td>NAD(P)H bispecific</td>
<td>200 uM</td>
<td>6.5&gt;7.5&gt;5.5</td>
</tr>
<tr>
<td>ZM-NR1</td>
<td>Maize</td>
<td>NADH-specific</td>
<td>300 uM</td>
<td>6.5&gt;7.5&gt;5.5</td>
</tr>
</tbody>
</table>

**FIGURE 4**
**Ubi:** *Porphyra perforata* NR

**Ear Wt**

![Graph showing Ear Wt for different samples](image1)

**Plant Wt**

![Graph showing Plant Wt for different samples](image2)

**FIGURE 5**
### INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

<table>
<thead>
<tr>
<th>Inv.</th>
<th>C12N9/02</th>
<th>C12N15/82</th>
<th>A01H5/00</th>
<th>C12N5/10</th>
</tr>
</thead>
</table>

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

- C12N  AOIH

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

- Electronic database consulted during the international search (name of database and, where practical, search terms used)
  - EPO-Internal, BIOSIS, WPI Data, PAJ, Sequence Search

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

- Special categories of cited documents:
  - 'A' document defining the general state of the art which is not considered to be of particular relevance
  - 'E' earlier document but published on or after the international filing date
  - 'I' 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

- 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

- 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

- 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**

1 October 2008

**Date of mailing of the international search report**

10/10/2008

**Name and mailing address of the ISA**

- European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax (+31-70) 340-3016

**Authorized officer**

- Holtorf, Sönke

Form PCT/SA/21 B (second sheet) (April 2005)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>DATABASE EMBL [Onl] [22 September 2006 (2006-09-22)] EST00767_0906 Sporophyte cDNA Library for Porphyra haitanensis cDNA S', mRNA sequence. XP002495778 retrieved from EBI accession no. EMBL: EG018284</td>
<td>1-3, 11, 19,20</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-3, 11, 19,20</td>
</tr>
</tbody>
</table>

Form PCT/ISA/21.0 (continuation of second sheet) (April 2005)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WO 00/62601 A (UNIV NORTHEASTERN [US]; CHENEY DONALD P [US]) 26 October 2000 (2000-10-26) example 1</td>
<td>13</td>
</tr>
<tr>
<td>A</td>
<td>NEJID'T ALI ET AL: &quot;Increased protein content in transgenic Arabidopsis thaliana over-expressing nitrate reductase activity&quot; PLANT SCIENCE (SHANNON), vol. 130, no. 1, 5 December 1997 (1997-12-05), pages 41-49, XP002495775 ISSN: 0168-9452 cited in the application the whole document</td>
<td></td>
</tr>
</tbody>
</table>

-/--
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>US 2005044585 A1</td>
<td>24-02-2005</td>
<td>NONE</td>
</tr>
<tr>
<td>WO 0062601 A</td>
<td>26-10-2000</td>
<td>NONE</td>
</tr>
</tbody>
</table>