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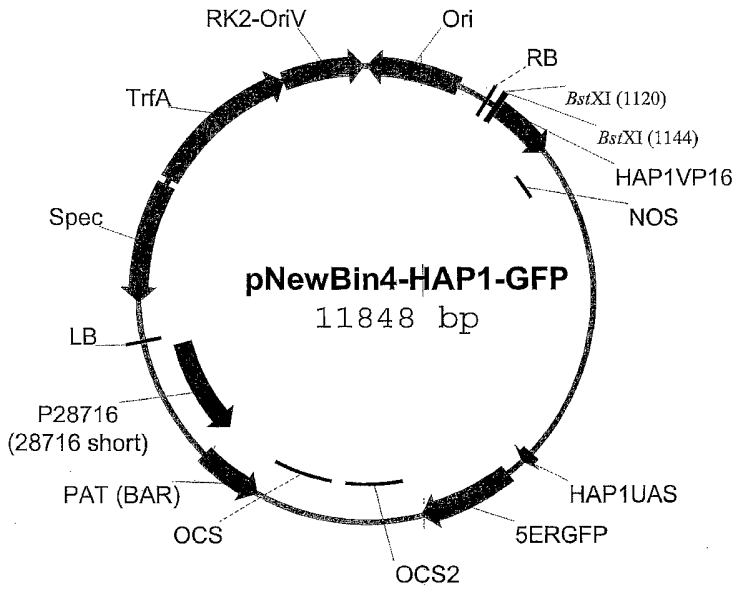
(54) **Title:** PROMOTER, PROMOTER CONTROL ELEMENTS, AND COMBINATIONS, AND USES THEREOF



(57) **Abstract:** The present invention is directed to nitrogen responsive promoter sequences and promoter control elements, polynucleotide constructs comprising the nitrogen responsive promoters and control elements and methods of identifying the nitrogen responsive promoters, control elements, or fragments thereof. The invention further relates to the use of the present nitrogen responsive promoters or promoter control elements to modulate transcript levels.

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PROMOTER, PROMOTER CONTROL ELEMENTS, AND COMBINATIONS, AND USES THEREOF

This Nonprovisional applications claims priority under 35 U.S.C. § 199 (e) on U.S. Provisional Application No: 60/612,603 filed on September 22, 2004, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to nitrogen responsive promoters and promoter control elements that are useful for modulating transcription of a desired polynucleotide. Such nitrogen responsive promoters and promoter control elements can be included in a polynucleotide construct, expression cassettes, vectors or inserted into the chromosome or used as an exogenous element to modulate *in vivo* and *in vitro* transcription of a polynucleotide. The invention also includes host cells and organisms, including plant cells and regenerated plants therefrom, with desired traits or characteristics obtained using polynucleotides comprising the nitrogen responsive promoters and promoter control elements of the present invention.

BACKGROUND OF THE INVENTION

Plants have a number of means to cope with nutrient deficiencies, such as poor nitrogen availability. They constantly sense nitrogen availability in the soil and respond accordingly by modulating gene expression. Although more is being discovered about nitrogen and the components involved in regulating its uptake and use, much is still unknown about many of these complex interactions. For this reason, it is interesting when a gene of known or unknown function is shown to have a nitrogen response, as it opens up new possibilities and insights into nitrogen use and nitrogen use efficiency in a competitive environment (i.e. low and/or high nitrogen).

Nitrogen regulated gene expression is an important aspect of a plant's response to changes in nitrogen availability. Nitrate acts as a signal to initiate a number of responses that serve to reprogram plant metabolism, physiology and development (Redinbaugh and Campbell, 1991; Forde, 2002). Nitrogen-inducible gene expression has been characterized for a number of genes in some detail. These include nitrate reductase, nitrite reductase, 6-

phosphoglucante dehydrogenase, and nitrate and ammonium transporters (Redinbaugh and Campbell, 1991; Huber et al., 1994; Hwang et al., 1997; Redinbaugh and Campbell, 1998; Gazzarrini et al., 1999; Glass et al., 2002; Okamoto et al., 2003). Investigations into the *cis* acting control elements and DNA binding factors involved in nitrate regulated gene expression have focused on the nitrate reductase gene from tobacco and spinach and have identified several putative regulatory elements (Rastogi et al., 1993; Lin et al., 1994; Hwang et al., 1997). Transcriptional profiling of nitrate-regulated gene expression has extended knowledge of genes and processes regulated by nitrate availability and also identified a number of genes with distinct spatial and temporal patterns of expression (Ceres unpublished; Wang et al., 2000; Wang et al., 2003).

Nitrogen is most frequently the rate limiting mineral nutrient for crop production. Plants have evolved complex signaling and regulatory mechanisms to enable rapid physiological and metabolic response to changes in the supply of inorganic nitrogen in the soil. Part of this regulation is achieved through transcriptional regulation of gene expression. This is an important mechanism for allowing plants to adjust nitrogen uptake, reduction and transport in response to changing environmental conditions. Inefficiencies in nitrogen use efficiency may be overcome through the use of nitrogen regulated gene expression to modify the response of rate limiting enzymes and metabolic pathways to changes in nitrogen availability.

The ability to modify plant gene expression and ultimately the phenotype of a plant using nitrogen-inducible promoters can be a powerful method for deploying nitrogen transgene product concepts in the field. We have identified promoters that are induced in nitrogen starved *Arabidopsis* plants in response to nitrate provision as well as promoters that are induced by decreases in nitrate concentration.

SUMMARY OF THE INVENTION

The present invention is directed to isolated polynucleotide sequences that comprise nitrogen responsive promoters and promoter control elements from plants, especially *Arabidopsis thaliana, Glycine max, Oryza sativa,* and *Zea mays* used alone or in combination with other promoters and promoter control elements functional in plants.

It is an object of the present invention to provide isolated polynucleotides that are nitrogen responsive promoter, promoter control element and motif sequences. These promoter sequences comprise, for example,

(1) a polynucleotide having a nucleotide sequence according to any one of SEQ ID NOs: 1-17 or a functional fragment thereof;

(2) a polynucleotide having a nucleotide sequence having at least 80% sequence identity to sequences shown in any one of SEQ ID NOs: 1-17 or a functional fragment thereof; and

(3) a polynucleotide having a nucleotide sequence which hybridizes to those shown in any one of SEQ ID NOs: 1-17 under a condition establishing at least a Tm-20°C.

Nitrogen responsive promoter and promoter control element sequences of the present invention are capable of modulating preferential transcription under varying nitrogen conditions.

In another embodiment, the present nitrogen responsive promoters and promoter control elements are capable of serving as or fulfilling the function of a core nitrogen responsive promoter, a nitrogen responsive initiator site, a nitrogen responsive transcription binding site, a nitrogen responsive enhancer, a nitrogen responsive inverted repeat, a nitrogen responsive locus control region or a nitrogen responsive scaffold/matrix attachment region.

It is yet another object of the present invention to provide a polynucleotide that includes at least a first and a second promoter control element. The first promoter control element is a nitrogen responsive promoter control element sequence as discussed above and the second promoter control element is heterologous to the first control element. Moreover, the first and second control elements are operatively linked. Such promoters may modulate transcript levels preferentially in a tissue or under particular conditions in addition to responding to nitrogen conditions.

In another embodiment, the present isolated polynucleotide comprises a nitrogen responsive promoter or promoter control element as described above, wherein the promoter or promoter control element is operatively linked to a polynucleotide to be transcribed.

In another embodiment of the present vector, the nitrogen responsive promoter or promoter control element of the instant invention is operatively linked to a heterologous polynucleotide that is a regulatory sequence.

It is another object of the present invention to provide a host cell comprising an isolated polynucleotide or vector as described above or fragment thereof. Host cells include bacterial, yeast, insect, mammalian, and plant. The host cell can comprise a nitrogen responsive promoter or promoter control element exogenous to the genome. Such a nitrogen responsive promoter can modulate transcription in cis- and/or in trans-.

In yet another embodiment, the present host cell is a plant cell capable of regenerating into a plant.

It is yet another embodiment of the present invention to provide a plant comprising an isolated polynucleotide or vector described above.

It is a further embodiment of the present invention to provide a plant comprising a nucleic acid encoding a nitrogen responsive promoter operatively linked to a coding sequence so that the coding sequence is ectopically overexpressed in the plant in response to sub-optimal, normal or abnormal nitrogen conditions, and the plant exhibits at least one of the following characteristics: improved performance, improved nitrogen responsiveness, faster rate of growth, greater fresh or dry weight at maturation, greater fruit or seed yield, higher tolerance to sub-optimal, normal or abnormal nitrogen conditions, greater germination rate under sub-optimal, normal or abnormal nitrogen conditions, reduced nitrogen needs or greater tolerance to excess nitrogen compred to a progenitor plant.

It is another object of the present invention to provide a method of modulating transcription in a sample that contains either a cell-free transcription system or a host cell. This method comprises providing a polynucleotide or vector according to the present invention as described above, and contacting the sample of the polynucleotide or vector with conditions that permit transcription.

In another embodiment of the present method, the polynucleotide or vector preferentially modulates nitrogen metabolism and utilization.

The present invention also provides a method of obtaining a plant enhanced in a product of a structural gene comprising growing a transformed plant resulting from transformation with a nitrogen responsive promoter or promoter control element selected from any one of SEQ ID NOs: 1-17 with or without at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom, wherein the enhanced product of the structural gene in the transformed plant results from transcription of a structural gene modulated by the introduced promoter or promoter control element of any one of SEQ ID

NOs: 1-17 with or without at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

It is a further embodiment of the invention to provide a method of reducing the amount and/or frequency of fertilizer application to crop plants by providing a plant with a nitrogen responsive promoter or promoter control element selected from SEQ ID Nos: 1-17 with or without at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom with improved characteristics over a progenitor plant.

Other and further objects of the present invention will be made clear or become apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1

Figure 1 is a schematic representation of the vector pNewBin4-HAP1-GFP. The definitions of the abbreviations used in the vector map are as follows:

Ori – the origin of replication used by an *E. coli* host

RB – sequence for the right border of the T-DNA from pMOG800

*Bst*XI – restriction enzyme cleavage site used for cloning

HAP1VP16 – coding sequence for a fusion protein of the HAP1 and VP16 activation domains

NOS – terminator region from the nopaline synthase gene

HAP1UAS – the upstream activating sequence for HAP1

5ERGFP – the green fluorescent protein gene that has been optimized for localization to the endoplasmic reticulum

OCS2 – the terminator sequence from the octopine synthase 2 gene

OCS – the terminator sequence from the octopine synthase gene

p28716 (a.k.a 28716 short) – promoter used to drive expression of the PAT (BAR) gene

PAT (BAR) – a marker gene conferring herbicide resistance

LB – sequence for the left border of the T-DNA from pMOG800

Spec – a marker gene conferring spectinomycin resistance

TrfA – transcription repression factor gene

RK2-OriV – origin of replication for *Agrobacterium*

FIGURE 2
Quantitative RT-PCR Data for Example 3

FIGURE 3
Quantitative RT-PCR Data for Example 4

FIGURE 4
Quantitative RT-PCR Data for Example 5

FIGURE 5
Quantitative RT-PCR Data for Example 8

FIGURE 6
Differential expression of selected genes in leaves from Example 9. A: Fibrillarin-2. B: Putative monodedydroascorbate reductase.

FIGURE 7
Nitrate Content in growth media experimental and control plants hydroponically cultivated from Example 9.

FIGURE 8
Differential Exapression of selected genes in roots and shoots from Example 9. A: Fibrillarin-2. B: Putative monodedydroascorbate reductase.

FIGURE 9
Differential expression in roots and shoots of T2 mature plants cultivated in hydroponic conditions from Example 9. A: Putative monodedydroascorbate reductase. B: Fibrillarin-2.

FIGURE 10
Schematic of a gene.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Abnormal Nitrogen Conditions: Plant species vary in their capacity to tolerate particular nitrogen conditions. Nitrogen-sensitive plant species, including many agronomically important species, can be injured by nitrogen conditions that are either low or high compared to the range of nitrogen needed for normal growth. At nitrogen conditions above or below the range needed for normal growth, most plant species will be damaged. Thus, "abnormal nitrogen conditions" can be defined as the nitrogen concentration at which a given plant species will be adversely affected as evidenced by symptoms such as decreased chlorophyll (for example, measured by chlorophyll a/b absorbance) decreased photosynthesis

(for example, measured by CO2 fixation, membrane damage (for example measured by electrolyte leakage) and chlorosis (for example, via visual inspection). Since plant species vary in their capacity to tolerate abnormal nitrogen conditions, the precise environmental conditions that cause nitrogen stress can not be generalized. However, nitrogen tolerant plants are characterized by their ability to retain their normal appearance or recover quickly from abnormal nitrogen conditions. Such nitrogen tolerant plants produce higher biomass and yield than plants that are not nitrogen tolerant. Differences in physical appearance, recovery and yield can be quantified and statistically analyzed using well known measurement and analysis methods.

Plant seeds vary considerably in their ability to germinate under abnormal nitrogen conditions. Generally, seeds of many plant species will not germinate at nitrogen concentration less than about 1 ppm or greater than about 2000 ppm. In addition, high concentrations of ammoniac nitrogen are also inhibitory to seed germination and can occur when ammonium based fertilizer is used (Brenner and Krogmeier (1989) PNAS 86:8185-8188).

Once seeds have imbibed water they become very susceptible to disease, water and chemical damage. Seeds that are tolerant to nitrogen stress during germination can survive for relatively long periods under which the nitrogen concentration is too high or too low to germinate. Since plant species vary in their capacity to tolerate abnormal nitrogen conditions during germination, the precise environmental conditions that cause nitrogen stress during germination can not be generalized. However, seeds and seedlings that are nitrogen tolerant during germination are characterized by their ability to remain viable or recover quickly from low or high nitrogen conditions. Such nitrogen tolerant plants germinate, become established, grow more quickly and ultimately produce more biomass and yield than plants that are not nitrogen tolerant. Differences in germination rate, appearance, recovery and yield can be quantified and statistically analyzed using well known measurement and analysis methods.

Chimeric: The term "chimeric" is used to describe polynucleotides or genes, as defined below, or constructs wherein at least two of the elements of the polynucleotide or gene or construct, such as the promoter and the polynucleotide to be transcribed and/or other regulatory sequences and/or filler sequences and/or complements thereof, are heterologous to each other.

Chimera: The term "chimera" refers to a cell or organism containing at least one chimeric polynucleotide, gene or construct.

Constitutive Promoter: Promoters referred to herein as "constitutive promoters" actively promote transcription under most, but not necessarily all, environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcript initiation region and the 1' or 2' promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes, such as the maize ubiquitin-1 promoter, known to those of skill.

Core Promoter: This is the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the RNA polymerase II machinery (for review see: Struhl, 1987, Cell 49: 295-297; Smale, 1994, In *Transcription: Mechanisms and Regulation* (eds R.C. Conaway and J.W. Conaway), pp 63-81/ Raven Press, Ltd., New York; Smale, 1997, Biochim. Biophys. Acta 1351: 73-88; Smale et al., 1998, Cold Spring Harb. Symp. Quant. Biol. 58: 21-31; Smale, 2001, Genes & Dev. 15: 2503-2508; Weis and Reinberg, 1992, FASEB J. 6: 3300-3309; Burke et al., 1998, Cold Spring Harb. Symp. Quant. Biol 63: 75-82). There are several sequence motifs, including the TATA box, initiator (Inr), TFIIB recognition element (BRE) and downstream core promoter element (DPE), that are commonly found in core promoters, however not all of these elements occur in all promoters and there are no universal core promoter elements (Butler and Kadonaga, 2002, Genes & Dev. 16: 2583-2592).

Domain: Domains are fingerprints or signatures that can be used to characterize protein families and/or parts of proteins. Such fingerprints or signatures can comprise conserved (1) primary sequence, (2) secondary structure, and/or (3) three-dimensional conformation. A similar analysis can be applied to polynucleotides. Generally, each domain has been associated with either a conserved primary sequence or a sequence motif. Generally these conserved primary sequence motifs have been correlated with specific *in vitro* and/or *in vivo* activities. A domain can be any length, including the entirety of the polynucleotide to be

transcribed. Examples of domains include, without limitation, AP2, helicase, homeobox, zinc finger, etc.

Endogenous: The term "endogenous," within the context of the current invention refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell or organisms regenerated from said cell. In the context of promoter, the term "endogenous coding region" or "endogenous cDNA" refers to the coding region that is naturally operatively linked to the promoter.

Enhancer/Suppressor: An "enhancer" is a DNA regulatory element that can increase the steady state level of a transcript, usually by increasing the rate of transcription initiation. Enhancers usually exert their effect regardless of the distance, upstream or downstream location, or orientation of the enhancer relative to the start site of transcription. In contrast, a "suppressor" is a corresponding DNA regulatory element that decreases the steady state level of a transcript, again usually by affecting the rate of transcription initiation. The essential activity of enhancer and suppressor elements is to bind a protein factor(s). Such binding can be assayed, for example, by methods described below. The binding is typically in a manner that influences the steady state level of a transcript in a cell or in an in vitro transcription extract.

Exogenous: As referred to within, "exogenous" is any polynucleotide, polypeptide or protein sequence, whether chimeric or not, that is introduced into the genome of a host cell or organism regenerated from said host cell by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation (of dicots - e.g. Salomon *et al.* EMBO J. 3:141 (1984); Herrera-Estrella *et al.* EMBO J. 2:987 (1983); of monocots, representative papers are those by Escudero *et al.*, Plant J. 10:355 (1996), Ishida *et al.*, Nature Biotechnology 14:745 (1996), May *et al.*, Bio/Technology 13:486 (1995)), biolistic methods (Armaleo *et al.*, Current Genetics 17:97 1990)), electroporation, *in planta* techniques, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T_0 for the primary transgenic plant and T_1 for the first generation. The term "exogenous" as used herein is also

intended to encompass inserting a naturally found element into a non-naturally found location.

Functional Equivalent: This phrase describes a polynucleotide of sufficient length to retain at least one activity of the nitrogen responsive promoter or promoter control element.

Gene: The term "gene," as used in the context of the current invention, encompasses all regulatory and coding sequence contiguously associated with a single hereditary unit with a genetic function (see Figure 10). Genes can include non-coding sequences that modulate the genetic function that include, but are not limited to, those that specify polyadenylation, transcriptional regulation, DNA conformation, chromatin conformation, extent and position of base methylation and binding sites of proteins that control all of these. Genes encoding proteins are comprised of "exons" (coding sequences), which may be interrupted by "introns" (non-coding sequences). In some instances complexes of a plurality of protein or nucleic acids or other molecules, or of any two of the above, may be required for a gene's function. On the other hand a gene's genetic function may require only RNA expression or protein production, or may only require binding of proteins and/or nucleic acids without associated expression. In certain cases, genes adjacent to one another may share sequence in such a way that one gene will overlap the other. A gene can be found within the genome of an organism, in an artificial chromosome, in a plasmid, in any other sort of vector, or as a separate isolated entity.

Heterologous sequences: "Heterologous sequences" are those that are not operatively linked or are not contiguous to each other in nature. For example, a promoter from corn is considered heterologous to an *Arabidopsis* coding region sequence. Also, a promoter from a gene encoding a growth factor from corn is considered heterologous to a sequence encoding the corn receptor for the growth factor. Regulatory element sequences, such as UTRs or 3' end termination sequences that do not originate in nature from the same gene as the coding sequence originates from, are considered heterologous to said coding sequence. Elements operatively linked in nature and contiguous to each other are not heterologous to each other.

Homologous: In the current invention, a "homologous" gene or polynucleotide or polypeptide refers to a gene or polynucleotide or polypeptide that shares sequence similarity with the gene or polynucleotide or polypeptide of interest. This similarity may be in only a fragment of the sequence and often represents a functional domain such as, examples including without limitation a DNA binding domain or a domain with tyrosine kinase activity. The functional activities of homologous polynucleotide are not necessarily the same.

Inducible Promoter: An "inducible promoter" in the context of the current invention refers to a promoter, the activity of which is influenced by certain conditions, such as light, temperature, chemical concentration, protein concentration, conditions in an organism, cell, or organelle, etc. A typical example of an inducible promoter, which can be utilized with the polynucleotides of the present invention, is PARSK1, the promoter from an *Arabidopsis* gene encoding a serine-threonine kinase enzyme, and which promoter is induced by dehydration, abscissic acid and sodium chloride (Wang and Goodman, Plant J. 8:37 (1995)). Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence or absence of a nutrient or other chemical compound or the presence of light.

Modulate Transcription Level: As used herein, the phrase "modulate transcription" describes the biological activity of a promoter sequence or promoter control element. Such modulation includes, without limitation, includes up- and down-regulation of initiation of transcription, rate of transcription, and/or transcription levels.

Motif: This phrase is used to describe a discrete sequence that is associated with a particular function. The sequence can be either nucleic acid or amino acid. It can also be either contiguous or capable of being aligned to certain positions that are invariant or conserved. For example, the motif GXGXXG is associated with nucleotide binding.

Mutant: In the current invention, "mutant" refers to a heritable change in nucleotide sequence at a specific location. Mutant genes of the current invention may or may not have an associated identifiable phenotype.

Normal Nitrogen Conditions: Plant species vary in their capacity to tolerate particular nitrogen conditions. Nitrogen-sensitive plant species, including many agronomically important species, can be injured by nitrogen conditions that are either low or high compared to the range of nitrogen needed for normal growth. At nitrogen conditions above or below the range needed for normal growth, most plant species will be damaged. Thus, "normal nitrogen conditions" can be defined as the nitrogen concentration at which a given plant species will grow without damage. Since plant species vary in their capacity to tolerate nitrogen conditions, the precise environmental conditions that provide normal nitrogen conditions can not be generalized. However, the normal growth exhibited by nitrogen intolerant plants is characterized by the inability to retain a normal appearance or to recover quickly from abnormal nitrogen conditions. Such nitrogen intolerant plants produce lower biomass and yield less than plants that are nitrogen tolerant. Differences in physical appearance, recovery and yield can be quantified and statistically analyzed using well known measurement and analysis methods.

Plant seeds vary considerably in their ability to germinate under nitrogen conditions. Generally, seeds of many plant species will not germinate at nitrogen concentration less than about 1 ppm or greater than about 2000 ppm. In addition, high concentrations of ammoniac nitrogen are also inhibitory to seed germination and can occur when ammonium based fertilizer is used (Brenner and Krogmeier (1989) PNAS 86:8185-8188).

Once seeds have imbibed water they become very susceptible to disease, water and chemical damage. Seeds that are intolerant to nitrogen stress during germination can only survive for relatively short periods under which the nitrogen concentration is too high or too low to germinate. Since plant species vary in their capacity to tolerate nitrogen conditions during germination, the precise environmental conditions that cause nitrogen stress during germination can not be generalized. However, the normal growth associated with nitrogen intolerant plants is characterized by the inability to remain viable or recover quickly from low or high nitrogen conditions. Such nitrogen intolerant plants do not germinate, do not become established, do grow more slowly, if at all, and ultimately die faster or produce less biomass and yield than plants that are nitrogen tolerant. Differences in germination rate, appearance, recovery and yield can be quantified and statistically analyzed using well known measurement and analysis methods.

Operable Linkage: An "operable linkage" is a linkage in which a promoter sequence or promoter control element is connected to a polynucleotide sequence (or sequences) in such a way as to place transcription of the polynucleotide sequence under the influence or control of the promoter or promoter control element. Two DNA sequences (such as a polynucleotide to be transcribed and a promoter sequence linked to the 5' end of the polynucleotide to be transcribed) are said to be operatively linked if induction of promoter function results in the transcription of mRNA encoding the polynucleotide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter sequence to direct the expression of the protein, antisense RNA or ribozyme, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter sequence would be operatively linked to a polynucleotide sequence if the promoter was capable of effecting transcription of that polynucleotide sequence.

Optimal Nitrogen Conditions: The optimal nitrogen concentration range is known for many crop plants. For example, and without limitation to the crops disclosed, the following nitrate nitrogen concentrations in the soil at a depth of 6 inches are considered optimal for the following crop plants: maize, 20-40 ppm; wheat, 5-20 ppm; cotton, 20-60 ppm; tomato, 35-50 ppm.

Optional Promoter Fragments: The phrase "optional promoter fragments" is used to refer to any sub-sequence of the promoter that is not required for driving transcription of an operationally linked coding region. These fragments comprise the 5' UTR and any exon(s) of the endogenous coding region. The optional promoter fragments may also comprise any exon(s) and the 3' or 5' UTR of the gene residing upstream of the promoter (that is, 5' to the promoter). Optional promoter fragments also include any intervening sequences that are introns or sequence that occurs between exons or an exon and the UTR.

Orthologous: "Orthologous" is a term used herein to describe a relationship between two or more polynucleotides or proteins. Two polynucleotides or proteins are "orthologous" to one another if they serve a similar function in different organisms. In general, orthologous polynucleotides or proteins will have similar catalytic functions (when they encode enzymes)

or will serve similar structural functions (when they encode proteins or RNA that form part of the ultrastructure of a cell).

Percentage of sequence identity: "Percentage of sequence identity," as used herein, is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the polynucleotide or amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) 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. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (USA) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. The preceding references are hereby incorporated by reference in their entirety. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used.

Plant Promoter: A "plant promoter" is a promoter capable of initiating transcription in plant cells and can modulate transcription of a polynucleotide. Such promoters need not be of plant origin. For example, promoters derived from plant viruses, such as the CaMV35S promoter or from *Agrobacterium tumefaciens* such as the T-DNA promoters, can be plant promoters. A typical example of a plant promoter of plant origin is the maize ubiquitin-1 (ubi-1) promoter.

Plant Tissue: The term "plant tissue" includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, cotyledons, epicotyl,

hypocotyl, leaves, pollen, seeds, tumor tissue and various forms of cells in culture such as single cells, protoplast, embryos, and callus tissue. The plant tissue may be in plants or in organ, tissue or cell culture.

Preferential Transcription: "Preferential transcription" is defined as transcription that occurs in a particular pattern of cell types or developmental times or in response to specific stimuli or combination thereof. Non-limitive examples of preferential transcription include: high transcript levels of a desired sequence in root tissues; detectable transcript levels of a desired sequence in certain cell types during embryogenesis; and low transcript levels of a desired sequence under drought conditions. Such preferential transcription can be determined by measuring initiation, rate, and/or levels of transcription.

Promoter: A "promoter" is a DNA sequence that directs the transcription of a polynucleotide. Typically a promoter is located in the 5' region of a polynucleotide to be transcribed, proximal to the transcriptional start site of such polynucleotide. More typically, promoters are defined as the region upstream of the first exon; more typically, as a region upstream of the first of multiple transcription start sites; more typically, as the region downstream of the preceding gene and upstream of the first of multiple transcription start sites; more typically, the region downstream of a polyadenylation (polyA) signal and upstream of the first of multiple transcription start sites; even more typically, about 3,000 nucleotides upstream of the ATG of the first exon; even more typically, 2,000 nucleotides upstream of the first of multiple transcription start sites. The promoters of the invention comprise at least a core promoter as defined above. Frequently promoters are capable of directing transcription of genes located on each of the complementary DNA strands that are 3' to the promoter. Stated differently, many promoters exhibit bidirectionality and can direct transcription of a downstream gene when present in either orientation (i.e. 5' to 3' or 3' to 5' relative to the coding region of the gene). Additionally, the promoter may also include at least one control element such as an upstream element. Such elements include UARs and optionally, other DNA sequences that affect transcription of a polynucleotide such as a synthetic upstream element.

Promoter Control Element: The term "promoter control element" as used herein describes elements that influence the activity of the promoter. Promoter control elements include transcriptional regulatory sequence determinants such as, but not limited to, enhancers, scaffold/matrix attachment regions, TATA boxes, transcription start locus control regions, UARs, URRs, other transcription factor binding sites and inverted repeats.

Public sequence: The term "public sequence," as used in the context of the instant application, refers to any sequence that has been deposited in a publicly accessible database prior to the filing date of the present application. This term encompasses both amino acid and nucleotide sequences. Such sequences are publicly accessible, for example, on the BLAST databases on the NCBI FTP web site (accessible via the internet). The database at the NCBI FTP site utilizes "gi" numbers assigned by NCBI as a unique identifier for each sequence in the databases, thereby providing a non-redundant database for sequence from various databases, including GenBank, EMBL, DBBJ, (DNA Database of Japan) and PDB (Brookhaven Protein Data Bank).

Regulatory Sequence: The term "regulatory sequence," as used in the current invention, refers to any nucleotide sequence that influences transcription or translation initiation and/or rate, and/or stability and/or mobility of a transcript or polypeptide product. Regulatory sequences include, but are not limited to, promoters, promoter control elements, protein binding sequences, 5' and 3' UTRs, transcriptional start sites, termination sequences, polyadenylation sequences, introns, motifs, certain sequences within amino acid coding sequences such as secretory signals, protease cleavage sites, etc.

Related Sequences: "Related sequences" refer to either a polypeptide or a nucleotide sequence that exhibits some degree of sequence similarity with a reference sequence.

Specific Promoters: In the context of the current invention, "specific promoters" refers to a subset of promoters that have a high preference for modulating transcript levels in a specific tissue or organ or cell and/or at a specific time during development of an organism. By "high preference" is meant at least a 3-fold, preferably at least a 5-fold, more preferably at least a 10-fold still more preferably at least a 20-fold, 50-fold or 100-fold increase in

transcript levels under the specific condition over the transcription under any one reference condition considered. Typical examples of temporal and/or tissue or organ specific promoters of plant origin that can be used with the polynucleotides of the present invention, are: PTA29, a promoter which is capable of driving gene transcription specifically in tapetum and only during anther development (Koltonow *et al.*, Plant Cell 2:1201 (1990); RCc2 and RCc3, promoters that direct root-specific gene transcription in rice (Xu *et al.*, Plant Mol. Biol. 27:237 (1995); TobRB27, a root-specific promoter from tobacco (Yamamoto *et al.*, Plant Cell 3:371 (1991)). Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues or organs, such as root, ovule, fruit, seeds, or flowers. Other specific promoters include those from genes encoding seed storage proteins or the lipid body membrane protein, oleosin. A few root-specific promoters are noted above. See also "Preferential transcription".

Stringency: "Stringency" as used herein is a function of probe length, probe composition (G + C content), and salt concentration, organic solvent concentration, and temperature of hybridization or wash conditions. Stringency is typically compared by the parameter T_m, which is the temperature at which 50% of the complementary molecules in the hybridization are hybridized, in terms of a temperature differential from T_m. High stringency conditions are those providing a condition of T_m - 5°C to T_m - 10°C. Medium or moderate stringency conditions are those providing T_m - 20°C to T_m - 29°C. Low stringency conditions are those providing a condition of T_m - 40°C to T_m - 48°C. The relationship of hybridization conditions to T_m (in °C) is expressed in the mathematical equation

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - (600/N) \quad (1)$$

where N is the length of the probe. This equation works well for probes 14 to 70 nucleotides in length that are identical to the target sequence. The equation below for T_m of DNA-DNA hybrids is useful for probes in the range of 50 to greater than 500 nucleotides, and for conditions that include an organic solvent (formamide).

$$T_m = 81.5+16.6 \log \{[Na^+]/(1+0.7[Na^+])\}+ 0.41(\%G+C)-500/L\ 0.63(\%formamide) \quad (2)$$

where L is the length of the probe in the hybrid. (P. Tijessen, "Hybridization with Nucleic Acid Probes" in Laboratory Techniques in Biochemistry and Molecular Biology, P.C. vand der Vliet, ed., c. 1993 by Elsevier, Amsterdam, which is hereby incorporated by reference in its entirety). The T_m of equation (2) is affected by the nature of the hybrid; for DNA-RNA hybrids T_m is 10-15°C higher than calculated, for RNA-RNA hybrids T_m is 20-25°C higher. Because the T_m decreases about 1 °C for each 1% decrease in homology when a long probe is used (Bonner *et al.*, J. Mol. Biol. 81:123 (1973)), stringency conditions can be adjusted to favor detection of identical genes or related family members.

Equation (2) is derived assuming equilibrium and therefore, hybridizations according to the present invention are most preferably performed under conditions of probe excess and for sufficient time to achieve equilibrium. The time required to reach equilibrium can be shortened by inclusion of a hybridization accelerator such as dextran sulfate or another high volume polymer in the hybridization buffer.

Stringency can be controlled during the hybridization reaction or after hybridization has occurred by altering the salt and temperature conditions of the wash solutions used. The formulas shown above are equally valid when used to compute the stringency of a wash solution. Preferred wash solution stringencies lie within the ranges stated above; high stringency is 5-8°C below T_m, medium or moderate stringency is 26-29°C below T_m and low stringency is 45-48°C below T_m.

Substantially free of: A composition containing A is "substantially free of" B when at least 85% by weight of the total A+B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A+B in the composition, more preferably at least about 95% or even 99% by weight. For example, a plant gene can be substantially free of other plant genes. Other examples include, but are not limited to, ligands substantially free of receptors (and vice versa), a growth factor substantially free of other growth factors and a transcription binding factor substantially free of nucleic acids.

Suppressor: See "Enhancer/Suppressor"

TATA to start: "TATA to start" shall mean the distance, in number of nucleotides, between the primary TATA motif and the start of transcription.

Transgenic plant: A "transgenic plant" is a plant having one or more plant cells that contain at least one exogenous polynucleotide introduced by recombinant nucleic acid methods.

Translational start site: In the context of the present invention, a "translational start site" is usually an ATG or AUG in a transcript, often the first ATG or AUG. A single protein encoding transcript, however, may have multiple translational start sites.

Transcription start site: "Transcription start site" is used in the current invention to describe the point at which transcription is initiated. This point is typically located about 25 nucleotides downstream from a TFIID binding site, such as a TATA box. Transcription can initiate at one or more sites within the gene, and a single polynucleotide to be transcribed may have multiple transcriptional start sites, some of which may be specific for transcription in a particular cell-type or tissue or organ. "+1" is stated relative to the transcription start site and indicates the first nucleotide in a transcript.

Upstream Activating Region (UAR): An "Upstream Activating Region" or "UAR" is a position or orientation dependent nucleic acid element that primarily directs tissue, organ, cell type, or environmental regulation of transcript level, usually by affecting the rate of transcription initiation. Corresponding DNA elements that have a transcription inhibitory effect are called herein "Upstream Repressor Regions" or "URR"s. The essential activity of these elements is to bind a protein factor. Such binding can be assayed by methods described below. The binding is typically in a manner that influences the steady state level of a transcript in a cell or in vitro transcription extract.

Untranslated region (UTR): A "UTR" is any contiguous series of nucleotide bases that is transcribed, but is not translated. A 5' UTR lies between the start site of the transcript and the translation initiation codon and includes the +1 nucleotide. A 3' UTR lies between the translation termination codon and the end of the transcript. UTRs can have particular functions such as increasing mRNA message stability or translation attenuation. Examples of

3' UTRs include, but are not limited to polyadenylation signals and transcription termination sequences.

Variant: The term "variant" is used herein to denote a polypeptide or protein or polynucleotide molecule that differs from others of its kind in some way. For example, polypeptide and protein variants can consist of changes in amino acid sequence and/or charge and/or post-translational modifications (such as glycosylation, etc). Likewise, polynucleotide variants can consist of changes that add or delete a specific UTR or exon sequence. It will be understood that there may be sequence variations within sequence or fragments used or disclosed in this application. Preferably, variants will be such that the sequences have at least 80%, preferably at least 90%, 95, 97, 98, or 99% sequence identity. Variants preferably measure the primary biological function of the native polypeptide or protein or polynucleotide.

2. Introduction

The polynucleotides of the invention comprise nitrogen responsive promoters and promoter control elements that are capable of modulating transcription in response to nitrogen concentration, thereby enhancing the ability of a plant to grow under such nitrogen conditions.

Such nitrogen responsive promoters and promoter control elements can be used in combination with native or heterologous promoter fragments, control elements or other regulatory sequences to modulate transcription and/or translation.

Specifically, nitrogen responsive promoters and control elements of the invention can be used to modulate transcription of a desired polynucleotide, which include without limitation:

(a) antisense;
(b) ribozymes;
(c) coding sequences; or
(d) fragments thereof.

The nitrogen responsive promoter also can modulate transcription in a host genome in cis- or in trans-.

In an organism such as a plant, the nitrogen responsive promoters and promoter control elements of the instant invention are useful to produce preferential transcription which results in a desired pattern of transcript levels in a particular cell, tissue or organ, or under particular conditions.

3. Table of Contents

The following description of the present invention is outlined in the following table of contents.

A. Identifying and Isolating Promoter Sequences of the Invention

The nitrogen responsive promoters and promoter control elements of the present invention are presented in the Sequence Listing. In addition, Table 1 describes the optional promoter control element motifs of the invention. Additional promoter sequences encompassed by the invention can be identified as described below.

(1) Cloning Methods

Isolation from genomic libraries of polynucleotides comprising the sequences of the nitrogen responsive promoters and promoter control elements of the present invention is possible using known techniques.

For example, polymerase chain reaction (PCR) can amplify the desired polynucleotides using primers designed from sequences in the row titled "The spatial expression of the promoter-marker-vector". Polynucleotide libraries comprising genomic sequences can be constructed according to Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Press, Cold Spring Harbor, NY, which is hereby incorporated by reference in its entirety), for example.

Other procedures for isolating polynucleotides comprising the nitrogen responsive promoters and promoter control elements sequences of the invention include, without limitation, tail-PCR and 5' rapid amplification of cDNA ends (RACE). See, for tail-PCR, for example, Liu *et al.*, Plant J 8(3): 457-463 (Sept, 1995); Liu *et al.*, Genomics 25: 674-681 (1995); Liu *et al.*, Nucl. Acids Res. 21(14): 3333-3334 (1993); and Zoe *et al.*, BioTechniques 27(2): 240-248 (1999); ;for RACE, see, for example, PCR Protocols: A Guide to Methods and Applications, (1990) Academic Press, Inc. These publications are hereby incorporated by reference in their entirety.

(2) **Chemical Synthesis**

In addition, the nitrogen responsive promoters and promoter control elements of the invention can be chemically synthesized according to techniques in common use. See, for example, Beaucage *et al.*, Tet. Lett. (1981) 22: 1859 and U.S. Pat. No. 4,668,777.

Such chemical oligonucleotide synthesis can be carried out using commercially available devices, such as a Biosearch 4600 or 8600 DNA synthesizer (Applied Biosystems, a division of Perkin-Elmer Corp., Foster City, California, USA) and an Expedite (Perceptive Biosystems, Framingham, Massachusetts, USA).

Synthetic RNA, including natural and/or analog building blocks, can be synthesized on the Biosearch 8600 machines, see above.

Oligonucleotides can be synthesized and then ligated together to construct the desired polynucleotide.

B. Generating Reduced and "Core" Promoter Sequences

Included in the present invention are reduced and "core" nitrogen responsive promoter sequences. The reduced promoters can be isolated from the promoters of the invention by deleting at least one sequence present in the promoter sequence that is associated with a gene or coding region located 5' or 3' to the promoter sequence or on the complementary strand.

Similarly, the "core" nitrogen responsive promoter sequences can be generated by deleting all sequences present in the promoter sequence that are related to the gene or coding region 5' or 3' to the promoter region or on the complementary strand.

This data is presented in Table 1 which identifies the particular regions which can be deleted from the sequences of SEQ ID NOs: 1-17 to provide reduced or "core" promoters. One or more, including all, such optimal promoter fragments can be deleted from SEQ ID NOs: 1-17 to produce the reduced or "core" promoters..

C. Isolating Related Promoter Sequences

Included in the present invention are nitrogen responsive promoters and promoter control elements that are related to those described in the Sequence Listing. Such a related sequence can be isolated utilizing

(a) nucleotide sequence identity;

(b) coding sequence identity; or

(c) common function or gene products.

Such related sequences (or "relatives") include both naturally occurring promoters and non-natural promoter sequences. Non-natural related promoters include nucleotide substitutions, insertions or deletions of naturally-occurring promoter sequences that do not substantially affect transcription modulation activity. For example, the binding of relevant DNA binding proteins can still occur with the non-natural promoter sequences and promoter control elements of the present invention.

According to current knowledge, promoter sequences and promoter control elements exist as functionally important regions, such as protein binding sites and spacer regions. These spacer regions are apparently required for proper positioning of the protein binding sites. Thus, nucleotide substitutions, insertions and deletions can be tolerated in these spacer regions to a certain degree without loss of function.

In contrast, less variation is permissible in the functionally important regions since changes in the sequence can interfere with protein binding. Nonetheless, some variation in the functionally important regions is permissible so long as function is conserved.

The effects of substitutions, insertions and deletions to the nitrogen responsive promoter sequences or promoter control elements may be to increase or decrease the binding of relevant DNA binding proteins to modulate transcript levels of a polynucleotide to be transcribed. Effects may include tissue-specific or condition-specific modulation of transcript levels of the polypeptide to be transcribed. Polynucleotides representing changes to the nucleotide sequence of the DNA-protein contact region by insertion of additional nucleotides, changes to identity of relevant nucleotides, including use of chemically-modified bases, or deletion of one or more nucleotides are considered encompassed by the present invention.

(1) Relatives Based on Nucleotide Sequence Identity

Included in the present invention are nitrogen responsive promoter and promoter control elements exhibiting nucleotide sequence identity to those described in the Sequence Listing.

Definition

Typically, such related promoters exhibit at least 80% sequence identity, at least 85%, at least 90%, or at least 95%, including, at least 96%, 97%, 98% or 99% sequence identity

compared to those shown in the Sequence Listing. Such sequence identity can be calculated by the algorithms and computer programs described above.

Usually, such sequence identity is exhibited in an alignment region that is at least 75% of the length of a sequence shown in any one of SEQ ID NOs: 1-17 with or without at least one of the optional promoter fragments identified in Table 1 deleted therefrom; more usually at least 80%; more usually, at least 85%, more usually at least 90%, and most usually at least 95%, even more usually, at least 96%, 97%, 98% or 99% of the length of a sequence shown in any one of SEQ ID NOs: 1-17 with our without at least one of the optional promoter fragments identified in Table 1 deleted therefrom.

The percentage of the alignment length is calculated by counting the number of residues of the sequence in region of strongest alignment, e.g., a continuous region of the sequence that contains the greatest number of residues that are identical to the residues between two sequences that are being aligned. The number of residues in the region of strongest alignment is divided by the total residue length of a sequence in the Sequence Listing.

These related promoters exhibit similar preferential transcription as those promoters described in the Sequence Listing.

Construction of Polynucleotides

Naturally occurring nitrogen responsive promoter and promoter control elements that exhibit nucleotide sequence identity to those shown in any one of SEQ ID NOs: 1-17 can be isolated using the techniques as described above. More specifically, such related promoters can be identified by varying stringencies, as defined above, in typical hybridization procedures such as Southern blots or hybridization of polynucleotide libraries, for example.

Non-natural nitrogen responsive promoter and promoter control element variants of those shown in any one of SEQ ID NOs: 1-17 with or without the optional promoter fragments of Table 1 deleted therefrom can be constructed using cloning methods that incorporate the desired nucleotide variation. See, for example, Ho, S. N., *et al.* Gene 77:51-59 1989, describing a procedure site directed mutagenesis using PCR.

Any related nitrogen responsive promoter and promoter control element showing sequence identity to those shown in any one of SEQ ID NOs: 1-17 with or without the optional promoter fragments of Table 1 deleted therefrom can be chemically synthesized as described above.

Also, the present invention includes non-natural nitrogen responsive promoter, promoter control elements and motifs that exhibit the above-sequence identity to those in any one of SEQ ID NOs: 1-17 with or without the optional promoter fragments of Table 1 deleted therefrom.

The nitrogen responsive promoter, promoter control elements and motifs of the present invention may also be synthesized with 5' or 3' extensions, to facilitate additional manipulation, for instance.

The present invention also includes reduced nitrogen responsive promoter sequences. These sequences have at least one of the optional promoter fragments deleted.

Core nitrogen responsive promoter sequences are another embodiment of the present invention. The core nitrogen responsive promoter sequences lack all of the optional promoter fragments.

Testing of Polynucleotides

Polynucleotides of the invention are tested for activity by cloning the sequence into an appropriate vector, transforming plants with the construct and assaying for marker gene expression. Recombinant DNA constructs are prepared which comprise the polynucleotide sequences of the invention inserted into a vector suitable for transformation of plant cells. The construct can be made using standard recombinant DNA techniques (Sambrook et al. 1989) and can be introduced to the species of interest by *Agrobacterium*-mediated transformation or by other means of transformation as referenced below.

The vector backbone can be any of those typical in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs and PACs and vectors of the sort described by

(a) BAC: Shizuya et al., Proc. Natl. Acad. Sci. USA 89: 8794-8797 (1992); Hamilton et al., Proc. Natl. Acad. Sci. USA 93: 9975-9979 (1996);

(b) YAC: Burke et al., Science 236:806-812 (1987);

(c) PAC: Sternberg N. et al., Proc Natl Acad Sci U S A. Jan;87(1):103-7 (1990);

(d) Bacteria-Yeast Shuttle Vectors: Bradshaw et al., Nucl Acids Res 23: 4850-4856 (1995);

(e) Lambda Phage Vectors: Replacement Vector, e.g., Frischauf et al., J. Mol Biol 170: 827-842 (1983); or Insertion vector, e.g., Huynh et al., In: Glover NM (ed) DNA Cloning: A practical Approach, Vol.1 Oxford: IRL Press (1985); T-DNA gene fusion vectors :Walden et al., Mol Cell Biol 1: 175-194 (1990); and

(g) Plasmid vectors: Sambrook et al., infra.

Typically, the construct comprises a vector containing a sequence of the present invention operationally linked to any marker gene. The polynucleotide is identified as a nitrogen responsive promoter by the expression of the marker gene under appropriate conditions. Although many marker genes can be used, Green Fluorescent Protein (GFP) is preferred. The vector may also comprise a marker gene that confers a selectable phenotype on plant cells. The marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or phosphinotricin.. Vectors can also include origins of replication, scaffold attachment regions (SARs), markers, homologous sequences, introns, etc.

Promoter Control Elements of the Invention

The nitrogen responsive promoter control elements and motifs of the present invention include those that comprise a sequence shown in any one of SEQ ID NOs: 1-17 and those that comprise fragments of those sequences shown in the Sequence Listing, but that still possess nitrogen responsive activity. The size of the fragments can range from 5 bases to 10 kilobases (kb). Typically, the fragment size is no smaller than 8 bases; more typically, no smaller than 12; more typically, no smaller than 15 bases; more typically, no smaller than 20 bases; more typically, no smaller than 25 bases; even more typically, no more than 30, 35, 40 or 50 bases.

Usually, the fragment size in no larger than 5 kb bases; more usually, no larger than 2 kb; more usually, no larger than 1 kb; more usually, no larger than 800 bases; more usually, no larger than 500 bases; even more usually, no more than 250, 200, 150 or 100 bases.

E. Constructing Promoters with Control Elements

(1) Combining Promoters and Promoter Control Elements

The nitrogen responsive promoters, promoter control elements and/or motif sequences of the present invention, both naturally occurring and synthetic, can be combined with each other to produce the desired preferential transcription. Also, the polynucleotides of the invention can be combined with other known sequences to obtain other useful promoters to modulate, for example, tissue transcription specific or transcription specific to certain conditions. Such preferential transcription can be determined using the techniques or assays described above.

Fragments and variants, as well as the full-length sequences of those shown in any one of SEQ ID NOs: 1-17 and relatives are useful alone or in combination.

The location and relation of promoter control elements and motifs within a promoter affect the ability of the nitrogen responsive promoter to modulate transcription. The order and spacing of control elements is a factor when constructing promoters.

(2) Number of Promoter Control Elements

Nitrogen responsive promoters contain any number of control elements. For example, a nitrogen responsive promoter contains multiple transcription binding sites or other control elements. One element may confer tissue or organ specificity; another element may limit transcription to specific time periods, etc. Typically, nitrogen responsive promoters contain at least a basal or core promoter as described above. Any additional element is included as desired. For example, a fragment comprising a nitrogen responsive basal or "core" promoter is fused with another fragment with any number of additional control elements.

(3) Spacing Between Control Elements

Spacing between control elements or the configuration or control elements is determined or optimized to permit the desired protein-polynucleotide or polynucleotide interactions to occur.

For example, if two transcription factors bind to a promoter simultaneously or relatively close in time, the binding sites are spaced to allow each factor to bind without steric hindrance. The spacing between two such hybridizing control elements is as small as a profile of a protein bound to a control element. In some cases, two protein binding sites are

adjacent to each other when the proteins bind at different times during the transcription process.

Further, when two control elements hybridize, the spacing between such elements is sufficient to allow the promoter polynucleotide to hairpin or loop to permit the two elements to bind. The spacing between two such hybridizing control elements is as small as a t-RNA loop, to as large as 10 kb.

Typically, the spacing is no smaller than 5 bases; more typically, no smaller than 8; more typically, no smaller than 15 bases; more typically, no smaller than 20 bases; more typically, no smaller than 25 bases; even more typically, no more than 30, 35, 40 or 50 bases.

Usually, the fragment size in no larger than 5 kb bases; more usually, no larger than 2 kb; more usually, no larger than 1 kb; more usually, no larger than 800 bases; more usually, no larger than 500 bases; even more usually, no more than 250, 200, 150 or 100 bases.

Such spacing between promoter control elements is determined using the techniques and assays described above.

(4) Other Promoters

The nitrogen responsive promoters and promoter control elements of the present invention can be combined in a construct with other known promoters to affect transcription in a desired manner. The following are promoters that are induced under stress conditions and can be combined with the polynucleotides of the present invention: ldh1 (oxygen stress; tomato; see Germain and Ricard. 1997. Plant Mol Biol 35:949-54), GPx and CAT (oxygen stress; mouse; see Franco *et al.* 1999. Free Radic Biol Med 27:1122-32), ci7 (cold stress; potato; see Kirch *et al.* 1997. Plant Mol Biol. 33:897-909), Bz2 (heavy metals; maize; see Marrs and Walbot. 1997. Plant Physiol 113:93-102), HSP32 (hyperthermia; rat; see Raju and Maines. 1994. Biochim Biophys Acta 1217:273-80); MAPKAPK-2 (heat shock; Drosophila; see Larochelle and Suter. 1995. Gene 163:209-14).

In addition, the following examples are promoters induced by the presence or absence of light can be used in combination with the polynucleotides of the present invention: Topoisomerase II (pea; see Reddy *et al.* 1999. Plant Mol Biol 41:125-37), chalcone synthase (soybean; see Wingender *et al.* 1989. Mol Gen Genet 218:315-22) mdm2 gene (human tumor; see Saucedo *et al.* 1998. Cell Growth Differ 9:119-30), Clock and BMAL1 (rat; see Namihira *et al.* 1999. Neurosci Lett 271:1-4, PHYA (Arabidopsis; see Canton and Quail

1999. Plant Physiol 121:1207-16), PRB-1b (tobacco; see Sessa *et al.* 1995. Plant Mol Biol 28:537-47) and Ypr10 (common bean; see Walter *et al.* 1996. Eur J Biochem 239:281-93).

The nitrogen responsive promoters and promoter control elements of the following genes can be used in combination with the polynucleotides of the present invention to confer tissue specificity: MipB (iceplant; Yamada *et al.* 1995. Plant Cell 7:1129-42) and SUCS (root nodules; broadbean; Kuster *et al.* 1993. Mol Plant Microbe Interact 6:507-14) for roots, OsSUT1 (rice ; Hirose *et al.* 1997. Plant Cell Physiol 38:1389-96) for leaves, Msg (soybean; Stomvik *et al.* 1999. Plant Mol Biol 41:217-31) for siliques, cel1 (Arabidopsis; Shani *et al.* 1997. Plant Mol Biol 34(6):837-42) and ACT11 (Arabidopsis; Huang *et al.* 1997. Plant Mol Biol 33:125-39) for inflorescence.

Still other promoters are affected by hormones or participate in specific physiological processes, which can be used in combination with the polynucleotides of present invention. Some examples are the ACC synthase gene that is induced differently by ethylene and brassinosteroids (mung bean; Yi *et al.* 1999. Plant Mol Biol41:443-54), the TAPG1 gene that is active during abscission (tomato; Kalaitzis *et al.* 1995. Plant Mol Biol 28:647-56), and the 1-aminocyclopropane-1-carboxylate synthase gene (carnation; Jones *et al.* 1995l Plant Mol Biol 28:505-12) and the CP-2/cathepsin L gene (rat; Kim and Wright. 1997. Biol Reprod 57:1467-77), both active during senescence.

F. Vectors

Vectors are a useful component of the present invention. In particular, the present nitrogen responsive promoters and/or promoter control elements are delivered to a system such as a cell by way of a vector. For the purposes of this invention, such delivery may range from simply introducing the nitrogen responsive promoter and/or promoter control element by itself randomly into a cell, to integration of a cloning vector containing the present nitrogen responsive promoter and/or promoter control element. Thus, a vector is not to be limited to a DNA molecule such as a plasmid, cosmid or bacteria phage that has the capability of replicating autonomously in a host cell. All other manner of delivery of the nitrogen responsive promoters and promoter control elements of the invention are envisioned. The various T-DNA vector types are preferred vectors for use with the present invention. Many useful vectors are commercially available.

It may also be useful to attach a marker sequence to the present nitrogen responsive promoter or promoter control element in order to determine activity of such sequences. Marker sequences typically include genes that provide antibiotic resistance, such as tetracycline resistance, hygromycin resistance or ampicillin resistance, or provide herbicide resistance. Specific selectable marker genes may be used to confer resistance to herbicides such as glyphosate, glufosinate or broxynil (Comai *et al.*, Nature 317: 741-744 (1985); Gordon-Kamm *et al.*, Plant Cell 2: 603-618 (1990); and Stalker *et al.*, Science 242: 419-423 (1988)). Other marker genes exist which provide hormone responsiveness.

(1) Modification of Transcription by Nitrogen Responsive Promoters, Promoter Control Elements

The nitrogen responsive promoters and promoter control elements of the present invention are operatively linked to a polynucleotide to be transcribed. In this manner, the nitrogen responsive promoter or promoter control element modifies transcription by modulating transcript levels of that polynucleotide when inserted into a genome.

However, prior to insertion into a genome, the nitrogen responsive promoter or promoter control element need not be linked, operatively or otherwise, to a polynucleotide to be transcribed. For example, the nitrogen responsive promoter or promoter control element is inserted alone into the genome in front of a polynucleotide already present in the genome. In this manner, the nitrogen responsive promoter or promoter control element modulates the transcription of a polynucleotide that was already present in the genome. This polynucleotide may be native to the genome or inserted at an earlier time.

Alternatively, the nitrogen responsive promoter or promoter control element is inserted into a genome alone to modulate transcription. See, for example, Vaucheret, H *et al.* (1998) Plant J 16: 651-659. Rather, the nitrogen responsive promoter or promoter control element is simply inserted into a genome or maintained extrachromosomally as a way to divert transcription resources of the system to itself. This approach may be used to down-regulate the transcript levels of a group of polynucleotides.

(2) Polynucleotide to be Transcribed

The nature of the polynucleotide to be transcribed is not limited. Specifically, the polynucleotide includes sequences that have activity as RNA as well as sequences that result

in a polypeptide product. These sequences include, but are not limited to, antisense sequences, ribozyme sequences, spliceosomes, amino acid coding sequences, and fragments thereof.

Specific coding sequences may include, but are not limited to endogenous proteins or fragments thereof, or heterologous proteins including marker genes or fragments thereof.

Nitrogen responsive promoters and promoter control elements of the present invention are useful for modulating metabolic or catabolic processes. Such processes include, but are not limited to, secondary product metabolism, amino acid synthesis, seed protein storage, oil development, pest defense and nitrogen usage. Some examples of genes, transcripts and peptides or polypeptides participating in these processes, which can be modulated by the present invention: are tryptophan decarboxylase (tdc) and strictosidine synthase (str1), dihydrodipicolinate synthase (DHDPS) and aspartate kinase (AK), 2S albumin and alpha-, beta-, and gamma-zeins, ricinoleate and 3-ketoacyl-ACP synthase (KAS), *Bacillus thuringiensis* (Bt) insecticidal protein, cowpea trypsin inhibitor (CpTI), asparagine synthetase and nitrite reductase. Alternatively, expression constructs are used to inhibit expression of these peptides and polypeptides by incorporating the nitrogen responsive promoters in constructs for antisense use, co-suppression use, RNAi suppression or for the production of dominant negative mutations.

(3) Other Regulatory Elements

As explained above, several types of regulatory elements exist concerning transcription regulation. Each of these regulatory elements may be combined with the present vector if desired.

(4) Other Components of Vectors

Translation of eukaryotic mRNA is often initiated at the codon that encodes the first methionine. Thus, when constructing a recombinant polynucleotide according to the present invention for expressing a protein product, it is preferable to ensure that the linkage between the 3' portion, preferably including the TATA box, of the promoter and the polynucleotide to be transcribed, or a functional derivative thereof, does not contain any intervening codons which are capable of encoding a methionine.

The vector of the present invention may contain additional components. For example, an origin of replication allows for replication of the vector in a host cell. Additionally, homologous sequences flanking a specific sequence allow for specific recombination of the specific sequence at a desired location in the target genome. T-DNA sequences also allow for insertion of a specific sequence randomly into a target genome.

The vector may also be provided with a plurality of restriction sites for insertion of a polynucleotide to be transcribed as well as the nitrogen responsive promoters and promoter control elements of the present invention. The vector may additionally contain selectable marker genes. The vector may also contain a transcriptional and translational initiation region, and a transcriptional and translational termination region functional in the host cell. The termination region may be native with the transcriptional initiation region, may be native with the polynucleotide to be transcribed, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.*, (199 1) Mol. Gen. Genet. 262:141-144; Proudfoot (199 1) Cell 64:671-674; Sanfacon *et al.* (199 1) Genes Dev. 5:141-149; Mogen *et al.* (1990) Plant Cell 2:1261-1272; Munroe *et al.* (1990) Gene 91:151-158; Ballas *et al.* 1989) Nucleic Acids Res. 17:7891-7903; Joshi *et al.* (1987) Nucleic Acid Res. 15:9627-9639.

Where appropriate, the polynucleotide to be transcribed may be optimized for increased expression in a certain host cell. For example, the polynucleotide can be synthesized using preferred codons for improved transcription and translation. See U.S. Patent Nos. 5,380,831, 5,436, 391; see also and Murray *et al.*, (1989) Nucleic Acids Res. 17:477-498.

Additional sequence modifications include elimination of sequences encoding spurious polyadenylation signals, exon intron splice site signals, transposon-like repeats, and other such sequences well characterized as deleterious to expression. The G-C content of the polynucleotide may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. The polynucleotide sequence may be modified to avoid hairpin secondary mRNA structures.

A general description of expression vectors and reporter genes can be found in Gruber, *et al.*, "Vectors for Plant Transformation, in Methods in Plant Molecular Biology & Biotechnology" in Glich *et al.*, (Eds. pp. 89-119, CRC Press, 1993). Moreover GUS

expression vectors and GUS gene cassettes are available from Clonetech Laboratories, Inc., Palo Alto, California while luciferase expression vectors and luciferase gene cassettes are available from Promega Corp. (Madison, Wisconsin). GFP vectors are available from Aurora Biosciences.

G. Insertion of Polynucleotide and Vectors Into A Host Cell

The polynucleotides according to the present invention can be inserted into a host cell. A host cell includes but is not limited to a plant, mammalian, insect, yeast, and prokaryotic cell, preferably a plant cell.

The method of insertion into the host cell genome is chosen based on convenience. For example, the insertion into the host cell genome may be accomplished either by vectors that integrate into the host cell genome or by vectors which exist independent of the host cell genome

(1) Polynucleotides Autonomous of the Host Genome

The polynucleotides of the present invention exist autonomously or independent of the host cell genome. Vectors of these types are known in the art and include, for example, certain type of non-integrating viral vectors, autonomously replicating plasmids, artificial chromosomes, and the like.

Additionally, in some cases transient expression of a polynucleotide is desired.

(2) Polynucleotides Integrated into the Host Genome

The nitrogen responsive promoters, promoter control elements or vectors of the present invention may be transformed into host cells. These transformations may be into protoplasts or intact tissues or isolated cells. Preferably expression vectors are introduced into intact tissue. General methods of culturing plant tissues are provided for example by Maki *et al.* "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biology & Biotechnology, Glich *et al.* (Eds. pp. 67-88 CRC Press, 1993); and by Phillips *et al.* "Cell-Tissue Culture and In-Vitro Manipulation" in Corn & Corn Improvement, 3rd Edition 10Sprague *et al.* (Eds. pp. 345-387) American Society of Agronomy Inc. *et al.* 1988.

Methods of introducing polynucleotides into plant tissue include the direct infection or co-cultivation of a plant cell with *Agrobacterium tumefaciens* (Horsch *et al.* (1985)

Science 227:1229). Descriptions of *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer provided by Gruber *et al.* supra.

Alternatively, polynucleotides are introduced into plant cells or other plant tissues using a direct gene transfer method such as microprojectile-mediated delivery, DNA injection, electroporation and the like. More preferably polynucleotides are introduced into plant tissues using the microprojectile media delivery with the biolistic device. See, for example, Tomes *et al.*, "*Direct DNA transfer into intact plant cells via microprojectile bombardment*" In: Gamborg and Phillips (Eds.) Plant Cell, Tissue and Organ Culture: Fundamental Methods, Springer Verlag, Berlin (1995).

In another embodiment of the current invention, expression constructs are used for gene expression in callus culture for the purpose of expressing marker genes encoding peptides or polypeptides that allow identification of transformed plants. Here, a nitrogen responsive promoter that is operatively linked to a polynucleotide to be transcribed is transformed into plant cells, and the transformed tissue is then placed on callus-inducing media. If the transformation is conducted with leaf discs, for example, callus will initiate along the cut edges. Once callus growth has initiated, callus cells are transferred to callus shoot-inducing or callus root-inducing media. Gene expression occurs in the callus cells developing on the appropriate media: callus root-inducing promoters will be activated on callus root-inducing media, etc. Examples of such peptides or polypeptides useful as transformation markers include, but are not limited to, barstar, glyphosate, chloramphenicol acetyltransferase (CAT), kanamycin, spectinomycin, streptomycin or other antibiotic resistance enzymes, green fluorescent protein (GFP), and β-glucuronidase (GUS), etc. Some of the exemplary nitrogen responsive promoters of any one of SEQ ID NOs: 1-17 with or without the optional promoter fragments of Table 1 deleted therefrom will also be capable of sustaining expression in some tissues or organs after the initiation or completion of regeneration. Examples of these tissues or organs are somatic embryos, cotyledon, hypocotyl, epicotyl, leaf, stems, roots, flowers and seed.

Integration into the host cell genome also can be accomplished by methods known in the art, for example, by the homologous sequences or T-DNA discussed above or using the cre-lox system (A.C. Vergunst *et al.*, *Plant Mol. Biol.* 38:393 (1998)).

H. Utility

Common Uses

In yet another embodiment, the nitrogen responsive promoters and/or promoter control elements of the present invention are used to further understand developmental mechanisms. For example, nitrogen responsive promoters and/or promoter control elements that are specifically induced during callus formation, somatic embryo formation, shoot formation or root formation are used to explore the effects of overexpression, repression or ectopic expression of target genes, or for isolation of trans-acting factors.

The vectors of the invention are used not only for expression of coding regions, but also in exon-trap cloning, or promoter trap procedures to detect differential gene expression in various tissues (K. Lindsey *et al.*, 1993 "*Tagging Genomic Sequences That Direct Transgene Expression by Activation of a Promoter Trap in Plants*", Transgenic Research 2:3347. D. Auch & Reth, *et al.*, "*Exon Trap Cloning: Using PCR to Rapidly Detect and Clone Exons from Genomic DNA Fragments*", Nucleic Acids Research, Vol. 18, No. 22, p. 674).

Entrapment vectors, first described for use in bacteria (Casadaban and Cohen, 1979, Proc. Nat. Aca. Sci. U.S.A., 76: 4530; Casadaban *et al.*, 1980, J. Bacteriol., 143: 971) permit selection of insertional events that lie within coding sequences. Entrapment vectors are introduced into pluripotent ES cells in culture and then passed into the germline via chimeras (Gossler *et al.*, 1989, Science, 244: 463; Skarnes, 1990, Biotechnology, 8: 827). Promoter or gene trap vectors often contain a reporter gene, e.g., lacZ, lacking its own promoter and/or splice acceptor sequence upstream. That is, promoter gene traps contain a reporter gene with a splice site but no promoter. If the vector lands in a gene and is spliced into the gene product, then the reporter gene is expressed.

Recently, the isolation of preferentially-induced genes has been made possible with the use of sophisticated promoter traps (e.g. IVET) that are based on conditional auxotrophy complementation or drug resistance. In one IVET approach, various bacterial genome fragments are placed in front of a necessary metabolic gene coupled to a reporter gene. The DNA constructs are inserted into a bacterial strain otherwise lacking the metabolic gene, and the resulting bacteria are used to infect the host organism. Only bacteria expressing the metabolic gene survive in the host organism; consequently, inactive constructs can be eliminated by harvesting only bacteria that survive for some minimum period in the host. At

the same time, constitutively active constructs can be eliminated by screening only bacteria that do not express the reporter gene under laboratory conditions. The bacteria selected by such a method contain constructs that are selectively induced only during infection of the host. The IVET approach can be modified for use in plants to identify genes induced in either the bacteria or the plant cells upon pathogen infection or root colonization. For information on IVET see the articles by Mahan *et al.* in Science 259:686-688 (1993), Mahan *et al.* in PNAS USA 92:669-673 (1995), Heithoff *et al.* in PNAS USA 94:934-939 (1997), and Wang*et al.* in PNAS USA. 93:10434 (1996).

Particular Uses

Nitrogen is often the rate-limiting element in plant growth, and all field crops have a fundamental dependence on exogenous nitrogen sources. Nitrogenous fertilizer, which is usually supplied as ammonium nitrate, potassium nitrate, or urea, typically accounts for 40% of the costs associated with crops, such as corn and wheat, in intensive agriculture. Increased efficiency of nitrogen use by plants enables the production of higher yields with existing fertilizer inputs and/or enables existing yields of crops to be obtained with lower fertilizer input, or provide for better yields on soils of poorer quality. Also, higher amounts of proteins in the crops are produced more cost-effectively. "Nitrogen responsive" promoters and/or promoter control elements are used to alter or modulate plant growth and development.

In addition, high concentrations of nitrogen are known to be toxic to plants, especially at the seedling stage (Brenner and Krogmeier (1989) PNAS 86:8185-8188). Here, abnormally high nitrogen creates toxic nitrogen effects ("burning") and/or leads to the inhibition of germination, reducing yield as a consequence. This is a particular problem during the application of urea and other ammonium based fertilizers since segments of a planting field can vary widely in terms of the available nitrogen present and high ammonium levels are toxic to plants. Currently, because most crop plants are severely damaged by high nitrogen conditions, yield can be significantly reduced.

Such deleterious effects can be avoided when the nitrogen responsive promoters and/or promoter control elements of the instant invention are used to direct expression of genes involved in ammonium assimilation and ion transport, as well as pH maintenance. As an example, the nitrogen responsive promoters and/or promoter control elements of the instant invention can be operatively linked to genes such as a ammonium transport Amt1

gene (Sonoda et al. (2003) Plant Cell Phys. 44:726-734) or to nitrate reductase (Loque et al. (2003) Plant Phys. 132:958-967; Gansel et al. (2001) Plant J. 26:143-155) in order to mitigate the effects of inadvertent over-application of urea fertilizer.

Nitrogen responsive promoter and/or promoter control element sequences are used in combination with gene coding sequences, either gDNA or cDNA, to induce the expression of proteins and enzymes during conditions of high or low soil or solution nitrogen concentration. Increased mRNA expression via one of the nitrogen responsive promoters and/or promoter control elements described herein is used to overcome rate limiting steps in nitrogen assimilation, transport and metabolism. General reviews of these processes can be found in: Derlot, S. et al., 2001, Amino Acid Transport. In *Plant Nitrogen* (eds. P. Lea and J.-F. Morot-Gaudry), pp. 167-212. Springer-Verlag, Berlin, Heidelberg, Glass, A.D.M et al., 2002,. *J. Exp. Bot.* **53:** 855-864, Krapp, A. et al., 2002, Nitrogen and Signaling. In *Photosynthetic Nitrogen Assimilation and Associated Carbon Respiratory Metabolism* (eds. C.H. Foyer and G. Noctor), pp. 205-225. Kluwer Academic Publisher, Dordrecht, The Netherlands, and Touraine, B. et al., 2001, Nitrate uptake and its regulation. In *Plant Nitrogen* (eds. P. Lea and J.-F. Morot-Gaudry), pp. 1-36. Springer-Verlag, Berlin, Heidelberg. Overcoming the rate limiting steps in nitrogen assimilation, transport and metabolism has the effect of increasing the yield, reducing the nitrogen content and reducing the protein content of plants grown under nitrogen limiting conditions.

Nitrogen responsive promoters and/or promoter control elements are also used to turn off the expression of genes that are not beneficial to nitrogen uptake, utilization and/or transport. Here, the nitrogen responsive promoter and/or promoter control element is operatively linked to the antisense orientation of a non-beneficial gene sequence. Expression of this antisense gene sequence has the effect of decreasing the amount of the non-beneficial sequence such that the expression of the protein encoded by the non-beneficial sequence is reduced. The reduction in expression of the non-beneficial sequence leads to a reduction in the genetic function of the protein, thus allowing for more efficient nitrogen uptake, utilization and transport (Hamada et al. 1996, Modification of fatty acid composition by over- and antisense-expression of a microsomal omega-3 fatty acid desaturase gene in transgenic tobacco. *Transgenic Res* **5:** 115-121; Takahashi et al. 2001, Nitrite Reductase Gene Enrichment Improves Assimilation of NO2 in Arabidopsis. *Plant Physiol.* **126:** 731-741; Temple et al. 1998, Down-regulation of specific members of the glutamine synthetase gene

family in alfalfa by antisense RNA technology. *Plant Mol Biol* **37:** 535-547). Alternatively, suppression of a non-beneficial gene sequence can be accomplished via co-suprression or RNAi suppression.

Nitrogen responsive promoters and/or promoter control elements are further used to express a non-beneficial sequence in inverted orientation, thus producing a double stranded RNA molecule. Double stranded RNAs are recognized in plant cells as foreign and are targeted for degradation (Vance and Vaucheret 2001, RNA Silencing in Plants--Defense and Counterdefense. *Science* **292:** 2277-2280; Wesley et al. 2001, Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27:** 581-590.). The end result is reduced expression of the mRNA of the non-beneficial sequence, which leads to reduced gene function (Tang et al. 2003, A biochemical framework for RNA silencing in plants. *Genes Dev* **17:** 49-63).

Nitrogen responsive promoters and/or promoter control elements that are expressed in the root are used to modify root architecture by increasing or decreasing the expression of genes involved in primary and lateral root formation. For example the ANR1 gene is involved in nitrogen dependent lateral root formation (Zhang and Forde 2000, Regulation of Arabidopsis root development by nitrate availability. *J. Exp. Bot.* **51:** 51-59). Antisense inhibition of ANR1 gene expression results in a decrease in lateral root formation at inducing concentrations of nitrate (Zhang and Forde 1998, An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279:** 407-409.). Conversely, increased expression of ANR1 and other proteins involved in lateral root formation are used to increase lateral root number and length and thus increase nitrogen uptake from the soil or solution by increasing surface area contact between soil or solution and root absorbing surface.

The nitrogen responsive promoters and promoter control elements of the present invention are useful for modulating nitrogen metabolism and utilization. For example, the promoters and promoter control elements of the invention are used to increase the expression of nitrate and ammonium transporter gene products. These transporter gene products increase the uptake of nitrogen and transport of nitrogen from roots to shoots, which leads to an increase in the amount of nitrogen available for reduction to ammonia. As a consequence, such transgenic plants require less fertilizer, leading to reduced costs for the farmer and less nitrate pollution in ground water.

The nitrogen responsive promoters and promoter control elements of the invention also down-regulate genes which lead to feedback inhibition of nitrogen uptake and reduction. An example of such genes are those encoding the 14-3-3 proteins, which repress nitrate reductase (Swiedrych A et al., 2002, J Agric Food Chem 27;50(7):2137-41.*Repression of the 14-3-3 gene affects the amino acid and mineral composition of potato tuber*). Here the nitrogen responsive promoters and promoter control elements described herein can be used to drive expression of an antisense copy of a 14-3-3 protein. The resulting transgenic plants have an increase in amino acid content and protein content in the seed and/or leaves. Such plants are especially useful for livestock feed. For example, an increase in amino acid and/or protein content in alfalfa provides an increase in forage quality and thus enhanced nutrition.

Generally, the nitrogen responsive promoters and/or promoter control elements of the invention can be used to improve plant performance when plants are grown under sub-optimal, normal or abnormal nitrogen conditions. For example, the transgenic plants of the invention can be grown without damage on soils or solutions containing at least 1, 2, 3, 4 or 5 percent less nitrogen, more preferably at least 5, 10, 20, 30, 40 or percent less nitrogen, even more preferably at least 60, 70 or 80 percent less nitrogen and most preferably at least 90 or 95 percent less nitrogen than normal, depending on the coding region operatively linked to the nitrogen responsive promoter or promoter control element of the invention. Similarly, the transgenic plants of the invention can be grown without damage on soils or solutions containing at least 1, 2, 3, 4 or 5 percent more nitrogen, more preferably at least 5, 10, 20, 30, 40 or 50 percent more nitrogen, even more preferably at least 60, 70 or 80 percent more nitrogen and most preferably at least 90 or 95 percent more nitrogen than normal, depending on the coding region operatively linked to the nitrogen responsive promoter or promoter control element of the invention.

GFP EXPERIMENTAL PROCEDURES AND RESULTS

PROCEDURES

The polynucleotide sequences of the present invention are tested for promoter activity using Green Fluorescent Protein (GFP) assays in the following manner.

Approximately 1-2 kb of genomic sequence occurring immediately upstream of the ATG translational start site of the gene of interest is isolated using appropriate primers tailed

with *Bst*XI restriction sites. Standard PCR reactions using these primers and genomic DNA are conducted. The resulting product is isolated, cleaved with BstXI and cloned into the BstXI site of an appropriate vector, such as pNewBin4-HAP1-GFP (see Figure 1).

Transformation

The following procedure is used for transformation of plants

1. **Seed Preparation and Plant Growth.**

A homogeneous mixture of *Arabidopsis thaliana* seed in a 0.2% Phytagar solution is inclubated at 4°C in the dark for 3 days. Seed is planted in 4 inch pots in a soil miture of Sunshine Mix, Vermiculite, Marathon and Osmocote. Pots are placed in flats, covered with plastic domes and subsequently subirrigated. After 3 to 4 days, the domes are removed.

Seven to ten days after planting, seedlings are thinned to 20 plants per pot. When 5-10 cm long bolts appear, they are clipped between the first node and the stem base to induce secondary bolts. Six to 7 days after clipping, the plants are transformed via dipping infiltration.

2. **Preparation of *Agrobacterium*.**

Each 4 inch pot is inverted and the aerial portion of the plants submerged into a 16 oz. polypropylene container holding 200 mls of *Agrobacterium tumefaciens* (1 x10^7 bacteria) in Infiltration media (2.2 g MS salts, 50 g sucrose, 110 µg BAP and 0.02% Silwet L-77 per liter). After 5 minutes, the *Agrobacterium* solution is removed while keeping the polypropylene containiner in place and the pots returned to an upright position. Pots are then placed in flats (10 pots per flat) containing approximately 1 inch of water and covered with shade cloth. After 24 hours, the shade cloth and polypropylene containers are removed.

After flowering, each pot is covered with a ciber plant sleeve. When plants are completely dry, seed is collected and stored.

3. **High Throughput Screening - T1 Generation**

Transformed seed are placed in pots containing a water saturated soil miture of Sunshine Mix, Vermiculite, Marathon and Osmocote. Pots are then placed in flats and stored in the dark at 4oC for at least 2 days. After transferring the flats from the cooler to the greenhouse, they are covered with 55% shade cloth and propagation domes. When the cotyledons are fully expanded the cloth and domes are removed.

Plants are sprayed with a solution of 3 ml concentrated Finale in 48 oz water. Spraying is repeated every 3-4 days until only transformants remain. Transformants are thinned to a maximum of 5 plants per pot.

4. *GFP Assay*

Tissues are dissected by eye or under magnification using INOX 5 grade forceps and placed on a slide with water and coversliped. An attempt is made to record images of observed expression patterns at earliest and latest stages of development of tissues listed below. Specific tissues will be preceded with High (H), Medium (M), Low (L) designations.

Flower	Pedicel, receptacle, nectary, sepal, petal, filament, anther, pollen, carpel, style, papillae, vascular, epidermis, stomata, trichome
Silique	Stigma, style, carpel, septum, placentae, transmitting tissue, vascular, epidermis, stomata, abscission zone, ovule
Ovule	**Pre-fertilization:** inner integument, outer integument, embryo sac, funiculus, chalaza, micropyle, gametophyte **Post-fertilization:** zygote, inner integument, outer integument, seed coat, primordial, chalaza, miccropyle, early endosperm, mature endosperm, embryo
Embryo	Suspensor, preglobular, globular, heart, torpedo, late, mature, provascular, hypophysis, radicle, cotyledons, hypocotyl
Stem	Epidermis, cortex, vascular, xylem, phloem, pith, stomata, trichome
Leaf	Petiole, mesophyll, vascular, epidermis, trichome, primordial, stomata, stipule, margin

T1 Mature: These are the T1 plants resulting from independent transformation events. These are screened between stage 6.50-6.90 (means the plant is flowering and that 50-90% of the flowers that the plant will make have developed) which is 4-6 weeks of age. At this stage the mature plant possesses flowers, siliques at all stages of development, and fully expanded leaves. We do not generally differentiate between 6.50 and 6.90 in the report but rather just indicate 6.50. The plants are initially imaged under UV with a Leica Confocal microscope. This allows examination of the plants on a global level. If expression is present, they are imaged using scanning laser confocal micsrocopy.

T2 Seedling: Progeny are collected from the T1 plants giving the same expression pattern and the progeny (T2) are sterilized and plated on agar-solidified medium containing M&S salts. In the event that there is no expression in the T1 plants, T2 seeds are planted from all lines. The seedlings are grown in Percival incubators under continuous light at 22°C for 10-12 days. Cotyledons, roots, hypocotyls, petioles, leaves, and the shoot meristem region of individual seedlings are screened until two seedlings are observed to have the same pattern. Generally found the same expression pattern is found in the first two seedlings. However, up

to 6 seedlings are screened before "no expression pattern" is recorded. All constructs are screened as T2 seedlings even if they did not have an expression pattern in the T1 generation.

T2 Mature: The T2 mature plants are screened in a similar manner to the T1 plants. The T2 seeds are planted in the greenhouse, exposed to selection and at least one plant screened to confirm the T1 expression pattern. In instances where there are any subtle changes in expression, multiple plants are examined and the changes noted in the tables.

T3 Seedling: This is done similar to the T2 seedlings except that only the plants for which we are trying to confirm the pattern are planted.

IMAGE DATA:

Images are collected by scanning laser confocal microscopy. Scanned images are taken as 2-D optical sections or 3-D images generated by stacking the 2-D optical sections collected in series. All scanned images are saved as TIFF files by imaging software, edited in Adobe Photoshop, and labeled in Powerpoint specifying organ and specific expressing tissues.

Instrumentation:

An Inverted Leica DM IRB microscope is used with two Fluorescence filter blocks: (1) Blue excitation BP 450-490; long pass emission LP 515 and (2) Green excitation BP 515-560; long pass emission LP 590. The following objectives are used: HC PL FLUOTAR 5X/0.5,
HCPL APO 10X/0.4 IMM water/glycerol/oil, HCPL APO 20X/0.7 IMM water/glycerol/oil and
HCXL APO 63X/1.2 IMM water/glycerol/oil. A Leica TCS SP2 confocal scanner with a Spectral range of detector optics of 400-850nm was used with a variable computer controlled pinhole diameter, an Optical zoom 1-32X and four simultaneous detectors: three channels for collection of fluorescence or reflected light and one channel for transmitted light detector. The laser sources are: (1) Blue Ar 458/5mW, 476nm/5mW, 488nm/20mW, 514nm/20mW, (2) Green HeNe 543nm/1.2mW and (3)Red HeNe 633nm/10mW.

4. Quantitative PCR

Plants are staged according to Boyes et al. (2001) Plant Cell 13:1499-1510.

For experiments analyzing the response to changes from low to high Nitrogen concentrations, *Arabidopsis thaliana* (ecotype Wassilewskija) seeds are sown on flats containing 4 L of a 1:2 mixture of Grace Zonolite vermiculite and soil. Flats are watered with 3 L of water and vernalized at 4°C for five days. Flats are placed in a Conviron growth chamber having 16 hr light/8 hr dark at 20°C, 80% humidity and 17,450 LUX. Flats are watered with approximately 1.5 L of water every four days. Mature, bolting plants (24 days after germination) are bottom treated with 2 L of either a control (100 mM mannitol pH 5.5) or an experimental (50 mM ammonium nitrate, pH 5.5) solution. Roots, leaves and siliques are harvested separately 30, 120 and 240 minutes after treatment, flash frozen in liquid nitrogen and stored at ⁻80°C.

Hybrid maize seed (Pioneer hybrid 35A19) are aerated overnight in deionized water. Thirty seeds are plated in each flat, which contained 4 liters of Grace zonolite vermiculite. Two liters of water are bottom fed and flats were kept in a Conviron growth chamber with 16 hr light/8 hr dark at 20°C and 80% humidity. Flats are watered with 1 L of tap water every three days. Five day old seedlings are treated as described above with 2 L of either a control (100 mM mannitol pH 6.5) solution or 1 L of an experimental (50 mM ammonium nitrate, pH 6.8) solution. Fifteen shoots per time point per treatment are harvested 10, 90 and 180 minutes after treatment, flash frozen in liquid nitrogen and stored at ⁻80°C.

Alternatively, plants were cultivated hydroponically and submitted to low-to-high nitrate treatment. Plants were cultivated in a modified Hoagland's solution containing 15ppm of nitrogen as KNO3 (1.7mM KNO3) as the sole nitrogen (N) source. Plants were grown in a walk-in Conviron growth chamber under long day light cycle until they developed siliques and then transferred to 0.0 ppm N media for 3 days to adapt them to low nitrogen conditions. Nitrate induction was carried out by transferring experimental plants to 200ppm of N (14.3mM KNO3) and controls to 28.6mM mannitol. Root and rosette tissue from experimental and control plants (2 plants each) were harvested at 0.25, 1, 2, 4, 6 and 24 hours after treatment.

For experiments analyzing the response to changes from high to low Nitrogen conditions, wild type *Arabidopsis thaliana* seeds (ecotype Wassilewskija) are surface sterilized with 30% Clorox, 0.1% Triton X-100 for 5 minutes. Seeds are then rinsed with 4-5 exchanges of sterile double distilled deionized water. Seeds are vernalized at 4°C for 2-4 days in darkness. After cold treatment, seeds are plated on modified 1X MS media (without NH_4NO_3 or KNO_3), 0.5% sucrose, 0.5g/L MES pH5.7, 1% phytagar and supplemented with

KNO_3 to a final concentration of 60 mM (high nitrate modified 1X MS media). Plates are then grown for 7 days in a Percival growth chamber at 22°C with 16 hr. light/8 hr dark.

Germinated seedlings are then transferred to a sterile flask containing 50 mL of high nitrate modified 1X MS liquid media. Seedlings are grown with mild shaking for 3 additional days at 22°C in 16 hr. light/8 hr dark (in a Percival growth chamber) on the high nitrate modified 1X MS liquid media.

After three days of growth on high nitrate modified 1X MS liquid media, seedlings are transferred either to a new sterile flask containing 50 mL of high nitrate modified 1X MS liquid media or to low nitrate modified 1X MS liquid media (containing 20 μM KNO_3). Seedlings are grown in these media conditions with mild shaking at 22°C in 16 hr light/ 8 hr dark for the appropriate time points and whole seedlings harvested for total RNA isolation via the Trizol method (LifeTech.). The time points used for the microarray experiments are 10 min. and 1 hour time points for both the high and low nitrate modified 1X MS media.

Alternatively, seeds that are surface sterilized in 30% bleach containing 0.1% Triton X-100 and further rinsed in sterile water, are planted on MS agar, (0.5% sucrose) plates containing 50 mM KNO_3 (potassium nitrate). The seedlings are grown under constant light (3500 LUX) at 22°C. After 12 days, seedlings are transferred to MS agar plates containing either 1mM KNO_3 or 50 mM KNO_3. Seedlings transferred to agar plates containing 50 mM KNO_3 are treated as controls in the experiment. Seedlings transferred to plates with 1mM KNO_3 are rinsed thoroughly with sterile MS solution containing 1 mM KNO_3. There are ten plates per transfer. Root tissue was collected and frozen in 15 mL Falcon tubes at various time points which included 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 9 hours, 12 hours, 16 hours, and 24 hours.

Maize 35A19 Pioneer hybrid seeds are sown on flats containing sand and grown in a Conviron growth chamber at 25°C, 16 hr light/8 hr dark, ~13,000 LUX and 80% relative humidity. Plants are watered every three days with double distilled deionized water. Germinated seedlings are allowed to grow for 10 days and are watered with high nitrate modified 1X MS liquid media (see above). On day 11, young corn seedlings are removed from the sand (with their roots intact) and rinsed briefly in high nitrate modified 1X MS liquid media. The equivalent of half a flat of seedlings is then submerged (up to their roots) in a beaker containing either 500 mL of high or low nitrate modified 1X MS liquid media (see above for details).

At appropriate time points, seedlings are removed from their respective liquid media, the roots separated from the shoots and each tissue type flash frozen in liquid nitrogen and stored at
$^{-}80^{o}$C. This is repeated for each time point. Total RNA is isolated using the Trizol method (see above) with root tissues only.

Corn root tissues isolated at the 4 hr and 16 hr time points are used for the microarray experiments. Both the high and low nitrate modified 1X MS media are used.

Quantitative RNA PCR (qt-PCR) was conducted according to standard procedures, for example using the Bio-Rad SYBR® Green qRT-PCR system.

EXAMPLES

The following Examples include various information about each nitrogen responsive promoter and/or promoter control element of the invention including the nucleotide sequence, the spatial expression promoted by each promoter and the corresponding results from different expression experiments.

Example 1

Promoter Expression Report #166.PT0625.FPNUE
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype
Spatial expression summary: **Primary Root** **H** epidermis
Observed expression pattern: **T1 mature:** No expression **T2 seedling:** Root specific GFP expression. High expression in root epidermal cells.
Expected expression pattern: Shoots, Roots - Nitrogen inducible
Selection Criteria: Microarray
Gene: Arabidopsis thaliana LOB domain protein 38
GenBank: NM_114854 Arabidopsis thaliana LOB domain protein 38 / lateral organ boundaries domain protein 38 (LBD38) (At3g49940) mRNA, complete cds gi|18408982| ref| NM_114854.1
Source Promoter Organism: *Arabidopsis thaliana*, Columbia (Col) ecotype
Vector: pNewbin4-HAP1-GFP
Marker Type: GFP-ER
Generation Screened: **X**T1 Mature **X**T2 Seedling T2 Mature T3 Seedling

Inductions completed.					
Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. Minus N to 60mM N (MS)	12 d.	T2	2 Hr 6 Hr	3/3 3/0	Low No
2. 100µM KNO3 to	12 d.	T2	24 Hr	3/0	No

60mM KNO3			48 Hr	3/0	No
Inducible expression summary: **Treatment:** **Time point induced:** **Organs induced:** **Tissues induced:**					
1. Minus N to 60mM N (MS)	**2 Hr**		Root	vascular	

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=3	**Events Expressing: n=0**
No GFP Expression Detected	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=3	**Events Expressing: n=3**
Seedlings expressing / Seedlings screened Event-01: 5/6 Event-02: 5/6 Event-03: 5/6	
GFP Expression Detected	
Hypocotyl	epidermis cortex vascular xylem phloem stomata
Cotyledon	mesophyll vascular epidermis margin stomata hydathode
Rosette Leaf	mesophyll vascular epidermis trichome petiole primordia stomata stipule margin hydathode
X Primary Root	**H** epidermis trichoblast atrichoblast cortex endodermis vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials flanking cells vascular lateral root cap
Shoot apical meristem	Shoot apical meristem
X in the Epidermis **(Ep)** of the Root Transition zone and the Root **(Rt)**	

Induction Screens
1. Minus N to 60mM N (MS) 2 Hr and 6 Hr At 2 Hrs, induction under 60mM total Nitrogen (MS) conditions, no induction under Minus N conditions. At 6 HRs, induction under 60mM total Nitrogen (MS) conditions, no induction under Minus N conditions.
2. 100µM KNO3 to 60mM KNO3 24 Hr, 48 Hr At 24 Hrs, induction under 60mM KNO3 conditions, induction in 1 of three samples under 120mM Mannitol conditions. At 48 Hrs, induction under 60mM KNO3 conditions, induction in 1 of three samples under 120mM Mannitol conditions.

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen

utilization by increasing the expression of nitrogen use efficiency genes in root epidermal tissue. The promoter can also promoter greater uptake in response to locally high concentrations of nitrate. The target genes could be in involved in processes that increase transport of nitrate, ammonium and amino acids into the root e.g. nitrogen transporter proteins such as NRT2.1, NRT1.1 or AMT1.1. This promoter can also be used to regulate the development of root hairs. Increasing the number of root hairs can improve nutrient uptake.

Construct:	PT0625
Promoter candidate I.D:	13148207
cDNA I.D:	23643047
Events expressing:	PT0625 01-03 5(6)

Promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence. In every case, the sequences of the 2-3 events have matched.

Promoter sequence

>166.PT0625 predicted (Ceres cDNA_13492462; SEQ ID NO:1)

ttaaccctaaacaaaacaatctcattggtttcataaataaattgtttacaaagtatacgtac
tgcatgaacgaatgaaccatatctatatttataaaactcatagagaccaatagtttaagaga
ggcacttatatagctcaacaaataatagcgaactagagagaatatgatctaattagttataa
atctcaattttgaaattgaagtgcgttatttcatttgagaatctatgtgtttttttttgttgt
tgttagatgagaagctaggtttttttctttttctttacaccgataatcgataatatatgttaa
tcacactgatttttgtttgagacatgaagattcgaaaaatttgtcaacgaataaacactgga
tagatagaattgagatctgccatcaaataatcgagatcgttcatgcatgacgcaaacattta
tatagaaatgaagcaagtaaagaatatgaaaaagaatagaaatgagaaatttataaagaaag
aaaaaaagaaccaatggttgaggaggcaactattcgcggggacacggagccgttcgcaccca
tcaccttggaatctctctttcttcctctctcctcatcaccaactagtcaacaaccacacacc
atttttaactttcataattaaacctaacataacatttttttttgtataaactatagcataaa
ttaaattcagttaatgataaaataaatatattttgtagcaatcattctattttgtaatttgg
tagggctctttaaactttgattattatccaatttttattaaaatataataaaatctcaaagc
catgacccattccttcactcaagtatcaatgtctattgtctataaatattacataactcttc
ttcttcaaccaaacattgaaacactttgtcccactctctctttctctttcttgtaccaaa
agctttttgaatctccaagattatagcaaaaccaaagataaaatactaacttaaaagatttc
tgaaaata

>166.PT0625 experimental (Ceres cDNA 23643047; SEQ ID NO:2)

gtaggcaaaaaaacgcctctatctttcttctaaaacatttttcatattaaattatcaaaacc
cttaaggttgatttaagggtcaggtagtggatttgtttcgttgaagggtcagcttagcctta
accctaaacaaaacaatctcattggtttcataaataaattgtttacaaagtatacgtactgc
atgaacgaatgaaccatatctatatttataaaactcatagagaccaatagtttaagagaggc
acttatatagctcaacaaataatagcgaactagagagaatatgatctaattagttataaatc
tcaattttgaaattgaagtgcgttatttcatttgagaatctatgtgtttttttttgttgttgt
tagatgagaagctaggtttttttctttttctttacaccgataatcgataatatatgttaatca
cactgatttttgtttgagacatgaagattcgaaaaatttgtcaacgaataaacactggatag
atagaattgagatctgccatcaaataatcgagatcgttcatgcatgacgcaaacatttatat
agaaatgaagcaagtaaagaatatgaaaaagaatagaaatgagaaatttataaagaaagaaa
aaaagaaccaatggttgaggaggcaactattcgcggggacacggagccgttcgcacccatca
ccttggaatctctctttcttcctctctcctcatcaccaactagtcaacaaccacacaccatt
tttaactttcataattaaacctaacataacatttttttttgtataaactatagcataaatta

aattcagttaatgataaaataaatatattttgtagcaatcattctattttgtaatttggtag
ggctctttaaactttgattattatccaatttttattaaaatataataaaatctcaaagccat
gacccattccttcactcaagtatcaatgtctattgtctataaatattacataactcttcttc
ttcaacca

Example 2

Promoter Expression Report #169.PT0669.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary:	
Flower	**H** nectary
Silique	**H** stomata
Ovule	**Post-fertilization: H** early endosperm **H** embryo
Embryo	**H** radicle **H** cotyledons **H** mature
Rosette Leaf	**H** petiole
Primary Root	**H** epidermis **H** cortex **H** endodermis **H** vascular **H** pericycle **H** root cap **L** root hairs
Lateral root	**H** epidermis **H** cortex **H** endodermis **H** initials **H** primordia **H** vascular **H** lateral root cap
Observed expression pattern: **T1 Mature expression:** GFP is highly expressed throughout the female gametophyte, early endosperm and mature embryos. GFP is also expressed in nectarines of developing flowers, pollen, and guard cells in some siliques. **T2 Seedling expression:** GFP is highly expressed throughout roots of seedlings. GFP also expressed in petioles of emerging rosette leaves.	
Expected expression pattern:	Shoots, Roots - Nitrogen inducible
Selection Criteria:	Microarray
Gene:	Arabidopsis thaliana ferredoxin, putative
GenBank: NM_128311 Arabidopsis thaliana ferredoxin, putative (At2g27510) mRNA, complete	
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype
Vector:	pNewbin4-HAP1-GFP
Marker Type:	GFP-ER
Generation Screened:	**X**T1 Mature **X**T2 Seedling T2 Mature T3 Seedling

Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. 100µM KNO3 to 20 mM KNO3	8 days	T2	24 Hr 48 Hr	3/2 3/1	Yes
2. 0.566 mM KNO3 to 30mM KNO3	4 weeks	T2	48 Hr	3/2	Yes

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=2	**Events Expressing: n=2**
GFP Expression Detected	
X Flower	pedicel receptacle **H** nectary sepal petal filament anther pollen carpel style papillae vascular epidermis stomata trichome

	silique
X Silique **X Ovule** **X Embryo**	stigma style carpel septum placentae funiculus transmitting tissue vascular epidermis **H** stomata abscission zone ovule **Pre-fertilization:** primordia inner integument outer integument **H** embryo sac funiculus chalaza micropyle gametophyte **Post-fertilization:** zygote suspensor embryo sack funiculus inner integument outer integument endothelium seed coat primordia chalaza micropyle **H** early endosperm mature endosperm **H** embryo suspensor preglobular globular heart torpedo late **H** mature provascular hypophysis **H** radicle **H** cotyledons root meristem shoot meristem
Stem	epidermis cortex interfascicular region vascular xylem phloem pith stomata trichome
Leaf	petiole mesophyll vascular epidermis trichome primordia stomata stipule margin
Shoot apical meristem	Shoot apical meristem Flower primordium
X in the Nectary **(Ne)** of the flower, the Ovule/Ovary **(Ov)** and Pollen **(Po)** of the Silique **(Si)** and the Embryo sac **(Es)** of the prefertilized ovule. **X** in the Guard cells **(Gc)** and Endosperm **(En)** of the Silique **(Si)**. **X** in the Root cap **(Rc)** of the embryo root. **X** in the Seed.	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=3	**Events Expressing: n=3**
Seedlings expressing / Seedlings screened Event-01: 6/6 Event-02: 6/6 Event-03: 6/6	
GFP Expression Detected	
Hypocotyl	epidermis cortex vascular xylem phloem stomata
Cotyledon	mesophyll vascular epidermis margin petiole stomata hydathode
X Rosette Leaf	mesophyll vascular epidermis trichome **H** petiole primordia stomata stipule margin hydathode
X Primary Root	**H** epidermis trichoblast atrichoblast **H** cortex **H** endodermis **H** vascular xylem phloem **H** pericycle quiescent columella **H** root cap **L** root hairs
X Lateral root	**H** epidermis trichoblast atrichoblast **H** cortex **H** endodermis **H** initials **H** primordia flanking cells **H** vascular **H** lateral root cap
Shoot apical meristem	Shoot apical meristem
X in all seedlings **X** in the Petiole **(Pt),** Lateral root **(Lr)** and Vasculature **(Vs),** Cortex **(Cr),** Endodermis **(Eo),** Epidermis **(Ep)** and Stele **(Sl)** of the root. **X** in the Root cap **(Rc)** of the root tip.	

Induction Screens
1. 100μM KNO3 to 20 mM KNO3 Seedlings **24 Hrs** Induction in roots under 20 mM KNO3 conditions, no induction under 40 mM Mannitol control conditions. **48 Hrs** Induction in roots under 20 mM KNO3 conditions, no induction under 40 mM Mannitol control conditions.

Induction Screens
0.566 mM KNO3 to 30mM KNO3 Mature **48 Hrs** Induction in flowers and roots under 20 mM KNO3 conditions, no induction under 60 mM Mannitol control conditions. **48 Hrs** Increased GFP expression observed in petals, stamens and in embryos in event -01 under 30 mM KNO3 conditions. Petal **(Pe),** Pollen **(Po),** Sepal **(Se),** Root **(Rt),** Silique **(Si),** Stamen **(St)** No expression under 60 mM Mannitol control conditions.

qRT-PCR Data
Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. The QPCR results do not show highly inducible expression at either six hours or 48 hours after nitrate induction with the exception of events -02 and -03 at six hours after treatment in shoot and root. Event 1 also shows strong GFP induction at 48 hours after treatment. This pattern is consistent with the observed expression in flowers at 48 hours after treatment.

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in root tissue in response to nitrogen fertilizer application. These genes can be in involved in processes that improve transport of nitrate, ammonium and amino acids. This promoter can also be used to increase expression of genes in seeds after nitrate fertilization. This can be useful for increasing transport of sucrose and amino acids to seeds and thereby increasing plant vigor and yield.

Construct:	PT0669
Promoter candidate I.D:	15372193
cDNA I.D:	23373586
Events expressing:	PT0669 –01, -02, -03

Promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.*) Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence.

Promoter sequence

>169.PT0669.FPNUE predicted (Ceres cDNA 12340498; SEQ ID NO: 3)

aactatatttatatccgatttcattttcgcgaaacgagaaaatccaatgaaaaattaactcaagaa
aaaaaaaagttacgaaaacattttatttgtaattaaatgaatcatatataaaatcaaaaacagcag
aataatggaaacaaataatctggtaggaaaaataatcaaataattaagacgtctcaggtgacacaa
gttgggccgtcacggccttccaaaagccacactgctctctccttttatatattttgcttccacctc
tcaagactcctccaccaaccccctctcgcactctccgccaccttcttccctaattctctctctctc
gctacctctctacgtaagtttcagatttgactttattagcttcgattctctctgatatttgtttct
agaatttgatctgatcagcgatgtttacttgttccttgtttttttgtttttttcattgacttcttgtg
gggacaaaaaaaaacaatcaaatatctttcgatttcgttgttcttctctttttcgttatctgatag
tgaccgatttgatcctgtatcgttgctattcagatgctaatcatctccttaattgtgaatttttttt
gttgttatttagtgaatcttgttacaagtctgttgtaggtttatttttgccattaagctactttga
tcgactttagaatctatttgatgataagtaattaaacatgttttagtgattgttaagtaagtcatt
tagtcatgtttttggagcatcgagtgaagatctaatatagctttaagcttgcatcttctcattacg
ctccatacactaattttcacatcatatttgctattggaaacagataagtttttggttcttgtttcc
attgctacttgtgatgcacatcctcacaattttctctcagttttggttcttatttctctggaacag
tttgatttgttagattgtatcactatgaagaaaccctgaagctaaacttgtttataaacgcaggtg
ataaacaaga

>169.PT0669.FPNUE experimental (Ceres cDNA 23373586; SEQ ID NO:4)

aactatatttatatccgatttcattttcgcgaaacgagaaaatccaatgaaaaattaactcaagaa
aaaaaaaagttacgaaaacattttatttgtaattaaatgaatcatatataaaatcaaaaacagcag
aataatggaaacaaataatctggtaggaaaaataatcaaataattaagacgtctcaggtgacacaa
gttgggccgtcacggccttccaaaagccacactgctctctccttttatatattttgcttccacctc
tcaagactcctccaccaaccccctctcgcactctccgccaccttcttccctaattctctctctctc
gctacctctctacgtaagtttcagatttgactttattagcttcgattctctctgatatttgtttct
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tgaccgatttgatcctgtatcgttgctattcagatgctaatcatctccttaattgtgaatttttttt
gttgttatttagtgaatcttgttacaagtctgttgtaggtttatttttgccattaagctactttga
tcgactttagaatctatttgatgataagtaattaaacatgttttagtgattgttaagtaagtcatt
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attgctacttgtgatgcacatcctcacaattttctctcagttttggttcttatttctctggaacag
tttgatttgttagattgtaacactatgaagaaaccctgaagctaaacttgtttataaacgcaggtg
ataaacaaga

Example 3

Promoter Expression Report #170.PT0668.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary:	
Flower	**H** filament
Silique	**H** vascular **H** ovule
Ovule	**Pre-fertilization: H** outer integument **H** chalaza
	Post-fertilization: H outer integument **H** chalaza
Hypocotyl	**L** epidermis **H** vascular
Rosette Leaf	**H** epidermis
Observed expression pattern:	
T1 Mature expression: GFP is preferentially expressed in chalazal region of the outer integument in developing ovules and seed coats. In flowers, GFP is also expressed in vasculature	

of carpels and connective region of anther filament. GFP is highly expressed in mesophyll and vasculature of leaves with weak expression in epidermal cells. Not expressed in cells of the stem. **T2 Seedling expression:** GFP is highly expressed in epidermis and cortex cells of root, vascular cells of the hypocotyl and in the epidermis of leaves.
Expected expression pattern: Shoots, Roots - Nitrogen inducible
Selection Criteria: Microarray
Gene: 5'-adenylylsulfate reductase 2, chloroplast (APR2) (APSR) / adenosine 5'-phosphosulfate 5'-adenylylsulfate (APS) sulfotransferase, At1g62180.
GenBank: AK221838 Arabidopsis thaliana gene for putative adenosine-5'-phosphosulfate reductase, complete cds, clone: RAFL22-02-P09 gi|62321019|dbj| AK221838.1| [62321019]
Source Promoter Organism: *Arabidopsis thaliana*, Columbia (Col) ecotype
Vector: pNewbin4-HAP1-GFP
Marker Type: GFP-ER
Generation Screened: **X** T1 Mature **X** T2 Seedling T2 Mature T3 Seedling

Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. 100µM KNO3 to 60mM KNO3	7 days	T2	24 Hr 48 Hr	4/1 4/2	Yes Yes
2. 0.566 mM KNO3 to 30mM KNO3	4 weeks	T2	48 Hr	2/1	Yes

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=2	**Events Expressing: n=2**
GFP Expression Detected	
X Flower	pedicel receptacle nectary sepal petal **H** filament anther pollen carpel style papillae vascular epidermis stomata trichome
X Silique	silique stigma style carpel septum placentae funiculus transmitting tissue **H** vascular epidermis stomata abscission zone **H** ovule
X Ovule	**Pre-fertilization:** primordia inner integument **H** outer integument embryo sac funiculus **H** chalaza micropyle gametophyte **Post-fertilization:** zygote suspensor embryo sack funiculus inner integument **H** outer integument endothelium seed coat primordia
Embryo	**H** chalaza micropyle early endosperm mature endosperm embryo suspensor preglobular globular heart torpedo late mature provascular hypophysis radicle cotyledons root meristem shoot meristem
Stem	epidermis cortex interfascicular region vascular xylem phloem pith stomata trichome
X Leaf	petiole **H** mesophyll **L** vascular epidermis trichome primordia stomata stipule margin
Shoot apical meristem	Shoot apical meristem Flower primordium

X in the Filament **(Fi)** and Ovule/Ovary **(Ov)** of the Flower. **X** in the Vasculature **(Vs)** of the Silique **(Si).** **X** in the Chalaza **(Ch),** Funiculus **(Fn)** and Outer integumenta **(Oi)** of the ovule. **X** in the Mesophyll **(Me)** and Vasculature **(Vs)** of the leaf **X** in the Seed coat **(Sc)** of the seed.

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=3	**Events Expressing: n=3**
Seedlings expressing / Seedlings screened Event-01: 2/6 Event-02: 2/6 Event-03: 7/7	
GFP Expression Detected	
X Hypocotyl	**L** epidermis cortex **H** vascular xylem phloem stomata
Cotyledon	mesophyll vascular epidermis margin petiole stomata hydathode
X Rosette Leaf	mesophyll vascular **H** epidermis trichome petiole primordia stomata stipule margin hydathode
Primary Root	epidermis trichoblast atrichoblast cortex endodermis vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials primordia flanking cells vascular lateral root cap
Shoot apical meristem	Shoot apical meristem
X in the Epidermis **(Ep)** and Vasculature **(Vs)** of the leaf, seedling and hypocotyl-root transition zone	

Induction Screens
1. 100µM KNO3 to 60mM KNO3 11/19/2004 **24 Hr and 48 Hr** Nitrate induced GFP expression is observed in cotyledons at 24 Hr and 48 Hr. under 60mM KNO3 conditions.
2. 0.566 mM KNO3 to 30mM KNO3 5/11/2005 Increase in GFP observed relative to control in the leaves of plants hydroponically grown at 0.566 mM KNO3 and treated in 30mM KNO3

qRT-PCR Data
Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. Little to no nitrate-induced mRNA accumulation is observed at 6 hrs after nitrate induction except for event 4 in roots (the large values reported for 6 hour shoots could be due to very low levels of mRNA) Both events show strong nitrate induced mRNA accumulation of At1g62180, HAP1 and GFP transcripts in shoots and event -01 shows induction in roots. See Figure 2

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in leaf and seed tissue in response to nitrogen fertilizer application. These genes could be in involved in processes that increase photosynthesis, improve transport of nitrate, ammonium and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield.

Construct:	PT0668
Promoter candidate I.D:	15372190
cDNA I.D:	23547574
Events expressing:	-01, -02, -03

Predicted promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence.

Promoter sequence (1000bp).

>170.PT0668.predicted (Ceres cDNA 13610771; SEQ ID NO:5)

atagagttttactatgcttttggaatctttcttctaatgtgccaactacagagaaatacatg
tattaccactaggaatcggaccatatcatagatatcaggattagataactagttctcgtcgc
tatcacttcgcattaagttctagtaattgttaaagattctaatttttttactaaacaaaaact
aaatcaacatcaaatatgcaaagtgtgtgttgtccacacaagtgactcaaagtatacgcagg
tgggattggaccatattattgcaaatcgtttccgaaccactcatatttctttttttctctcc
ttttttttatccggagaattatggaaccacttcatttcaacttcaaaactaatttttttggttc
agtgatcaaatacaaaaaaaaaaaaaaagttatagatattaaatagaaaactattccaatct
taaaaatacaaatgaaaccataattttaatttatacaaaactatttaattagctaagggttg
tcttaacgtttagaaaataaaaaattatgattgtctgtttaaaattacaatgaatgaataaa
aaaaatatgcaatgaatgaaagaataaattttgtacatccgatagaatgagaaaatgaattt
tgtacaaaccactcaagaattcaaaacaattgtcaaagttttcttctcagccgtgtgtcctc
ctctcctagccgccacatctcacacactaatgctaaccacgcgatgtaaccgtaagcgctga
gtttttgcatttcagatttcacttccaccaaacaaaactcgccacgtcatcaatacgaatca
ttccgtataaacgtctagattctttacagcctacaatgttctcttctttggtcggccattat
ttaacgctttgaacctaaatctagcccagccaacgaagaagacgaagcaaatccaaaccaaa
gttctccattttcgtagcttctttaagcttttcagtatcatagagacacttttttttttt
gattagaa

>170.PT0668.experimental (Ceres cDNA 23547574; SEQ ID NO:6)

atagagttttactatgcttttggaatctttcttctaatgtgccaactacagagaaatacatg
tattaccactaggaatcggaccatatcatagatatcaggattagataactagttctcgtcgc
tatcacttcgcattaagttctagtaattgttaaagattctaatttttttactaaacaaaaact
aaatcaacatcaaatatgcaaagtgtgtgttgtccacacaagtgactcaaagtatacgcagg
tgggattggaccatattattgcaaatcgtttccgaaccactcatatttctttttttctctcc
ttttttttatccggagaattatggaaccacttcatttcaacttcaaaactaatttttttggttc
agtgatcaaatacaaaaaaaaaaaaaaagttatagatattaaatagaaaactattccaatct
taaaaatacaaatgaaaccataattttaatttatacaaaactatttaattagctaagggttg
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aaaaatatgcaatgaatgaaagaataaattttgtacatccgatagaatgagaaaatgaattt
tgtacaaaccactcaagaattcaaaacaattgtcaaagttttcttctcagccgtgtgtcctc
ctctcctagccgccacatctcacacactaatgctaaccacgcgatgtaaccgtaagcgctga
gtttttgcatttcagatttcacttccaccaaacaaaactcgccacgtcatcaatacgaatca
ttccgtataaacgtctagattctttacagcctacaatgttctcttctttggtcggccattat
ttaacgctttgaacctaaatctagcccagccaacgaagaagacgaagcaaatccaaaccaaa
gttctccattttcgtagcttctttaagctttttcagtatcatagagacacttttttttttt
gattagaa

Example 4

Promoter Expression Report #174.PT0664.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary:	
Stem	**H** phloem
Leaf	**L** vascular
Hypocotyl	**H** vascular
Cotyledon	**H** vascular
Primary Root	**L** epidermis **H** vascular
Observed expression pattern: **T1 Mature expression:** GFP expression specific to phloem cells within the vascular bundles of stem. Low GFP expression in vasculature of leaf. **T2 Seedling expression:** GFP expressed in vasculature of hypocotyl and cotyledons and roots. Low root epidermal expression near transitions zone.	
Expected expression pattern:	Shoots - Nitrogen inducible
Selection Criteria:	Microarray
Gene:	adenylate isopentenyltransferase 3 / cytokinin synthase (IPT3)
GenBank: NM_116176 Arabidopsis thaliana adenylate isopentenyltransferase 3 / cytokinin synthase (IPT3) (At3g63110) mRNA, complete cds gi|30695727|ref|NM_116176.2 |[30695727]	
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype
Vector:	pNewbin4-HAP1-GFP
Marker Type:	GFP-ER
Generation Screened:	**X**T1 Mature **X**T2 Seedling T2 Mature T3 Seedling

Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. 100uM KNO3 to 20mM	7 days	T2	24 Hrs 48 Hrs	4/2 4/0	Yes No
2. 0.566 mM KNO3 to 30mM KNO3	28 days	T2	48 Hrs	2/0	No

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=2	**Events Expressing: n=6**
GFP Expression Detected	
Flower	pedicel receptacle nectary sepal petal filament anther pollen carpel style papillae vascular epidermis stomata trichome

	silique
Silique	stigma style carpel septum placentae funiculus transmitting tissue vascular epidermis stomata abscission zone ovule
Ovule	**Pre-fertilization:** primordia inner integument outer integument embryo sac funiculus chalaza micropyle gametophyte **Post-fertilization:** zygote suspensor embryo sack funiculus inner integument outer integument endothelium seed coat primordia chalaza micropyle early endosperm mature endosperm embryo
Embryo	suspensor preglobular globular heart torpedo late mature provascular hypophysis radicle cotyledons root meristem shoot meristem
X Stem	epidermis cortex interfascicular region vascular xylem **H** phloem pith stomata trichome
X Leaf	petiole mesophyll **L** vascular epidermis trichome primordia stomata stipule margin
Shoot apical meristem	Shoot apical meristem Flower primordium
X in the Phloem **(Ph)** of the Stem **X** in the Vascular **(Vs)** of the Leaf	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=3	**Events Expressing: n=3**
Seedlings expressing / Seedlings screened Event-01: 3/6 Event-02: 4/6 Event-02: 6/6	
GFP Expression Detected	
X Hypocotyl	epidermis cortex **H** vascular xylem phloem stomata
X Cotyledon	mesophyll **H** vascular epidermis margin petiole stomata hydathode
Rosette Leaf	mesophyll vascular epidermis trichome petiole primordia stomata stipule margin hydathode
X Primary Root	**L** epidermis trichoblast atrichoblast cortex endodermis **H** vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials primordia flanking cells vascular lateral root cap
Shoot apical meristem	Shoot apical meristem
X in the Vasculature **(Vs)** of the Seedling, Cotyledon, Hypocotyl-root Transition zone and the root. **X** in the Epidermis **(Ep)** of the root.	

Induction Screens **1. 100uM KNO3 to 20mM**
Increased GFP response in roots relative to control in events 01 and 02.

Nitrate induced GFP expression was not observed in seedlings 48 hrs after treatment.
2.Mature plants Shoots and Roots : 0.566 mM KNO3 to 30mM KNO3 Nitrate induced GFP expression was not observed in mature plants 48 hrs after treatment.

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in vascular tissue in response to nitrogen fertilizer application. These genes can be in involved in processes that increase photosynthesis, improve transport of nitrate and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield.

qRT-PCR Data
Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. Little to no nitrate-induced mRNA accumulation is observed at 6 hrs after nitrate induction. Both events show mRNA induction of At3g63110, HAP1 in shoots 48 hrs after nitrate induction but GFP levels remain low. Event 4 shows high levels of induction of all three mRNA transcripts in 48hrs after treatment of root tissue. The data are broadly consistent with the GFP imaging results. See Figure 3

Construct:	PT0664
Promoter candidate I.D:	15372148
cDNA I.D:	23500661
Events expressing:	-01, -02, -05

Predicted promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence.

Promoter sequence (1000bp).

>PT0664.FPNUE predicted (Ceres cDNA 12663481; SEQ ID NO: 7)

tccaatagctatgacttgtcgctgtaagaataatctttttaaaggccctttctcggaccatt
atatttcttatctcatgtgaataattataatgtaataaaaaacaaaagttttctttgtgttt
tttttcgtcttcagatttatatgtaagtggggagagtaataagagacgttcccgggggtctt
tggccattgcaggtcgacaaacaattttgcctctccgtttcattaatggacggtccaataga
acctttatattattctacaaatataaacaactctatgataatatcaaaatatgagatagaat
cacatctgcataacttttttcttatgaaattagggaatacagaatatctatatacatataata
tttgatagaccgatcatgaggaggaagcatcataacctaatttcttaaatgtttttagttaa
ataatgtcaatccatccaaggtaattgccgagttttcattgcgactgctctaataacatga
taaaatctattaaaaacaaatatactatgagcttagacaataacccatcaaaaaaaaataac
ccatatatatttttattaaaaagaagagaaatgcttcttaaaactttctgcctcgcatataa
tcgttattttcctagaaaaaaaatcgtatcttaacttcacatcaaacgtaatagaagtttac
gtttgattgtgacattatcaatatatatcatctgcattgcacgcggatcaaatatttggcca
gtctaaatagaattagaggagaataaagtaaaataaaacaacaggtttgaccaattaattaa
aaaaggggcgagccaacttgtcgtatatcattcgtacagtggccatttactaagtgtgtgac
cctatatatataaatcatatccttcatgcaaagtcacctgaacatttcatatataagaagat

atacaagcctaccaaacataacaaaacatattttaaacaccagcaagtttatattgcaaagc
gtttcatc

>PT0664.FPNUE experimental (Ceres cDNA 23500661; SEQ ID NO: 8)
tccaatagctatgacttgtcgctgtaagaataatctttttaaaggccctttctcggaccattatat
ttcttatctcatgtgaataattataatgtaataaaaaacaaaagttttctttgtgtttttttttcgt
cttcagatttatatgtaagtggggagagtaataagagacgttcccgggggtctttggccattgcag
gtcgacaaacaattttgcctctccgtttcattaatggacggtccaatagaacctttatattattct
acaaatataaacaactctatgataatatcaaaatatgagatagaatcacatctgcataacttttc
ttatggaattagggaatacagaatatctatatacatataatatttgatagaccgatcatgaggagg
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cgagtttttcattgcgactgctctaataacatgataaaatctattaaaaacaaatatactatgagc
ttagacaataacccatcaaaaaaaaataacccatatatatttttattaaaaagaagagaaatgctt
cttaaaactttctgcctcgcatataatcgttattttcctagaaaaaaaatcgtatcttaacttcac
atcaaacgtaatagaagtttacgtttgattgtgacattatcaatatatatcatctgcattgcacgc
ggatcaaatgcttggccagtctaaatagaattagaggagaataaagtaaaataaacaacaggtttg
accaattaattaaaaaaggggcgagccaacttgtcgtatatcattcgtacagtggccatttactaa
gtgtgtgaccctatatatataaatcatatccttcatgcaaagtcacctgaacatttcatatataag
aagatatacaagcctaccaaacataacaaaacatattttaaacaccagcaagtttatattgcaaag
cgtttcatc

Example 5

Promoter Expression Report #182.PT0663.FPNUE		
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype		
Spatial expression summary:		
Flower	**H** receptacle **H** pollen **L** vascular	
Silique	**H** ovule	
Ovule	**Post-fertilization:**	**H** zygote **H** suspensor **H** embryo
	Pre-fertilization:	**H** embryo sac
Embryo	**H** suspensor **H** preglobular **H** globular **H** late **H** mature **H** hypophysis **H** radicle	
Stem	**H** vascular	
Observed expression pattern: **T1 mature:** High GFP expression in receptacle cells of flowers. GFP also expressed in vasculature of petals and stamens and in pollen. GFP expressed within the egg sac of prefertilized ovules and in 2 cell zygote through mature stage embryos. GFP preferentially expressed at the root cap in mature embryos. GFP also expressed in vasculature of stem. **T2 seedling:** Standard screen not completed.		
Expected expression pattern:	Shoots - Nitrogen inducible	
Selection Criteria:	Microarray	
Gene: two-component responsive regulator / response regulator 4 (ARR4)		
GenBank: NM_100921 Arabidopsis thaliana two-component responsive regulator / response regulator 4 (ARR4) (At1g10470) mRNA, complete cds gi|30681723|ref| NM_100921.2		
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype	
Vector:	pNewbin4-HAP1-GFP	
Marker Type:	GFP-ER	
Generation Screened:	**X** T1 Mature T2 Seedling T2 Mature T3 Seedling	

Treatment:	Age:	Gen:	Time points:	Events Screened / Response	Response:
1. 100µM KNO3 to 20mMKNO3	7 days	T2	24 Hrs 48 Hrs	4/3 4/3	Yes Yes
2. 0.566 mM KNO3 to 30mM KNO3	4 weeks	T2	48 Hrs	2/2	Yes

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=2	**Events Expressing: n= 3**
GFP Expression Detected	
X Flower	pedicel **H** receptacle nectary sepal petal filament anther **H** pollen carpel style papillae **L** vascular epidermis stomata trichome silique
X Silique	stigma style carpel septum placentae transmitting tissue vascular epidermis stomata abscission zone **H** ovule
X Ovule	**Pre-fertilization:** primordia inner integument outer integument **H** embryo sac funiculus chalaza micropyle gametophyte **Post-fertilization: H** zygote **H** suspensor embryo sack inner integument outer integument endothelium seed coat primordia chalaza micropyle early endosperm mature endosperm **H** embryo
X Embryo	**H** suspensor **H** preglobular **H** globular heart torpedo **H** late **H** mature provascular **H** hypophysis **H** radicle cotyledons hypocotyl
X Stem	epidermis cortex **H** vascular xylem phloem pith stomata trichome
Leaf	petiole mesophyll vascular epidermis trichome primordia stomata stipule margin
Shoot apical meristem	Shoot apical meristem Flower primordium
X in the Receptacle **(Re)** of the inflorescence meristem. **X** in the Receptacle **(Re)** and Vasculature **(Vs)** of the Flower. **X** in the Ovule / Ovary **(Ov)** of the Silique **(Si),.** **X** in the Embryo sac **(Es)** of the Silique and prefertilized ovule. **X** in the Suspensor **(Su)** of the fertilized ovule and 2 cell globular embryo. **X** in the Root **(Rt)** of the mature embryo **X** in the Root cap **(Rc)** of the embryo root **X** In the Vasculature **(Vs),** Phloem **(Ph)** and Xylem **(Xy)** of the stem.	

Induction Screens
1. 100µM KNO3 to 20mMKNO3 **24 Hrs.** Increased GFP response relative to control in events 02, 03 and 04, 24 hrs after nitrate induction. **48 Hrs.** Increased GFP expression in cotyledon tissues in events 02, 03 and 04, 48 hrs after nitrate induction

2. Mature plants Shoots and Roots - 0.566 mM KNO3 to 30mM KNO3
Axillary meristem **(Ax),** Leaf **(Lf)** and Stem **(Sm)** tissues show response
Increased levels of GFP in epidermis and cortex cells of stem and epidermis and mesophyll cells of leaf. Cortex **(Cr),** Epidermis **(Ep),** Mesophyll **(Me),** Sepal **(Se)**

qRT-PCR Data
Results. Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. Both events show mRNA induction in roots and shoots 48hrs after treatment for the At1g10470, HAP1 and GFP transcripts. One of two events also show induction of At1g10470, HAP1 and GFP transcripts in root and shoot of 6 hour treated plants. The results are broadly correlated with the GFP imaging data. See Figure 4

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in leaf tissue in response to nitrogen fertilizer application. These genes can be in involved in processes that increase photosynthesis, improve transport of nitrate, ammonium and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield. The promoter also shows expression in embryo sac and developing embryo and can be useful for modifying reproduction and seed characteristics.

Construct:	**PT0663**
Promoter candidate I.D:	15372136
cDNA I.D:	12574427
Events expressing:	01, 02, 04, 05

Predicted promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence

Promoter sequence (1000bp).
>182.PT0663 predicted (Ceres cDNA 12574427; SEQ ID NO: 9)
gggtccctcttttagatttccctgggtcccgcggatccaaattttaatgtggacgtcaaatc
ctttttttttattattatttgtccactttcctcttcttctttttttttttttttgccatttga
aaacgatataaataaaagtgtttggataacataaaatttctagagtcatatggatggatata
ctactagttaggcgtatactaattttctcgtcaacccacaaaacccgatcttaatattattc
tatgaattgcatttgaaccataaattttaaattagaaactgaccaatcacatggaacaatat
aaaattgtcttagtggttagtacttaatacaaataagaccaatccgaagaaccgagccggtt
aagtttaaacacgctactatgaattgtaatggtgtatgaccaaaattagcttctttaatctt
ctggtttattattcttaacagtgagtgattccattttcagtttttttttccaatcacacta
atgagtaatgacgagattttgactaagaagttgtatatatctcacgatggtatatttttatt
ttttggattcctttgtacggatttcttctcctctattatttattcgattttaggaatattat
tttctctatgatattcgcataggccctccaccggattttccataaaatctctatttattaat
actattgttttcaaagataaaagttcaatttttcaaccctaaaagcacggcacataaaaat

atataattttcacattaataggaaccaaagattttgttggattttcctcgctggagattttt
caaaataaaaattgaaaaaaccaaaaagacacactcataaaagatttatttagagaacaaa
aaaatcagaaatataaaaaactgtcttaaggaagagaaaggaacaaaagaaaacagatgtga
gctcttcttcttcgtcttcttctctctatttattctcatcctctcctcacagttactataa
gctcgtct

>182.PT0663 experimental (Ceres cDNA 23457514; SEQ ID NO: 10)
gggtccccttttagatttccctgggtcccgcggatccaaattttaatgtggacgtcaaatcc
ttttttttattattatttgtccactttcctcttcttctttttttttttttttgccatttga
aaacgatataaataaaagtgtttggataacataaaatttctagagtcatatggatggatata
ctactagttaggcgtatactaattttctcgtcaaccccacaaaccccgatcttaatattatt
ctatgaattgcatttgaaccataaattttaaattagaaactgaccaatcacatggaacaata
taaaattgtcttagtggttagtacttaatacaaataagaccaatccgaagaaccgagccggt
taagtttaaacacgctactatgaattgtaatggtgtatgaccaaaattagcttctttaatct
tctggtttattattcttaacagtgagtgattccattttcagtttttttttccaatcacact
aatgagtaatgacgagattttgactaagaagttgtatatatctcacgatggtatatttttat
ttttggattcctttgtacggatttcttctcctctattatttattcgattttaggaatatta
ttttctctatgatattcgcataggccctccaccggattttccataaaatctctatttattaa
tactattgttttcaaagataaaagttcaatttttcaaccctaaaagcacggcacataaaaa
tatataattttcacattaataggaaccaaagattttgttggattttcctcgctggagatttt
tcaaaataaaaattgaaaaaaccaaaaagacacactcataaaagatttatttagagaacaa
aaaaatcagaaatataaaaaactgtcttaaggaagagaaaggaacaaaagaaaacagatgtg
agctcttcttcttcgtcttcttctctctatttattctcatcctctcctcacagttactata
agctcgtct

Example 6

Promoter Expression Report 282.#PT0863.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary: **Primary Root** **H** epidermis	
Observed expression pattern: **T1 Mature expression:** No observed expression. **T2 Seedling expression:** GFP expression specific to root. Preferentially expressed in epidermal cells of primary roots in seedlings. Not observed in lateral roots.	
Expected expression pattern:	Shoots - Nitrogen inducible
Selection Criteria:	Microarray
Gene: glucose-6-phosphate 1-dehydrogenase, putative / G6PD, putative	
GenBank: NM_102274 Arabidopsis thaliana glucose-6-phosphate 1-dehydrogenase, putative / G6PD, putative (At1g24280) mRNA, complete cds	
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype
Vector:	pNewbin4-HAP1-GFP
Marker Type:	GFP-ER
Generation Screened:	**X** T1 Mature **X** T2 Seedling T2 Mature T3 Seedling

Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. 100µM KNO3 to 20mM KNO3	7 days	T2	24 Hrs 48 Hrs	4/0 4/1	No Yes

2. 0.566 mM KNO3 to 30mM KNO3	28 days	T2	48 Hrs	4/2	Yes

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=3	**Events Expressing: n=0**
No GFP Expression Detected	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=3	**Events Expressing: n=2**
Seedlings expressing / Seedlings screened Event-01: 3/6 Event-02: 3/6 Event-03: 0/6	
GFP Expression Detected	
Hypocotyl	epidermis cortex vascular xylem phloem stomata
Cotyledon	mesophyll vascular epidermis margin petiole stomata hydathode
Rosette Leaf	mesophyll vascular epidermis trichome petiole primordia stomata stipule margin hydathode
X Primary Root	**H** epidermis trichoblast atrichoblast cortex endodermis vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials primordia flanking cells vascular lateral root cap
Shoot apical meristem	Shoot apical meristem
X in the Epidermis **(Ep)** of the Root transition zone, root and root tip.	

Induction Screens

1. 100µM KNO3 to 20mM KNO3

24 Hrs.

No response observed after 24 Hrs. for line PT0863 treated under Low to High Nitrate (0.566 mM KNO3 to 30mM KNO3) conditions.

48 Hrs.

An increased response in GFP level relative to control observed in roots of event 04 after 48 Hrs when line PT0863 treated under Low to High Nitrate (0.566 mM KNO3 to 30mM KNO3) conditions.

2. Mature Plants – 0.566 mM KNO3 to 30mM KNO3 – 48 Hrs

An increased response in GFP level relative to control is observed in roots of events 02 and 04 from line PT0863 treated under Low to High Nitrate (0.566 mM KNO3 to 30mM KNO3) conditions.

Increase in GFP response observed in Epidermis **(Ep)** and Vascular **(Vs)** cells of roots from lines 02 and 04.

Quantitative PCR

Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. Little to no nitrate-induced mRNA accumulation is observed at 6 hrs after nitrate induction except

for event 4 in roots (the large values reported for 6 hour shoots could be due to very low levels of mRNA).All 4 events show nitrate induction of the endogenous At2g24280 and HAP1 gene but modest or no induction of GFP in roots. The data indicates that the promoter can drive nitrogen induced expression of the HAP1-VP16 gene.

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in root and seed tissue in response to nitrogen fertilizer application. These genes can be in involved in processes that improve transport of nitrate, ammonium and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield. The promoter can also be used to express insecticidal, fungicidal and/or bactericidal proteins in order to prevent biotic root damage.

Construct:	PT0863
Promoter candidate I.D:	15372139
cDNA I.D:	23494405
Events expressing:	01 -04

Predicted promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence.

Promoter sequence

>282.PT856.FPNUE predicted A (Ceres cDNA 12667371; SEQ ID NO: 11)

aatgagctaaatcacaatagctccagcgaaaatgcatgatttttaaaatgcttctttcaatg
atatagttttattgtaatggaaaaatatttagcaaatagattataaacttacatgagacaag
tataaataattattataaacttattaagtttaagatcaaggcttttgtgcaatgtatcaatg
aatgttagatgtgatatgatgaaagcaatgttttaaacacatacatagtcattgatcggaat
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tagccaaatttcgaatctaattcttccaatatatatttttttttttttgcttagggccacta
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ctttcactaaaccataccaacaaaaccacacagatttggcaaagacataacctttgggagac
gtgaaaaggctcaaaatttgacaattgtccttacaaattcgctcattagtgcaattgtgaga
tttgtttgcatccaaatccaattcataactcacactcgtctcaaattcgaaaa

>282.PT856.FPNUE experimental (Ceres cDNA 23494405; SEQ ID NO: 12)

gattataaacttacatgagacaagtataaataattattataaacttattaagtttaagatcaaggc
ttttgtgcaatgtatcaatgaatgttagatgtgatatgatgaaagcaatgttttaaacacatacat
agtcattgatcggaatgtgtgttattagaaatgcatgcctaagccgatagggttatctatgtttgg
tcttggacattatagccaaatttcgaatctaattcttccaatatatatttttttttttttgcttag
ggccactactagtattgcttatcaatttaagagctcatgaaaatgcaacaatatagtagttgcaa
atccttgtttcaagagaaatcaaagggccacttgtgaattgaataataataatatttgcaaataac
ctttcactaaaccataccaacaaaaccacacagatttggcaaagacataacctttgggagacgtga
aaaggctcaaaatttgacaattgtccttacaaattcgctcattagtgcaattgtgagatttgtttg
catccaaatccaattcataactcacactcgtctcaaattcgaaaa

Example 7

Promoter Expression Report #302.PT0886.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary: **Hypocotyl** **H** epidermis **H** vascular **Cotyledon** **H** epidermis **H** mesophyll **H** vascular **Primary Root** **H** epidermis **H** cortex	
Observed expression pattern: **T1 mature:** No expression observed. **T2 seedling:** High GFP expression in epidermis, mesophyll, and vasculature of cotyledons and in epidermis and vasculature in hypocotyl. High GFP expression in epidermis and cortex cells of roots.	
Expected expression pattern:	Shoots - Nitrogen inducible (Low to High)
Selection Criteria:	Microarray data
Gene:	Ferredoxin-nitrite reductase, putative
GenBank: NM_127123 Arabidopsis thaliana ferredoxin-nitrite reductase, putative (At2g15620) mRNA, complete cds gi|30679484| ref|NM_127123.2|[30679484]	
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype
Vector:	pNewbin4-HAP1-GFP
Marker Type:	GFP-ER
Generation Screened:	**X** T1 Mature **X**T2 Seedling **X**T2 Mature T3 Seedling

Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
0.566 mM KNO3 to 30mM KNO3	4 wks	T2	48 Hrs	4/2	Low

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: n=3	**Events Expressing: n=0**
No GFP Expression Detected	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n=6	**Events Expressing: n=5**
GFP Expression Detected	
X Hypocotyl	**H** epidermis cortex **H** vascular xylem phloem stomata
X Cotyledon	**H** epidermis **H** mesophyll **H** vascular margin stomata hydathode
Rosette Leaf	epidermis mesophyll vascular trichome petiole primordia stomata stipule margin hydathode
X Primary Root	**H** epidermis trichoblast atrichoblast **H** cortex endodermis vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials flanking cells vascular lateral root cap
Shoot apical	Shoot apical meristem

meristem	
Seedlings of line PT0886 at 7 days old show six events with detectable expression in the Epidermis **(Ep)**, Cortex **(Cr),** Mesophyll **(Me),** Root **(Rt)**. Seedlings of line PT0886 at 14 days old show 4 seedlings for each of 6 events with GFP expression intensity highly variable in aerial organs.	

Induction Screens
1. 0.566 mM KNO3 to 30mM KNO3 (Low to High) Increased GFP expression detected in roots of events 05 and 06 of plants transferred to 30mM KN03 relative to mannitol control plants. Root **(Rt)** for 4 events of line PT0886

qRT-PCR
Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically. PT0886 lines -02, 05 and 06 showed strong induction of endogenous Fd-Nitrite reductase gene, Hap1 transgene and GFP transgene in shoots by 48 hrs after induction. PT0889-03 did not show endogenous gene induction but did show Hap1 and GFP induced expression in 48 hr shoots. PT0866 events -02 and -03 showed induced expression at 6 hrs in both shoots and roots while events -05 and -06 did not or showed modest levels (-06). The data are largely consistent with the GP imaging results.

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in vascular tissue in response to nitrogen fertilizer application. These genes can be in involved in processes that increase photosynthesis, improve transport of nitrate and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield.

Construct:	PT0886
Promoter candidate I.D:	15372145
cDNA I.D:	23446949
Lines expressing:	PT0886 -03, 04, 05, 06 N-inducible GFP 05, 06

Predicted promoter region was PCR amplified from the Columbia ecotype of *A. thaliana.* Promoter construct sequence is 5' verified in T1 mature plants and confirmed in the following generation by 5'and 3' sequencing of the entire promoter of two or 3 events. Sequences from all events are used to generate a consensus sequence.

Promoter sequence (397 bp).

>302.PT0886.experimental (Ceres cDNA 12558510; SEQ ID NO: 13)

agtgtatttgaaaacgacattgaagaattaatatatttttttttaattttagtttttttatag
tacaaatattaaaacaaacaatcctaccatatcataacatttgtaaataacattttaagttt
tgttttgagttttaattaattttctatgacaaaaaaatgaagtcaatagactaagtgaatca
tatagtataaataaacacaatttaaatagtttcaaataaatttagaaagaataaaacaaata
gaaatcagaaggtgtctgtttcctcctcgcaacatacgatcaaagagaaacaacttgaccct

ttacattgctcaagagctcatctcttccctctacaaaaatggccgcacgtctccaaccttct
cccaactccttcttccgccatcatc

Example 8

Promoter Expression Report #275.PT0959.FPNUE	
Promoter Tested In: *Arabidopsis thaliana*, Wassilewskija (WS) ecotype	
Spatial expression summary:	
Flower	**L** pedicel **H** sepal **H** abscission zone
Ovule	**Post-fertilization: H** Seed coat **L** embryo
Embryo	**L** cotyledons
Stem	**L** epidermis
Cotyledon	**L** epidermis **L** petiole
Observed expression pattern: **T1 Mature expression:** High GFP expression at abscission zone of developing flowers and seed coats. **T2 Seedling expression:** Low GFP expression in epidermis of cotyledons and petioles.	
Expected expression pattern:	Nitrogen-Inducible in leaf (High to Low)
Selection Criteria:	Microarray
Gene:	expressed protein
GenBank: NM_106662 Arabidopsis thaliana expressed protein (At1g80130)	
Source Promoter Organism:	*Arabidopsis thaliana*, Columbia (Col) ecotype
Vector:	pNewbin4-HAP1-GFP
Marker Type:	GFP-ER
Generation Screened:	**X**T1 Mature **X**T2 Seedling T2 Mature T3 Seedling

Inductions completed.					
Treatment:	**Age:**	**Gen:**	**Time points:**	**Events Screened / Response**	**Response:**
1. 14.3mM KNO3 to 28.6mM Mannitol	4 wks	T2	72 hrs post transfer	5/4	Low
Inducible expression summary:					
Treatment:	**Time point induced:**		**Organs induced:**		**Tissues induced:**
1. 14.3mM KNO3 to 28.6mM Mannitol	72 hrs post transfer		Flowers Siliques Ovules		Abscission zone, Sepals Epidermis Endosperm

T1 Mature Plant Expression	**Organs/Tissues screened**
Events Screened: **n= 6**	**Events Expressing: n= 6**
GFP Expression Detected	
X Flower	**L** pedicel receptacle nectary **H** sepal petal filament anther tapetum pollen carpel style papillae vascular epidermis stomata trichome silique **H** abscission zone
Silique	stigma style carpel septum placentae funiculus transmitting

X Ovule **X Embryo**	tissue vascular epidermis stomata abscission zone ovule **Pre-fertilization:** primordia inner integument outer integument embryo sac funiculus chalaza micropyle gametophyte **Post-fertilization:** zygote suspensor embryo sack funiculus inner integument outer integument endothelium **H** seed coat primordia chalaza micropyle early endosperm mature endosperm embryo suspensor preglobular globular heart torpedo late mature provascular hypophysis radicle **L** cotyledons root meristem shoot meristem
X Stem	**L** epidermis cortex interfascicular region vascular xylem phloem pith stomata trichome
Leaf	petiole mesophyll vascular epidermis trichome primordia stomata stipule margin
Shoot apical meristem	Shoot apical meristem Flower primordium
X in the Flower **(Fl)** and Silique **(Si)** **X** in the Abscission zone **(Az)** of the inflorescence meristem, the Stem, Cotyledon **(Co)** and Seed coat **(Sc)**	

T2 Seedling Expression	**Tissues Screened**
Events Screened: n= 5	**Events Expressing: n= 3**
GFP Expression Detected	
Hypocotyl	epidermis cortex vascular xylem phloem stomata
X Cotyledon	mesophyll vascular **L** epidermis margin **L** petiole stomata hydathode
Rosette Leaf	mesophyll vascular epidermis trichome petiole primordia stomata stipule margin hydathode
Primary Root	epidermis trichoblast atrichoblast cortex endodermis vascular xylem phloem pericycle quiescent columella root cap root hairs
Lateral root	epidermis trichoblast atrichoblast cortex endodermis initials primordia flanking cells vascular lateral root cap
Shoot apical meristem	Shoot apical meristem

Induction Screens
1. 14.3mM KNO3 to 28.6mM Mannitol Expression in the Silique **(Si)** of **PT0959 event -02** under low nitrate conditions compared to control Expression after 72 Hrs in the flower and flower buds, silique, ovules and carpels of **PT0959 event -04** under low nitrate conditions compared to the control

qRT-PCR Data
Results: Tissues for QPCR were collected from stage 6.3 - 6.5 plants grown hydroponically as described in report "NE040615C_Nitrogen Promoter Report 12-29-2004". Event -02 shows the expected induction pattern of the endogenous gene in leaf tissue. The expression patterns of HAP1 and GFP in leaves are broadly similar to the endogenous gene in events -02

and -04 especially at the 72 hour time point, whereas event 3 shows no induced expression of HAP1 or GFP at 72 hours even though the endogenous gene shows induction. These data correlate well with the GFP imaging data above showing that the promoter construct drives GFP expression induced by nitrogen deficiency in events -02 and -04. See Figure 5

Promoter utility
Trait Area: Nutrient
Sub-trait Area: Nitrogen utilization, Low nitrogen tolerance, Nitrogen use efficiency
Utility: Among other uses this promoter sequence could be useful to improve: nitrogen utilization by increasing the expression of nitrogen use efficiency genes in leaf and seed tissue in response to nitrogen deficiency. These genes could be in involved in processes that increase photosynthesis, improve transport of nitrate, ammonium and amino acids and increase export of sucrose to sink tissues, thereby increasing plant vigor and yield.

Construct:	PT0959
Promoter candidate I.D:	22254782
cDNA I.D:	23546169
Events expressing:	02-04

Promoter sequence (1000bp).

>PT0959 (Ceres cDNA 23546169; SEQ ID NO: 14)

aagacctttcgcaagtcatcaaagcacaatcccacaccgtacgttttggtttacctgtctgtcag
ataacgaccgtctcaatatcggatcttaattacatttatgaataactcgactgcgcctccgcaaaa
taagaagaaattgaatatcgaacatttcaacctcaggcatcacatccaagtgattccttatgttga
tgtaaaaatgggatatataggaccaatcagattcatataataatattcataaatcagattcgtaat
gcagtatttatcagctccataaatgatcctagagaatcttatgtaaagtggatcatgcacgtatct
ttatcttctcaaaccttcgaaagaaaccctcaaaacgttattatctaccgaatacatttaatccat
atagcgtgacaaaagaacagagcccgtagttgataaaaagcatgagagtgatgatgaatgtgaagc
actgagagagatctcaccgcttgccgtataacgtctccgtctccgtctttgtcggcattcgtcagc
tgaactcttaaacgtgtcgactgttgtctcgatccaagataacactgtagctgacagttacattta
gagtttgtctccatctcatgcgcaacgcagcaccgtcaattttctgtgaggatactaaactactat
gtaatgatgtcgacaaaagagtgaaaggtgggtcccgcatttgcccatgtggttatggtcaacgtg
tcaaagtactagcggctgtgttttaatccgatcttttttctatcaatccatggtcccgtagaataat
ttcactatttttttcacttggctggtgtcaacttagagaccaataatatatacacttatcttttaca
gtctaaatttaattatgcggcttaccattatataagactctggtagactactctcattatatacat
tataaagatactgatgagtggttcttgtttaatggagttttaaatttaaaaatatttggtaaccga
gtggatcatc

Example 9

Report #	**NE040615C Nitrogen Inducible Promoters.**
Trait area	**Nitrogen Use Efficiency**
Subtract Area	**Low Nitrogen Tolerance**
Promoter Sequences	**Promoters corresponding to the following genes; putative monodehydroascorbate Reductase (At1g63940), fibrillarin-2 (At4g25630),.**
Comments	**This report describes the promoters selected for nitrogen inducible gene expression.**

MATERIALS AND METHODS:

Gene expression that is consistently induced by low-to-high nitrogen treatment is used as the primary selection criterion to obtain promoter candidates. In short, *Arabidopsis thaliana* (ecotype Wassilewskija) seeds are sown on flats containing 4 L of a 1:2 mixture of Grace Zonolite vermiculite and soil. Flats are watered with 3 L of water and vernalized at 4°C for five days. Flats are placed in a Conviron growth chamber having 16 hr light/8 hr dark at 20°C, 80% humidity and 17,450 LUX. Flats are watered with approximately 1.5 L of water every four days. Mature, bolting plants (24 days after germination) are bottom treated with 2 L of either a control (100 mM mannitol pH 5.5) or an experimental (50 mM ammonium nitrate, pH 5.5) solution. Roots, leaves and siliques are harvested separately 30, 120 and 240 minutes after treatment, flash frozen in liquid nitrogen and stored at ⁻80°C.

Hybrid maize seed (Pioneer hybrid 35A19) are aerated overnight in deionized water. Thirty seeds are plated in each flat, which contained 4 liters of Grace zonolite vermiculite. Two liters of water are bottom fed and flats were kept in a Conviron growth chamber with 16 hr light/8 hr dark at 20°C and 80% humidity. Flats are watered with 1 L of tap water every three days. Five day old seedlings are treated as described above with 2 L of either a control (100 mM mannitol pH 6.5) solution or 1 L of an experimental (50 mM ammonium nitrate, pH 6.8) solution. Fifteen shoots per time point per treatment are harvested 10, 90 and 180 minutes after treatment, flash frozen in liquid nitrogen and stored at ⁻80°C.

Alternatively, seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) are left at 4°C for 3 days to vernalize. They are then sown on vermiculite in a growth chamber having 16 hours light/8 hours dark, 12,000-14,000 LUX, 70% humidity, and 20°C. They are bottom-watered with tap water, twice weekly. Twenty-four days old plants are sprayed with either water (control) or 0.6% ammonium nitrate at 4 $\mu L/cm^2$ of tray surface. Total shoots and some primary roots are cleaned of vermiculite, flash-frozen in liquid nitrogen and stored at ⁻80°C.

Any method of quantization of expression in the treated samples versus controls, such as microarray analysis can be used. Those genes showing increased expression under treatment conditions as compared to controls are identified as having candidate nitrogen-inducible promoters.

Quantitative PCR Validation of Nitrogen Inducible Gene Expression.

Expression profiles of the selected genes were verified by qRT-PCR with RNA samples. In addition, plants were cultivated hydroponically and submitted to low-to-high nitrate treatment. Plants were cultivated in a modified Hoagland's solution containing 15ppm of nitrogen as KNO3 (1.7mM KNO3) as the sole nitrogen (N) source. Plants were grown in a walk-in Conviron growth chamber under long day light cycle until they developed siliques and then transferred to 0.0 ppm N media for 3 days to adapt them to low nitrogen conditions. Nitrate induction was carried out by transferring experimental plants to 200ppm of N (14.3mM KNO3) and controls to 28.6mM mannitol. Root and rosette tissue from experimental and control plants (2 plants each) were harvested at 0.25, 1, 2, 4, 6 and 24 hours after treatment.

Analysis of Nitrate-Inducible Promoter:GFP fusions and Two-Component reporter gene constructs.

The promoter regions of five selected nitrate-inducible genes include 1000bp upstream of the first nucleotide 5' to the predicted ATG of the open reading frame. The promoter regions were shortened if a neighboring CDS overlapped the upstream 1000bp (Table 1). The sequences of the promoter regions are listened below. Primers including the restriction site BstX1 were designed to isolate these promoters by PCR (Table 2). The products were directly fused to mGFP5-ER in the vector Newbin4-35S-GFP. The selected promoters were also cloned into the two-component vector CRS815, upstream of VP16-HAP1. Transgenic T1 plants generated with these constructs were cultivated on soil and analyzed for expression of GFP in all aerial tissues under normal growth conditions.

Nitrogen-induced expression was analyzed in T2 generation plants. Seeds of each line were germinated on vertical MS minus N plates. Nitrogen induction was performed on seven days old seedlings by adding 3 ml/plate of 60 mM KNO_3 and Control plates were treated with 120 mM mannitol. GFP expression was visualized with Confocal laser scanning microscopy 6 and 24 hours after induction. In some cases, the induction time was extended to 48 hours.

Table 1. Nitrogen induced promoter candidates selected for GFP fusion and 2 component expression analyses constructs.

Locus ID	CDNA_ID	Gene Name	ANNOT_ID	Promoter pipeline_ID	PFAM_DESC	Genbank NR-DB Description
5847	12577385	At1g63940	520887	15372142	Pyridine nucleotide-disulphide oxidoreductase	putative monodehydroascorbate Reductase
21911	13497685	At4g25630	566416	15372151	Fibrillarin	Fibrillarin

Table 2. Oligonucleotides used for cloning into Newbin4-35S-GFP direct fusion construct.

			Oligos for cloning into CRS815		Oligos for cloning into Newbin4-35S-GFP	
Gene Name	**Promoter ID**	**Size (bp)**	**oligo 5' sequence**	**oligo 3' sequence**	**oligo 5' sequence**	**oligo 3' sequence**
At1g63940	15372142	921	(SEQ ID NO: 18) TTCACCAGTCG ATTGGCCCGAT CGGCCaaagttttg aattattggga	(SEQ ID NO: 19) CATGCCATTG CACTGGCCCT GCAGGCCtagttt ataagaagagccaa	(SEQ ID NO: 20) CCGGCGCCAG TCGATTGGGT TTTGTAATTCT TTGGGGG	(SEQ ID NO: 21) CGCGCGCCAG TGCAATGGGA CTCTACGAAC TGTAACAA
At4g25630	15372151	1000	(SEQ ID NO: 22) TTCACCAGTCG ATTGGCCCGAT CGGCCaaaaagg atgggtaatggga	(SEQ ID NO: 23) CATGCCATTG CACTGGCCCT GCAGGCCctttg cgttaagactctaaa	(SEQ ID NO: 24) CCGGCGCCAG TCGATTGGAA AAAGGATGGG TAATGGGA	(SEQ ID NO: 25) CGCGCGCCAG TGCAATGGCT TTGCGTTAAG ACTCTAAA

Analysis of nitrate induced promoters

Further analysis of GFP expression was carried out on mature plants cultivated in similar hydroponic conditions as described above. Nitrate induction was done by transferring plants to Hoagland's solution supplemented with 30mM KNO3. Plants were analyzed for GFP expression after 24, 48 and 72 hours of induction. Shoot and root tissues were collected for QRT-PCR analysis. A modification of this procedure was also implemented in order to avoid the adaptation period at 0.0 N. In this case, plants were cultivated in Hoagland's solution supplemented with 5 ppm N (600μM KNO3) and then transferred to media containing 30 mM KNO3. All experimental and control plants were genotyped for the presence of the promoter construct.

Promoter Sequences of Nitrogen Inducible Promoter Candidates. The ATG of predicted full length protein coding sequence occurs immediately downstream of the 3' nucleotide.

15372142 – At1g63940 predicted (Ceres cDNA_12577385; SEQ ID NO: 15)
gttttgtaattctttgggggctaataggatattttatttttcttggtttcgtctattgttgtttttctatttatggttgggcttttagaactctggacaggc
ccatgtcatatgttttcccttctccttatatttttcatttttcattttgttaaattaatgcataatatccaaaaacaatttaaatttttgaaggaaccccttt
agttacggctccgaagctttcacaagtgagaatgtgagatcaaagaaggcaaatggaggattttaaaagttaaaatcatcttttatctgcaaa
agttgacaatttttttgtatcaaatctaaatcatcaaactctcttaaactacaagagcataacaacctctatgtaatccatgaaataatctgcttg
aaggacataacataaatcattatggctagagtgactaacttcaatcaaatcctcttaactctagctcccttacaatggtatcgtaaaacattatg
cattagggattgttgtcctaggaaaataaaataaaaatccccacagaccaactaccattttaacttaaaaataagcttcgtccgcgacgaatt
gttttccatcctaaaaatagaatggtgtaatctgctaatggtttagttccattaacttgcaagttctattgaaagcctaaatgtcaataaagatatt
aaaattcggagtcaaaagacaaatgaatcaaaagcaacaagacaagtcagctccattcttcactacccatcttttacaataaatcatctctctt
ttcacaaatttcaaactactctcattgccctttagctttgttatagagccaacactacagagagactcacacacttgtttcaataattaaatctga
atttggctcttcttataaactaatgtctgcaggtcttcttatctctctcactcaccaccatcttcttcctcgattgtcaaaaccctagatcgaaatct
tatctctctaatctgttgttacagttcgtagagtc

15372142 – At1g63940 experimental (Ceres cDNA 13611030; SEQ ID NO: 16)

5'aaagttttgaattattgggaatcaatttcgaagttttgtaattctttgggggctaataggatattttatttttcttggtttcgtctattgttgtttttcta
tttatggttgggcttttagaactctggacaggcccatgtcatatgttttcccttctccttatatttttcatttttcatttttgttaaattaatgcataatatc
caaaaacaatttaaatttttgaaggaacccttttagttacggctccgaagctttcacaagtgagaatgtgagatcaaagaaggcaaatggag
gattttaaaagttaaaatcatcttttatctgcaaaagttgacaatttttttgtatcaaatctaaatcatcaaactctcttaaactacaagagcataac
aacctctatgtaatccatgaaataatctgcttgaaggacataacataaatcattatggctagagtgactaacttcaatcaaatcctcttaactct
agctcccttacaatggtatcgtaaaacattatgcattagggattgttgtcctaggaaaataaaataaaaatccccacagaccaactaccatttt
aacttaaaaataagcttcgtccgcgacgaattgttttccatcctaaaaatagaatggtgtaatctgctaatggtttagttccattaacttgcaagt
tctattgaaagcctaaatgtcaataaagatattaaaattcggagtcaaaagacaaatgaatcaaaagcaacaagacaagtcagctccattct
tcactacccatcttttacaataaatcatctctcttttcacaaatttcaaactactctcattgccctttagctttgttatagagccaacactacagag
agactcacacacttgtttcaataattaaatctgaatttggctcttcttataaacta3'

15372151 – At4g25630 (Cers cDNA 13497685; SEQ ID NO: 17)
5'aaaaaggatgggtaatgggacctattttccccaacatcccacatgcacacttccctctccattctctcacatttatttctttcattctaatttat
ccattccgtgtgtaacatattcactaataatctcatctcactaactcattcattgattgtgatatgtttatctagaattagtgttttaacactgtgtct
acatatgatttccttttcattgtatgtgaacatgttaactcactaatcattttgtattttcgagttaacatgagtctccacttcggtagactaaagta
aagataggtttgagtataataaagtttaaaatttgctttaaaatcaatatttataaataagtttttatcataagtgatttttgtatgttatattggacctt
gtataaacagactacagaagaaaattatttatgagaacttgtaatgttagagtggacctcgtataaactaattatgtgggcttttaccataaact
atttatgaaaattattatggcccacaccactataactaaagcccacatatttagcagcccagtttcattgtaagagacatgttcgctctggaac
tagaattttctggtttttgggtatttgttttcttatgtgtagagaaatgatggtaacgattaaatgttgtgtattacaatttacaatggtaagacgatt
aatatatttacacacaattttgttgttgctgtaacacgttagtgtgtgtgatgatagaatttcataaagctttaactacgaggggcaaaatgttaa
ttctaaatagttgacagcagaaaaagatatgtatacataatataaggattaaaacgtaaataataataaataaggcgagttaaattaaaaccc
tgttaaaaccctagcttgaaacacatgtataaaaacacttgcgagcgcagcttcatcgccatcgccattctctctctcatcaaaagcttttctc
cttgattttcgcattctttagagtcttaacgcaaag3'

RESULTS:

Gene expression that was consistently induced by low-to-high nitrogen treatment was used as the primary selection criterion to obtain promoter candidates. Selections with consistent expression profiles in replicate and across several experiments in either roots, leaves or siliques were made for independent experiments and then cross-referenced to other expression profile experiments in order to select against genes with highly variable expression patterns across several experiments.

qRT-PCR ANALYSIS OF 5 PUTATIVE NITROGEN INDUCIBLE GENES:

To verify the expression patterns of the selected genes, qRT-PCR was carried out with shoot RNA samples. Figure 6 shows the differential expression ratios obtained with qRT-PCR and the ratios obtained with the corresponding RNA samples used in the microarray experiment. The trend of induction is similar between the two data sets for most time points while the magnitude of response is sometimes much higher or lower in the qRT-PCR data.

In order to examine expression of the candidate promoters over longer induction times and to analyze expression in roots and shoots we carried out an extended nitrogen induction experiment in hydroponic conditions. The nitrogen content in the growth media of experimental and control plants

was monitored during treatment as shown in Figure 7. The results of differential expression ratios determined by qRT-PCR in roots and shoots are shown in the Figure 8. Expression in both shoots and roots was observed for all genes including the nitrate transporter gene At1g08100, which was originally selected for root specific expression from the Wang et al. 2003 TxP data set. The expression of At1g08100 in both shoots and roots is consistent with data reported by Okamoto et al., 2003. The monodehydroascorbate reductase gene shows similar high levels of induction in both shoots and roots. Overall, the data show that both of the selected genes are nitrogen-inducible.

T1 generation GFP Expression Analysis of Two-Component:GFP transgenic plants:

GFP expression data was obtained for the fibrillarin-2 (At4g25630) two-component promoter construct.under normal growth conditions. GFP expression for the fibrillarin-2 promoter construct was observed in only one out of 3 independent lines tested. The fibrillarin-2 (At4g25630) promoter drives GFP expression the inflorescence stem and a number of floral tissues. Moderate levels of expression are seen in sepals, petals, style and in the valve margins. No expression was observed in stamens, immature ovules or leaves. The fibrillarin-2 (At4g25630) promoter in the direct fusion construct shows a comparable expression pattern to the Two-component construct, however much weaker.

Table 3: Updated results of GFP expression in T1 transgenic plants derived from constructs of promoter candidates in two-component and direct fusion constructs.

Nitrogen promoters for construction of direct-GFP fusion constructs

	STATUS - Direct fusion lines	**Locus ID**	**CDNA ID**	**Gene Name**	ANNOT ID	METHOD	Promoter pipeline ID	T1 Lines tested	T1 Lines expressing
SR 01690	T1mature screened	21911	13497685	At4g25630	566416	OCDS	15372151	8	1
	STATUS-Two component lines								
PT 0829	T1 scheduled: 3 weeks old	5847	12577385	At1g63940	520887	OCDNA	15372142	6	0
PT 0665	T1 Mature screened	21911	13497685	At4g25630	566416	OCDS	15372151	4	1

T2 generation GFP Expression Analysis of transgenic plants treated with KNO3.

Seedlings of T2 lines from direct promoter:GFP fusion and two-component:GFP constructs were analyzed under nitrate inducing conditions. Lines of the 2-component:GFP fusion constructs of Fibrillarin-2 (At4g25630) and the monodehydroascorbate reductase gene (At1g63940) promoters showed inducible GFP expression. Strong induction of the pyridine nucleotide-disulfide oxidoreductase promoter was observed in roots and at a significant level in cotyledons. Expression of this promoter increased in time, being more intense after 48 hours of induction. The fibrillarin-2 promoter showed strong induction in cotyledons, including hypocotyls and pedicel. Induction was also significant in emerging rosette leaves and lateral roots. The promoter showed stronger expression at 6 hours of induction with a noticeable decrease after 24 hours. None of the transgenic plants from direct promoter:GFP fusions showed detectable induction.

EXPRESSION ANALYSIS OF MATURE PLANTS TREATED WITH KNO_3

Mature transgenic plants carrying either a direct fusion promoter:GFP or two-component:GFP construct were analyzed in hydroponic culture for expression of GFP in nitrate inducing conditions. In the first experiment, plants cultivated in 15 ppm nitrate, adapted to 0 ppm nitrate for 3 days followed by induction with 200ppm nitrate. Under these conditions, one event of a Fibrillarin-2 promoter - two-component construct, PT0665-01, showed nitrate induction in roots at 24 and 48 hours, however, in floral tissue the expression seems to decrease in induced plants. The other event for the Fibrillarin-2 2componenet construct tested, PT0665-02, showed expression in root tips, but no detectable induction. Two events of the two-component construct of the monodehydroascorbate reductase promoter, PT0829-04 and PT0829-05, showed weak induction of GFP expression in root vascular tissue after 48 and 72 hours of nitrate induction. No GFP expression was observed in aerial tissue of these lines. A line of monodehydroascorbate reductase promoter fused directly to GFP, SR01688-01, showed induction of expression in root vascular tissue at 48 hours. The rest of the lines tested showed no detectable GFP expression in control or experimental plants.

To study the induction of Fibrillarin-2 and monodehydroascorbate reductase gene promoters at a molecular level, RNA from root and shoot tissues were analyzed by QRT-PCR. We analyzed the expression of GFP, HAP1-VP16 and the corresponding endogenous genes (At1g63940 or At4g25630). The results reflect the GFP expression observed by fluorescence microscopy. For example, QRT-PCR of GFP and the endogenous gene in the two lines of the monodehydroascorbate reductase promoter (At1g63940) showed stronger induction in roots than in shoots. In these lines,

we detected GFP expression only in roots. In the case of Fibrillarin-2, stronger expression was obtained in shoots. Interestingly, with the exception of Fibrillarin-2 in shoots, the activity of the endogenous promoters and the isolated promoters followed the same trend. The activity of monodehydroascorbate reductase promoter was reduced significantly in the line PT0829-04 from 24 to 48 hours. In roots of PT0829-05, reduction of expression was significant after 72 hours. The Fibrillarin-2 line showed similar behavior in roots, however the activity of the promoter was stimulated in shoots.

The hydroponic conditions used in the experiment described above proved useful to test the inducibility of the promoters. However, during the procedure, before nitrate induction, the plants undergo an adaptation period from relatively high nitrogen to no nitrogen conditions. This step might introduce unpredicted responses of the promoters, which could obscure the nitrate induction response. To bypass the adaptation period, we modified the procedure by cultivation the plants under constant low nitrogen before induction with nitrate. The lines PT0665-01 (fibrillarin-2, At4g25630) and PT0829-05 (monodehydroascorbate reductase, At1g63940) were tested under these new conditions. We observed strong expression of GFP in pedicels of nitrate induced plants of line PT0665-01 after 24 hours. The GFP expression was more pronounced in pedicels after 48 hours of induction and significant induction was evident in root tips and the valve margins. Similar GFP expression patterns in aerial tissue were observed in PT0665 T1 generation plants cultivated on soil. The line PT0829-05 showed clear induction of GFP expression in roots. No expression was observed in any other tissue.

DISCUSSION

Nitrogen is most frequently the rate limiting mineral nutrient for crop production. Plants have evolved complex signaling and regulatory mechanisms to enable rapid physiological and metabolic response to changes in the supply of inorganic nitrogen in the soil. Part of this regulation is achieved through transcriptional regulation of gene expression. This is an important mechanism for allowing plants to adjust nitrogen uptake, reduction and transport in response to changing environmental conditions. Inefficiencies in nitrogen use efficiency may be overcome through the use of nitrogen regulated gene expression to modify the response of rate limiting enzymes and metabolic pathways to changes in nitrogen availability.

We selected nitrogen-induced genes in which nitrogen-induced gene expression is triggered in nitrogen-starved plants after supply with either nitrate alone or with ammonium nitrate. One selected gene, monodehydroascorbate reductase, functions in processes related to nitrate signaling,

transport, assimilation, and energy production. The other gene, fibrillarin-2, does not have a well-defined role in nitrogen metabolism. These genes were selected for GFP analysis in direct fusion vectors and in VP16-HAP1 two-component system as well as for cloning into VP16-HAP1 2-component GFP constructs and characterization in transgenic *Arabidopsis* plants. We verified the expression patterns observed for these genes using qRT-PCR with the same RNA samples used for a microarray hybridization. All of the genes showed similar trends to the transcription expression profiling data set. The expression of the genes was further characterized in roots and shoots of hydroponically grown plants using qRT-PCR.

The genes exhibit nitrate inducible expression in both roots and shoots.. The highest and most sustained level of expression was observed for At1g63940 which encodes a monodehydroascorbate reductase coding sequence. Overall the results suggest that all both genes selected for promoter analysis are nitrate inducible with different temporal patterns of nitrate induced expression.

Analysis of the promoters in the 2-component vector system indicates that two promoters are expressed to some degree under standard growth conditions containing sufficient nitrogen levels for normal plant growth. The monodehydroascorbate reductase promoter showed increasing expression of GFP after induction. Strong GFP expression was detected in roots and cotyledons. These expression patterns are in good agreement with the expression profile obtained in transcription expression profiling and qRT-PCR experiments for the corresponding gene. The fibrillarin-2 promoter was observed to drive GFP expression in a number of floral tissues and the stem under regular conditions. This promoter is also inducible by nitrate. Strong expression of GFP was observed in lateral roots and in most of the green tissue. The expression activity of the promoter seems to decrease after 24 hours of induction. To some extent this behavior does not reflects the expression pattern showed by the fibrillin-2 gene in transcription expression profiling and qRT-PCR experiments, where expression is sustained after 24 hours.

The monodehydroascorbate reductase and fibrillin-2 promoters fused directly to GFP did not show a significant increase in expression of GFP under nitrate inducing conditions on plates. Similar results were obtained in hydroponic conditions for direct promoter:GFP fusions. Only one line of the direct fusion monodehydroascorbate reductase (At1g63940) promoter:GFP showed detectable induction. It is possible that, under these inducing conditions, the promoters are not sufficiently strong to stimulate expression of detectable levels of GFP, or that additional transgenic events need to be examined to select for stronger expression. Nitrate induction analysis of the lines in

hydroponics revealed that the Fibrillarin-2 and the monodehydroascorbate reductase promoters are inducible by nitrate. A clearer response was observed under modified inducing conditions. The GFP expression patterns observed, and gene expression determined by QRT-PCR, indicated that the fibrillarin-2 promoter is preferably induced in shoots (mostly in reproductive tissue), while the monodehydroascorbate reductase promoter is induced in roots.

APPLICABILITY OF PROMOTERS TO CORN AND OTHER SPECIES

The fibrilliarin-2 promoter will be useful for driving expression in flowers especially pedicels and silique vasculature and may be useful for increasing nutrient transport and/or utilization in reproductive organs. The monodehydroascorbate reductase promoter will be useful driving nitrate inducible expression in roots.

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phosphogluconate dehydrogenase activity, protein and transcript levels. Plant Science 134, 129-140.

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The invention being thus described, it will be apparent to one of ordinary skill in the art that various modifications of the materials and methods for practicing the invention can be made. Such modifications are to be considered within the scope of the invention as defined by the following claims.

Each of the references from the patent and periodical literature cited herein is hereby expressly incorporated in its entirety by such citation.

CLAIMS

What is claimed is:

1. An isolated nitrogen responsive promoter capable of modulating transcription comprising a nucleic acid molecule having at least 85% sequence identity to any one of SEQ ID NOs: 1-17, or a complement thereof.
2. The isolated promoter of claim 1, wherein said nucleic acid comprises a sequence corresponding to any one of SEQ ID NOs: 1-17 having at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.
3. A vector construct comprising:
   a) a nitrogen responsive promoter capable of modulating transcription comprising a first nucleic acid molecule having at least 80% sequence identity to any one of SEQ ID NOs: 1-17; and
   b) a second nucleic acid molecule having to be transcribed,

   wherein said first and second nucleic acid molecules are heterologous to each other and are operatively linked together.
4. The vector construct according to claim 3, wherein said nucleic acid comprises a sequence according to any one of SEQ ID NOs: 1-17 with at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.
5. A host cell comprising an isolated nitrogen responsive promoter according to claim 1, wherein said nucleic acid molecule is flanked by exogenous sequence.
6. A host cell comprising a vector construct of claim 3.
7. A method of modulating transcription by combining, in an environment suitable for transcription:
   a) a nitrogen responsive promoter capable of modulating transcription comprising a first nucleic acid molecule having at least 80% sequence identity to a sequence according to any one of SEQ ID NOs: 1-17; and
   b) a second molecule to be transcribed;

wherein the first and second nucleic acid molecules are heterologous to each other and operatively linked together.

8. The method according to claim 7, wherein said first nucleic acid molecule is inserted into a plant cell and said plant cell is regenerated into a plant.

9. A plant comprising a vector construct according to claim 3.

10. A method of introducing an isolated nucleic acid into a host cell comprising:

a) providing an isolated nucleic acid molecule according to claim 1; and

b) contacting said isolated nucleic acid with said host cell under conditions that permit insertion of said nucleic acid into said host cell.

11. A method of transforming a host cell that comprises contacting a host cell with a vector construct according to claim 3.

12. A method for detecting a nucleic acid in a sample which comprises:

a) providing an isolated nucleic acid molecule according to claim 1;

b) contacting said isolated nucleic acid molecule with a sample under conditions which permit a comparison of the sequence of said isolated nucleic acid molecule with the sequence of DNA in said sample; and

c) analyzing the result of said comparison.

13. A plant, plant cell, plant material or seed of a plant which comprises a nucleic acid molecule according to claim 1 which is exogenous or heterologous to said plant or plant cell.

14. A plant, plant cell, plant material or seed of a plant which comprises a vector construct according to claim 3.

15. A plant that has been regenerated from a plant cell or seed according to claim 13 or 14.

16. A plant, plant cell, plant material or seed of a plant which comprises a nucleic acid molecule according to claim 1, wherein said plant has improved nitrogen responsiveness characteristics as compared to a wild-type plant cultivated under the same conditions.

17. A method for increasing nitrogen responsiveness in a plant comprising transforming a plant with a nucleic acid sequence according to claim 1.

18. A plant having a gene construct comprising a nucleic acid encoding a nitrogen responsive promoter operatively linked to a coding sequence so that the coding sequence is ectopically overexpressed in the plant in response to abnormal nitrogen conditions, and the plant exhibits:

i) faster rate of growth,

ii) greater fresh or dry weight at maturation,

iii) greater fruit or seed yield,

iv) higher tolerance to abnormal nitrogen conditions,

v) greater germination rate under abnormal nitrogen conditions,

viii) reduced nitrogen needs,

ix), greater tolerance to excess nitrogen, or

x) improved performance

than a progenitor plant when the plant and the progenitor plant are cultivated under identical environmental conditions, wherein the nitrogen responsive promoter is promoter sequence according to claim 1.

19. A crop plant having a gene construct comprising a nucleic acid encoding a nitrogen responsive promoter operatively linked to a coding sequence so that the coding sequence is ectopically overexpressed in the crop plant in response to sub-optimal nitrogen conditions, and the crop plant exhibits:

i) faster rate of growth,

ii) greater fresh or dry weight at maturation,

iii) greater fruit or seed yield,

iv) higher tolerance to sub-optimal nitrogen conditions,

v) greater germination rate under sub-optimal nitrogen conditions,

viii) reduced nitrogen needs, or

ix), greater tolerance to excess nitrogen, or

x) improved performance

than a progenitor plant when the crop plant and the progenitor plant are cultivated under identical environmental conditions, wherein the nitrogen responsive promoter is a promoter sequence according to claim 1.

20. A plant having a gene construct comprising a nucleic acid encoding a nitrogen responsive promoter operatively linked to a coding sequence so that the coding sequence is ectopically overexpressed in the plant under normal nitrogen conditions, and the plant exhibits:

i) faster rate of growth,

ii) greater fresh or dry weight at maturation,

iii) greater fruit or seed yield,

iv) higher tolerance to normal nitrogen conditions,

v) greater germination rate under normal nitrogen conditions,

viii) reduced nitrogen needs,

ix) greater tolerance to excess nitrogen, or

x) improved performance

than a progenitor plant when the transgenic plant and the progenitor plant are cultivated under identical environmental conditions, wherein the nitrogen responsive promoter is a promoter sequence according to claim 1.

FIGURE 1

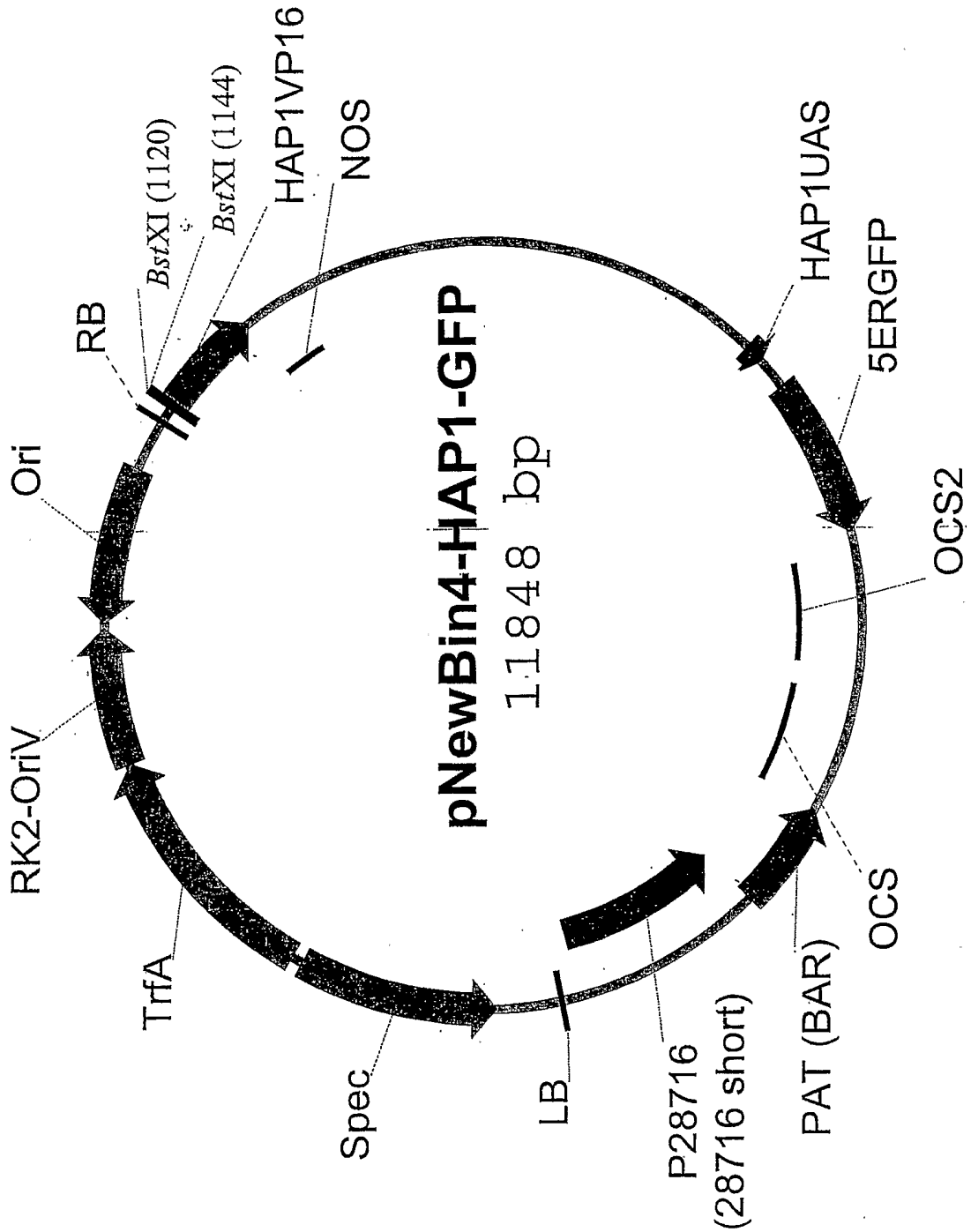
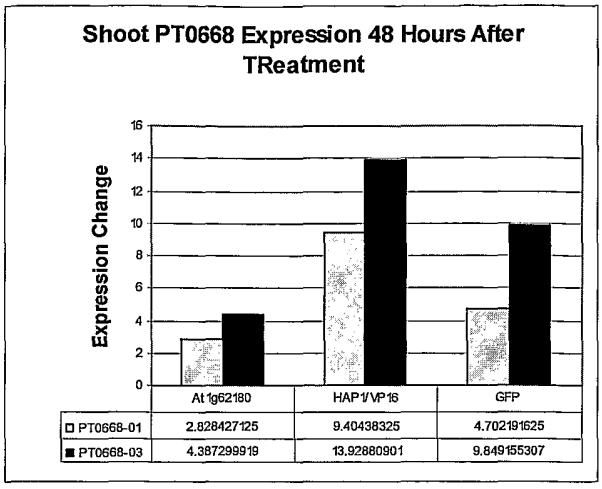

pNewBin4-HAP1-GFP
11848 bp
RB
BstXI (1120)
BstXI (1144)
HAP1VP16
NOS
HAP1UAS
5ERGFP
OCS2
OCS
PAT (BAR)
P28716
(28716 short)
LB
Spec
TrfA
RK2-OriV
Ori

Figure 2 qRT-PCR Data for Example 3



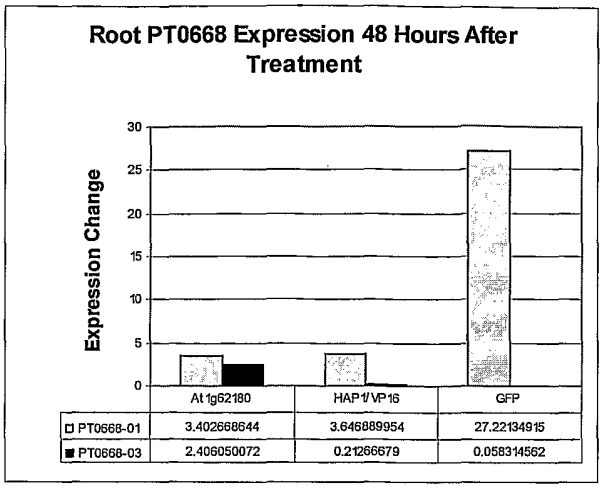


Figure 3. qRT-PCR Data for Example 4

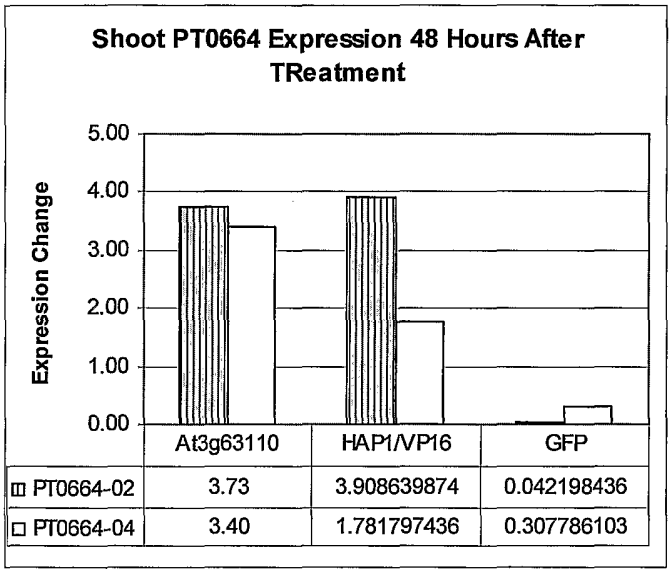
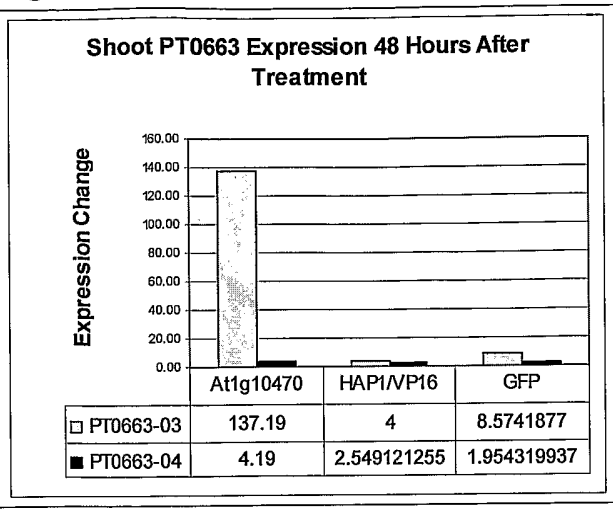
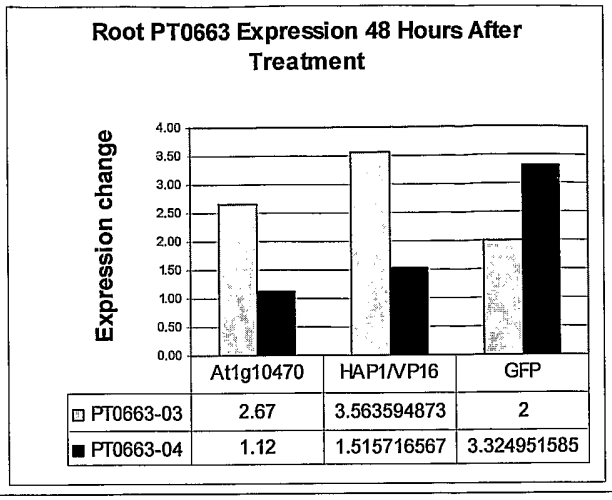


Figure 4. qRT-PCR Data for Example 5

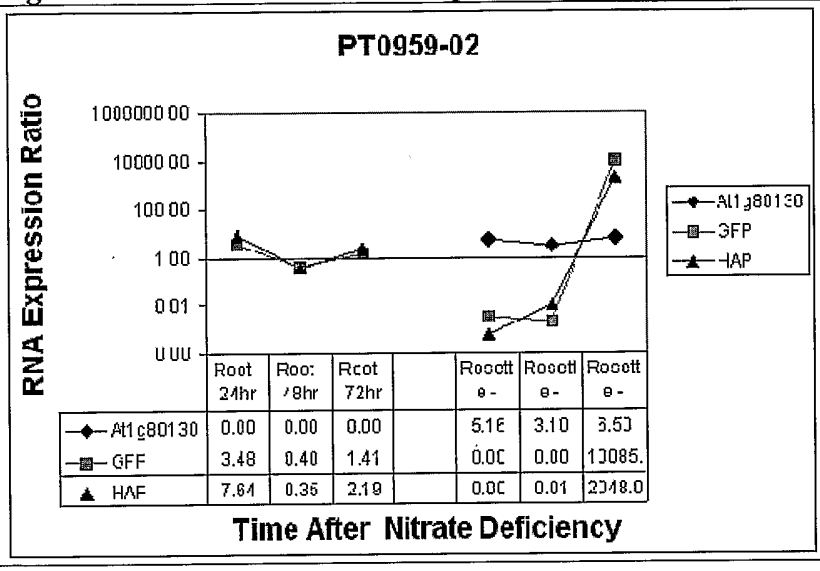


	At1g10470	HAP1/VP16	GFP
PT0663-03	137.19	4	8.5741877
PT0663-04	4.19	2.549121255	1.954319937



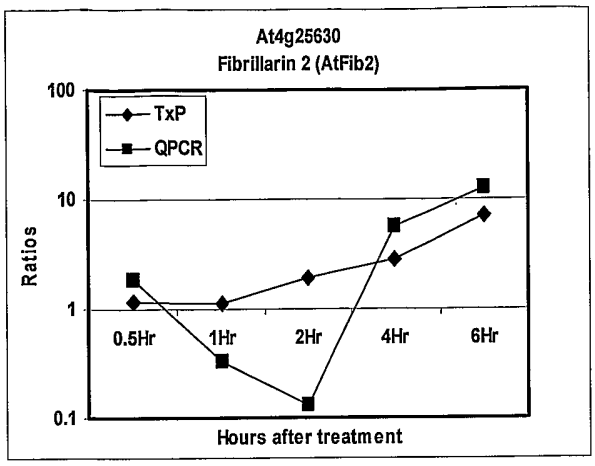
	At1g10470	HAP1/VP16	GFP
PT0663-03	2.67	3.563594873	2
PT0663-04	1.12	1.515716567	3.324951585

Figure 5: RT PCR Data for Example 8

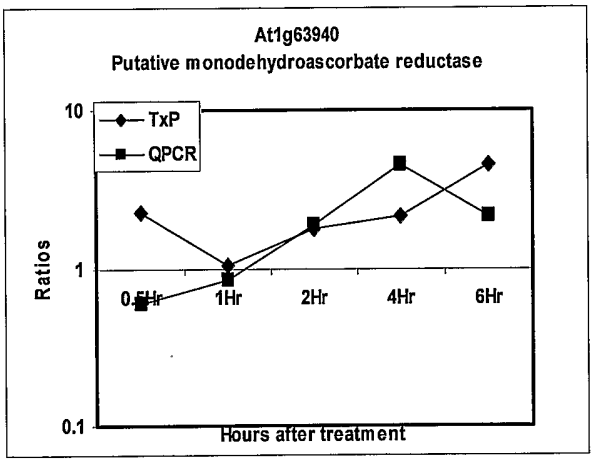


	Root 24hr	Roo: /8hr	Rcot 72hr		Roocti e-	Roocti e-	Roocti e-
At1g80130	0.00	0.00	0.00		5.16	3.10	3.53
GFF	3.48	0.40	1.41		0.00	0.00	10085.
HAF	7.64	0.36	2.19		0.00	0.01	2048.0

Figure 6 A and B. Differential Expression of Selected Genes in Leaves for Example 9. The graphs show comparison of ratios obtained with qRT-PCR and microarray. Y-axis is ratio of experimental to control signal. **A**: Fibrillarin-2. **B**: Putative monodehydoascorbate reductase



A



B

Figure 7. Nitrate (N) content in growth media experimental and control plants hydroponically cultivated for Example 9. L-H KNO3 - Experimental sample, L-H Mann – control sample. The striped bar indicates the value for a NO3 standard.

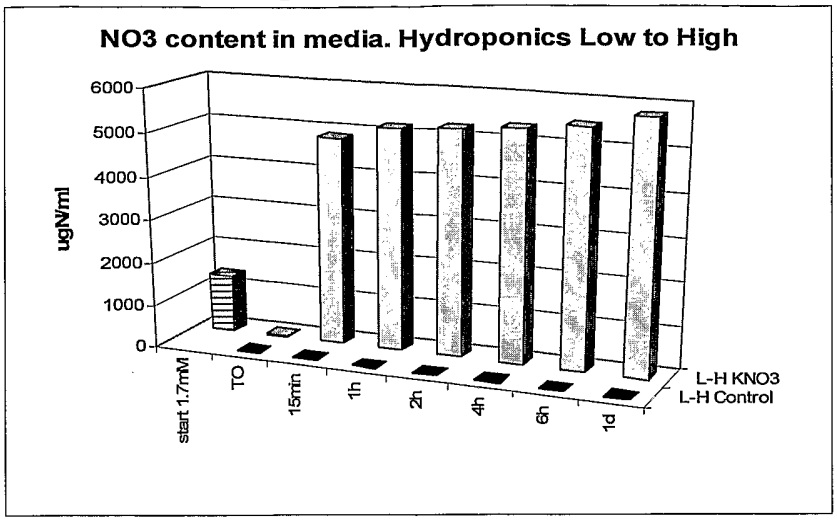
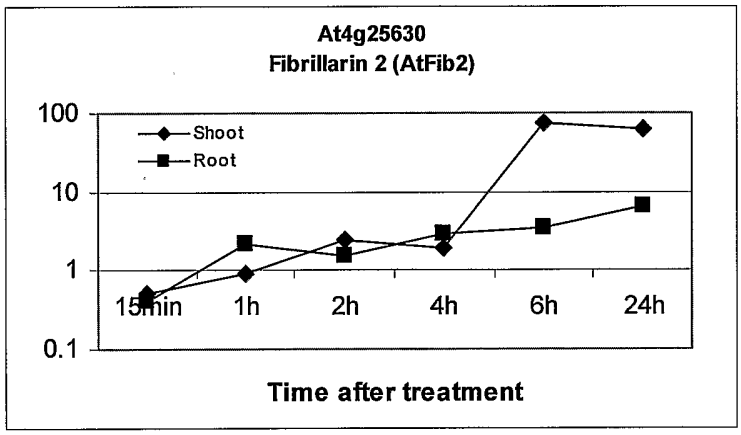
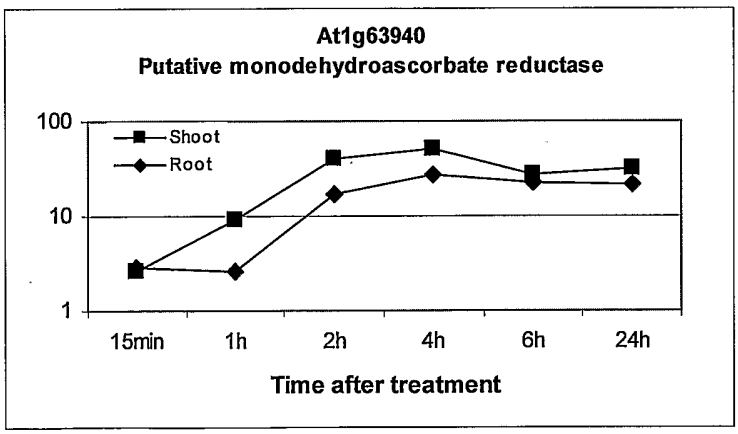


Figure 8. Differential Expression of Selected Genes in Roots and Shoots for Example 9. The nitrogen treated plants were cultivated in hydroponic conditions. Y-axis is ratio of experimental to control signal.

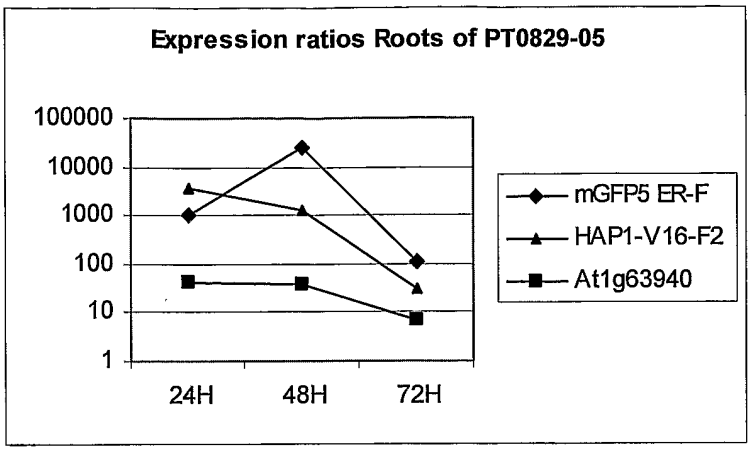


A

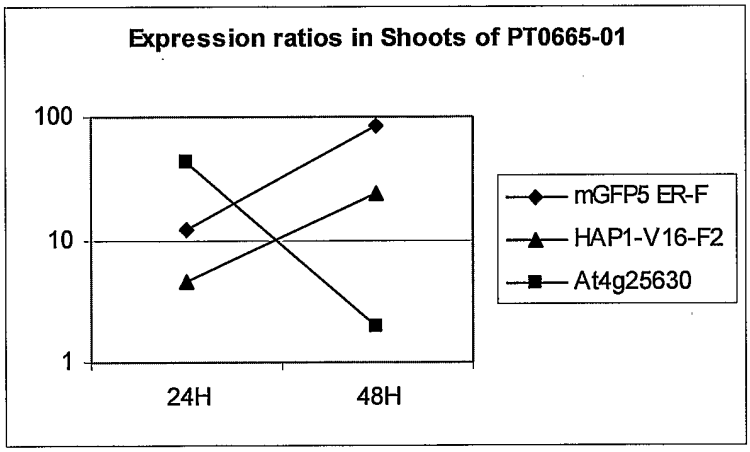


B

Figure 9 A and B. Differential expression in roots and shoots of T2 mature plants cultivated in hydroponic conditions for Example 9. Y-axis is the ratio of experimental to control signal. Fibrillarin-2 = At4g25630, monodehydroascorbate reductase =At1g63940. **A**: Putative monodehydroascorbate reductase. **B**: Fibrillarin-2



A



B

Figure 10

SCHEMATIC OF A GENE