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REVIEW ARTICLE

Identification of Biomarker for Determining Genotypic Potential of Nitrogen-Use-Efficiency and Optimization of the Nitrogen Inputs in Crop Plants

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Abstract

Worldwide, the nitrogen use efficiency (NUE) for crop plants is of great concern. The burgeoning world population needs crop genotypes that respond to higher nitrogen and show a direct relationship to yield with use of nitrogen inputs, i.e. high nitrogen-responsive genotypes. However, for fulfilling the high global demand of organic produce, it requires the low nitrogen responsive genotypes with greater NUE and grain yields. The lack of knowledge about precise regulatory mechanisms to explain NUE in crop plants hampers the goal of agricultural productivity. Understanding the molecular basis of NUE will enable to provide handle for crop improvement through biotechnological means. With the advent of modern genomics and proteomics approaches such as subtractive hybridization, differential display, and microarray techniques are revolutionizing to identify the candidate genes which play a pivotal role in the regulation of NUE. Beside it, quantitative real-time polymerase chain reaction technology is also being used to establish marker-trait association for NUE. The identification of potential candidate genes/proteins in the regulation of NUE will serve as biomarker(s) for screening genotypes for their nitrogen responsiveness for optimization of nitrogen input in agriculture. This paper describes the molecular basis of NUE with respect to nitrogen metabolism and its intimate relationship with carbon metabolism, use of molecular-physiological-genetics approaches for understanding the role of various genes/proteins, and their validation to use as biomarker(s) for determining genotypic potential for NUE. Since NUE in plants is a complex trait which not only involves the primary process of nitrogen uptake and assimilatory pathways but also a series of events, including metabolite partitioning, secondary remobilization, C-N interactions, as well as molecular signalling pathways and regulatory control outside the metabolic cascades. Therefore, identification of novel nitrogen responsive genes and their cis- and trans-acting gene elements is essential. Thus, fishing out a single gene, biomarker or a master regulator controlling complex trait of NUE could serve as an appropriate strategy for nitrogen management in agriculture.

Key words: Nitrogen use efficiency, nitrogen metabolism, biomarker, Dof transcription factor

Introduction

Although the "Green Revolution"-based modern agriculture helped in increasing crop production and averting hunger, it also had a number of negative ecological consequences such as depletion of lands, decline in soil fertility, soil salinization, soil erosion, deterioration of environment, health hazards, poor sustainability of agricultural lands, and degradation of biodiversity. The indiscriminate use of pesticides, irrigation, and imbalanced fertilization has threatened sustainability. In the last 50 years, the N fertilization of crop plants worldwide has increased more than 20-fold. However, use of this fertilizer is generally inefficient, as only about a third of the fertilizer applied is actually absorbed by crops, and 50-70% is lost from the plant-soil systems (Tabashnik 1994). Unused fertilizer can leach into the environment where it induces algal blooms, contaminates drinking water, and depletes aquatic oxygen to create dead zones, like those found in the Gulf of Mexico (Janmaat and Myers 2003).

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Elevated nutrient inputs into aquatic ecosystems due to heavy use of N and phosphorus leads to eutrophication and increases pathogenic infection in aquatic life forms. On the other hand, issues like declining use efficiency of inputs in the form of nutrients like nitrogen and dwindling output-input ratios have rendered crop production less remunerative. Demand for low-input sustainable crop cultivation is increasing to meet the need for environment-friendly agriculture. Hence, we need to reconsider our agricultural practices and adopt environmental-friendly practices. Consequently, developing genotypes with high NUE is one of the major objectives of crop breeding programs (Delmer 2005).

The question therefore arises, can we, based on our knowledge and the experimental techniques now available to us, improve the efficiency of nitrogen use by crop plants? Two possibilities appear, one to make best use of the available variation in nitrogen-use characteristics within the gene pool and second, to try to introduce new genes which might increase that variation. The best approach would be to identify and select cereal cultivars which absorb and metabolize nitrogen in the most efficient way (increased NUE) for grain or silage production, i.e. genotypic potential for high NUE. However, fulfilling the high global demand of organic produce requires low nitrogen-responsive genotypes with greater NUE and grain yields.

Nitrogen use efficiency (NUE) in crop plants

Nitrogen use efficiency (NUE) at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity. This includes nitrogen uptake and assimilatory processes, redistribution within the cell, and balance between storage and current use at the cellular and whole plant levels. In terms of agriculture, it is the optimal utilization of nitrogenous manures or fertilizers for plant growth, yield, and protein content, as atmospheric nitrogen gas is not utilized by higher plants, except symbiotic legumes. Nitrogen use efficiency is calculated as grain yield/ available nitrogen content (soil + fertilizer nitrogen or only fertilizer nitrogen). There are two components of nitrogen use efficiency: 1) the efficiency of absorption (uptake) and 2) the efficiency with which the N absorbed is utilized to produce grain (utilization) (Moll et al. 1982). Nitrogen uptake efficiency was calculated as plant nitrogen content/available nitrogen content (soil + fertilizer nitrogen or only fertilizer nitrogen), and nitrogen utilization efficiency was calculated as grain yield/plant nitrogen content. To increase agricultural production, several technologies and practices hold promise for improving NUE. Some possible opportunities are (Giller et al. 2004): i.) Increasing yield potential and yield stability through genetic improvement and crop management, ii.) Balanced nutrition to allow optimum utilization of available N, iii.) Split N applications to better match N requirements of crops through the growing season, iv.) More efficient fertilizer products that better synchronize N release and crop N demand (e.g. slow- and controlled-release fertilizers), v.) Fertilizer additives to reduce N losses (e.g. urease and nitrification inhibitors), vi.) Site-specific N-management prescriptive (before planting), corrective (Using in-season diagnostic tools), or both, vii.) Decision support systems: computer-based models or simple field assessment tools and interpretation aids, viii.) Genetic improvement in N recovery or N utilization efficiency of some crops (primarily those having received little attention by breeders in the past such as under-utilized crops), and ix.) Use of organic manures, green manuring of legumes in cropping systems.

While the amount of N available to the plant can be improved by using sustained-release fertilizers, split applications, minimizing fertilizer losses, and other nutrient and crop management strategies (Abrol 1993; Kanwar and Katyal 2000), the inherent efficiency of the plant to utilize available N-inputs for higher productivity needs to be tackled biologically. Since overuse of inorganic nitrogenous fertilizers is hazardous to the environment, unused fertilizers are washing off fields into rivers, poisoning coastal waters, and causing acid rain. Therefore, the creation of crops with high nitrogen efficiency is agriculturally important and might pose a big challenge for molecular breeding. Marker-assisted selection of genotypes responding to low and high nitrogen inputs will enable us to enhance agricultural productivity and production of organic produce. This in turn, led to the identification of novel nitrogen responsive genes and their cis- and *trans*-acting gene elements, which can be used as biomarkers for determining of genotypic potential of NUE. Such types of biomarkers would enable to help in optimization of nitrogen inputs in crop plants including cereal production.

Factors affecting NUE

Many ^{15}N recovery experiments have reported loss of N-fertilizer in cereal production from 20 to 50%. These losses have been attributed to the combined effects of denitrification, volatilization, and/or leaching (Francis et al. 1993; Olson and Swallow 1984). Loss of fertilizer N results from:

i.) Soil nitrification/denitrification: Certain soil bacteria that thrive in saturated (anaerobic) soil will convert nitrate N to oxygen and nitrogen gases. Reported gaseous N losses due to denitrification from applied fertilizer N include 9.5% in winter wheat (Aulakh et al. 1982), 10% in lowland rice (DeDatta et al, 1991), and 10 (conventional tillage) to 22% (no-till) in corn (Hilton et al. 1994). Incorporation of straw and/or application of straw on the surface of zero-till plots can double denitrification losses (Aulakh et al. 1984).

ii.) NO_3^--leaching: All applied N-fertilizer sources eventually convert completely to the nitrate N form. This form is not held tightly by soil particles and can be leached from the soil profile with excessive rains especially on lighter textured soil. When fertilizer N is applied at rates in excess of that needed for maximum yield in cereal crops, nitrate leaching can be significant (Olson and Swallow- 1984; Raun and Johnson- 1995). In cooler temperate climates, nitrate losses through tile drainage have approached 26 kg N ha^{-1} yr^{-1} under conventional tillage corn when only 115 kg N ha^{-1} was applied (Drury et al. 1996).

iii.) Volatilization of urea-based products: Urea-based fertilizers products are susceptible to volatilization losses of N if surface applied and then not incorporated. Urease enzyme in the

soil and plant residues converts the urea component to free ammonia gas. If this conversion occurs at the soil surface and is accompanied by warm sunny days, as much as 15-20% of the urea based nitrogen may volatilize within a week after application.

iv.) Inherent ability of genotypes.

v.) Presence of soil microflora.

vi.) Beside these, nitrogen metabolism is a major contributing factor which affects nitrogen use efficiency.

Molecular mechanism associated with nitrogen metabolism

Nitrogen uptake

Nitrogen is one of the important mineral nutrients needed in greatest abundance by plants. However, plants also compete for nitrogen in the soil with abiotic and biotic processes such as erosion, leaching, and microbial consumption. Soil nitrogen is also lost when crops are harvested and plant material is removed from the soil. To be competitive, plants have evolved several mechanisms to acquire nitrogen at low concentrations and to use a variety of forms of nitrogen. Plants can assimilate inorganic forms, such as nitrate and ammonia, and organic forms, such as urea. Some plants, including legumes, can fix dinitrogen gas in association with symbiotic bacteria (Mylona et al. 1995). Among the various forms of N available to the plant, nitrate (NO_3^-) is the major source of nitrogen for the vast majority of plants. In most plant species, only a proportion of the absorbed NO_3^- is assimilated in the root, the remainder being transported upwards through the xylem for assimilation in the shoot. In situations of excess NO_3^- supply, high concentrations of NO_3^- can accumulate in the vacuole and some of the NO_3^- may also be lost to the soil solution by efflux across the PM (Forde and Clarkson 1999). The vacuolar store of NO_3^- may be used to help maintain the concentration of the cytosolic NO_3^- pool, which has been reported to be held relatively constant under a wide range of external NO_3^- concentrations (Miller and Smith 1996). Thus, an essential element in the process of NO_3^- assimilation is the trafficking of the NO_3^- ion across membranes.

Nitrate Uptake

Nitrate uptake by root cells is a key step of nitrogen metabolism and has been widely studied at the physiological and molecular levels (Orsel et al. 2002). In the soil solution, nitrate is carried towards the root by bulk flow and is absorbed into the epidermal and cortical symplasm. Within the root symplasm, NO_3^- has four fates: (1) reduction to NO_2^- by the cytoplasmic enzyme nitrate reductase, (2) efflux back across the plasma membrane to the apoplasm, (3) influx and storage in the vacuole, or (4) transport to the xylem for long-distance translocation to the leaves (see Fig. 1). Following long-distance translocation, NO_3^- must leave the xylem and enter the leaf apoplasm to reach leaf mesophyll cells, where NO_3^- is again absorbed and either reduced to NO_2^- or stored in the vacuole. Little attention has been paid to the transport processes involved in leaf absorption of NO_3^-, however, the high NO_3^- concentrations of the xylem sap (5-40 mM) and the low levels of NO_3^- in the phloem (Schobert and Komor

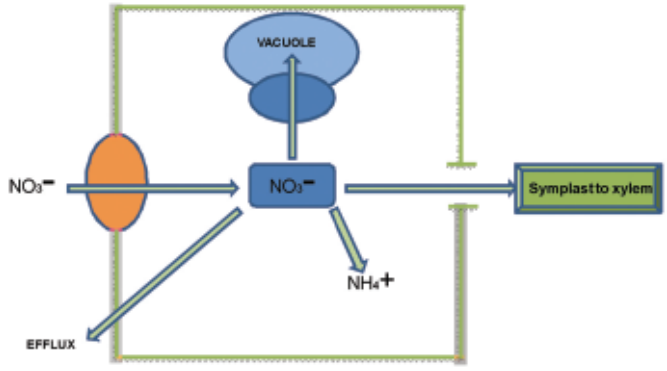

Fig. 1. Fate of nitrate within root

1992) indicate that NO_3^- must be efficiently absorbed by leaf cells.

The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy the total demand for nitrogen in the face of external nitrate concentrations that can vary by five orders of magnitude. To function efficiently in the face of such environmental variation, plants have evolved a transport system that is active, regulated, and multiphasic. The energy that drives nitrate uptake comes from the proton gradient maintained across the plasma membrane by the H^+-ATPase. In addition, NO_3^- uptake is associated with depolarization of the plasma membrane (an increase in the positive charge inside the cell). The accumulated evidence from kinetic studies indicates that roots have at least three distinct NO_3^- uptake systems (Forde and Clarkson 1999; Glass and Siddiqi 1995), two of which have a high affinity for NO_3^-, while the third has a low affinity. One of the high-affinity systems is strongly induced in the presence of an external NO_3^- supply and is known as the inducible high-affinity transport system (or iHATS), while the second high-affinity system (the cHATS) is constitutively expressed (Aslam et al. 1992). Typically, these systems have saturable kinetics. Constitutive high affinity transport systems (CHATS) are characterized by low values of both Km and Vmax (typically 6-20 μM and 0.3-0.82 μmol $g.h^{-1}$, respectively). High affinity transporters (IHATS) with higher Km and Vmax values (typically 20-100 μM and 3-8 μmol $g.h^{-1}$, respectively) are induced within hours to days of exposure to NO_3^- Finally, constitutive low affinity transporters (LATS), which can significantly contribute to nitrate uptake at concentrations above 250 mM, fail to saturate at NO_3^-concentrations as high as 50 mM. Thermodynamic evaluations demonstrate that NO_3^-uptake by LATS is also active, in spite of the linear response to concentration (Glass et al. 1992).

Ammonium assimilation

Since ammonium assimilation requires less energy than that of nitrate (Bloom et al. 1992), ammonium is the preferential form of nitrogen uptake when plants are subjected to nitrogen deficiency (Gazzarrini et al. 1999). However, excessive ammonium uptake into plants can be toxic (Britto et al. 2001; Kronzucker et al. 2001). In contrast to nitrate, plants in general tend not to accumulate high concentrations of ammonium ions. Toxicity symptoms frequently occur if crop plants are grown in

ammonium in the absence of nitrate (Britto and Kronzucker 2002), although rice is an exception. Therefore, ammonium uptake and metabolism in plants must be tightly regulated. In anaerobic agricultural soils, in particular in paddy fields, ammonium is the major form of inorganic nitrogen, making rice almost totally dependent on ammonium nutrition during a large part of the cropping season. Also, in terms of the efficiency of fertilizer utilization, ammonium is superior to nitrate in paddy soil (Yoshida 1981).

The ammonium uptake is facilitated by the presence of transporter proteins. Both HATS and LATS for ammonium uptake are present in plant roots that are constitutive and do not seem to be significantly induced by ammonium (Glass et al. 2002). However, rice is able to take up NH_4^+ ions from the soil solution when grown in a paddy field, through the action of ammonium transporters (AMTs). Ammonium transporters that have been isolated and partially characterized in several plant species, such as *Arabidopsis thaliana*, (AtAMT1;1, AtAMT1;2, AtAMT1;3, and AtAMT2; Gazzarrini et al. 1999; Kaiser et al. 2002; Ninnemann et al. 1994; Sohlenkamp et al. 2000; Sohlenkamp et al. 2002), *Brassica napus* (BnAMT1;2; Pearson et al. 2002), *Lotus japonicus* (LjAMT1;1, LJAMT2;1; Salvemini et al. 2001, Simon-Rosin et al. 2003), *Lycopersicon esculentum* (LeAMT1;1, LeAMT1;2, and LeAMT1;3; Becker et al. 2002; Lauter et al. 1996; von Wirén et al. 2000) or *Oryza sativa* (OsAMT1;1, OsAMT1;2, OsAMT1;3, OsAMT2;1, OsAMT2;2, OsAMT2;3, OsAMT3;1, OsAMT3;2, OsAMT3;3, and OsAMT4 (Loqué & von Wirén, 2004; Suenaga et al. 2003).

A family of five ammonium transporter genes designated AMT1;1 to AMT 1;5 were originally identified in *A. thaliana*, which were related to cyanobacterial ammonium transporters, while in tomato, only three AMT1 genes were isolated. Both plants had a second AMT2 gene whose sequence is more closely related to transporters isolated from *Saccharomyces cerevisiae* and *Escherichia coli* (Loqué and von Wirén 2004). However, in rice 10 different AMT genes have been identified which have been divided into two types of transporters i.e., (1) high efficiency ammonium transporters and (2) low efficiency ammonium transporters which are further classified into four different clades on the basis of protein sequence homology (Suenaga et al. 2003). Interestingly, rice is one of the few crop plants that is adapted to high ammonium nutrition. The AMT proteins have 11 transmembrane- spanning domains, with an extracytosolic N-terminus and a cytosolic C-terminus (Loqué and von Wirén 2004). Additional genes encoding tonoplast intrinsic proteins have been identified, which transport ammonium into the vacuole (Loqué et al, 2005).

The ammonium taken up by plant roots or produced by a reduction of nitrate is first assimilated by glutamine synthetase (GS) to yield the amino group of Gln. In higher plants, Gln serves as a major source transported from root to shoot through the xylem. GS is coupled to glutamate synthase (GOGAT) in a so-called GS/GOGAT cycle. GS produces Gln from ammonium and Glu, and GOGAT transfers the amino group of Gln to 2-oxoglutarate to generate two molecules of Glu in the cycle. Besides forming glutamate, glutamine can also donate its amide group to aspartic acid to form asparagines. This reaction is catalyzed by asparagine synthetase. These four amino acids are precursors of all nitrogen-containing organic biomolecules. The translocation from sources to sinks occurs in this form (Lea and Miflin 1980; Peoples and Gifford 1993). The fourth major enzyme in nitrogen assimilation is Glutamate dehydrogenase (GDH). This enzyme can catalyze both forward and backward biochemical reactions; the amination of 2-oxoglutarate into glutamate (anabolic reaction) and/or the deamination of glutamate into ammonia and 2-oxoglutarate (catabolic reaction) (Lea et al. 1990). These organic nitrogen compounds assimilate in leaves and stem of plants in the form of protein, amino acids, nucleotides, and chlorophyll (Lea 1993). Although, there is wide acceptance for the GS/GOGAT cycle, controversy still exists as to the role of the enzyme GDH in higher plants because the general characteristics of the enzyme offer conflicting evidence with regard to its role as an assimilating enzyme. *In vitro* the thermodynamically favored direction of the reaction is the production of glutamate, but the enzyme is reported as having a very high Michaelis constant with respect to ammonium, a characteristic that argues strongly against an assimilatory role (Milfin and Lea 1980).

Metabolite Partitioning and C: N Interactions

At first sight, nitrate is utilized in a linear pathway that involves the uptake and transport of nitrate within the plant, followed by nitrate assimilation, ammonium assimilation, amino acid biosynthesis, and protein synthesis. There are, however, complex interactions with many other aspects of nitrogen metabolism, including (i) the storage and remobilization of nitrate in different parts of the plant, (ii) *de novo* ammonium assimilation, (iii) the recycling of ammonium released during photorespiration (Hirel and Lea 2001), (iv) the distribution of nitrogen between the highly branched pathways of amino acid biosynthesis (Morot-Gaudry et al, 2001), and (v) the multifarious fates of amino acids, which can be exported, stored in the vacuole, used for protein synthesis, or diverted into secondary metabolic pathways leading to phenylpropanoids, alkaloids, and tetrapyrroles (Heldt 1996). There is also a complex interaction with carbon metabolism which provides (vi) malate as a counter-anion to prevent alkalinization (Martinioa and Rentsch 1994), (vii) 2-oxoglutarate as the primary acceptor of ammonium in the GOGAT pathway (Heldt 1996), and (viii) numerous other organic acids and phosphorylated intermediates that are required as carbon precursors in the various amino acid pathways (Morot-Gaudry et al. 2001). Further, (ix) reactions in photosynthesis or carbohydrate breakdown are required to generate the reducing equivalents that are consumed during the reduction of nitrate to ammonium (Foyer et al. 2001; Kaiser et al, 2000).

Enzymes and Genes involved in carbon and nitrogen metabolism

The most studied gene is that for NR, the first gene shown to be nitrate inducible (Cheng et al. 1986; Crawford et al. 1986; Tang and Wu 1957). NR mRNA accumulates in plants within minutes after treatment with nitrate at concentrations from 10

µM to 50 mM (Aslam et al. 1993; Cheng et al. 1991; Gowri et al. 1992; Melzer et al. 1989; Trischner et al. 1993). In higher plants, the expression of the NR genes is influenced by several external and endogenous factors and is highly regulated at the transcriptional as well as post-translational levels (Meyer and Smitt 2001). The over-expression of either the NR or the NiR gene in plants increases mRNA levels and often affects N-uptake. However, the increased uptake of N does not seem to increase the yield or growth of plants, regardless of the N source (Andrews et al. 2004; Good et al. 2004). This is believed to be due, in part, to the complex regulation of both NR and the pathway as a whole. Recently, Lea et al, 2006 demonstrated that post-translational regulation of NR strongly affects the levels of free amino acids, ammonium, and nitrate, whereas transcriptional regulation has only a minor influence. Plants expressing fully unregulated NR accumulate high concentrations of asparagine and glutamine in leaves; however these transgenic plants grow and developed normally, despite having an NR enzyme that is active during both light and dark periods.

Others genes involved in nitrate uptake or nitrite reduction include nitrate transporters (NRT1and NRT2), NiR, Fd $NADP^+$ oxidoreductase (FNR), 6-phosphogluconate dehydrogenase (6GDPH), and S-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase (UPM1). Genes involved in ammonium assimilation, encoding specific isoforms of GS and GOGAT, are also induced (reviewed in Koch 1997; Lam et al. 1996; Stitt 1999). In higher plants, glutamine synthetase (GS) is represented by two groups of proteins-the cytosolic and plastidic forms (Hirel et al. 1993). A large number of studies on various plant species including both monocots and dicots show that cytosolic GS (GS1) is encoded by a complex multigene family, whereas plastidic GS (GS2) is encoded by a single gene. In rice however, a third GS has also been identified designated as OsGS1;3, which specifically expresses in the spikelet (Tabuchi et al. 2007).

Glutamate synthase (GOGAT) occurs as two distinct isoforms-ferridoxin and NADH-dependent-both of which are located in the plastid. Since the discovery of the role of GS/GOGAT in ammonium assimilation in higher plants (Miflin and Lea 1976), there has been great interest in the understanding of mechanisms controlling the regulation of this pathway (Harrison et al. 2000). Mutants or transgenic plants with altered levels of GS/GOGAT are used to determine the effects of these proteins on plant development and to study the expression of the different members of the GS multigene family (Coschigano et al. 1998). Although several studies demonstrate that an increase in GS activity in transgenic plants has no effect on the phenotype, other researchers show a direct correlation between an enhanced GS activity in transgenic plants and an increase in biomass or yield, upon incorporating a novel *gs1*construct (Good et al. 2004, Hirel et al. 2007). In comparison to GS, few reports have described the production of transgenic plants overexpressing *GOGAT* genes. The most interesting results were obtained by Yamaya et al. 2002, who overexpressed *OsNADH-GOGAT1* in rice under the control of its own promoter and found that transgenic rice plants show an increase in spikelet weight (up to 80%). Plant height and spikelet number are unaffected. This study shows that overexpression of *NADH-GOGAT1* can be used as a key step for N use and grain filling in rice and other cereal crops. Over the past few years, attention was focused on the enzyme asparagine synthetase (AS), which catalyzes the formation of asparagine (Asn) and glutamate from glutamine (Gln) and aspartate. In higher plants, AS is encoded by a small gene family. For starch and organic acid metabolism, mRNA concentrations for phosphoenol pyruvate carboxylase (PEPC; involved in organic acid metabolism) increase and those for ADP-glucose pyrophosphorylase (AGPS2; involved in starch synthesis) decrease after 2 h of treatment with 12 mM nitrate (Sheible et al. 1997). Transcripts for other organic acid metabolism enzymes - cytosolic pyruvate kinase, citrate synthase and NADP+ - isocitrate dehydrogenase - were present in NR mutant plants in greater amounts than in wild type plants grown in 12 mM nitrate, implying that these genes also respond to the nitrate signal (Sheible et al. 1997).

Molecular signaling network for NUE

Plants constantly sense changes in nitrogen availability and respond appropriately by modulating gene expression. Plants employ multiple routes for the long-distance signaling and communication of nitrogen status. One of these depends on nitrate itself (nitrate-specific signaling), while another uses cytokinin as a messenger. Recent studies suggest that nitrate-specific signaling functions predominantly in the context of the synthesis of amino acids and nucleic acids (Fig. 2). This pathway includes the control of the expression of a wide variety of genes. On the other hand, cytokinin-mediated-signaling is related mainly to the control of nitrogen partitioning and development (Fig. 2). Nitrogen-dependent cytokinin accumulation and the involvement of His-Asp phosphorelay systems are characteristic of this pathway (Sugiyama and Sakakibara 2002). The coordination of

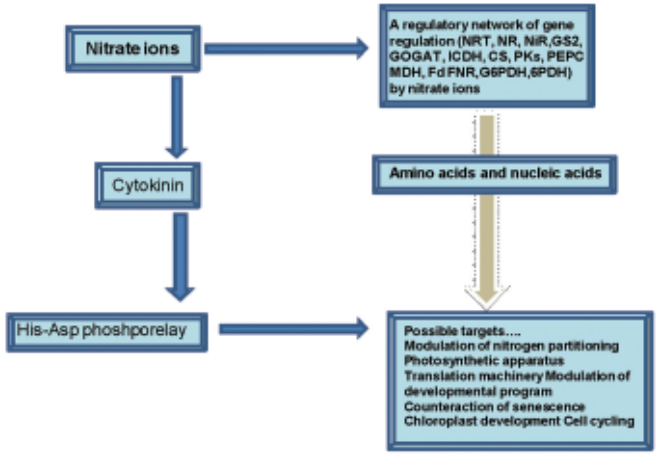

Fig. 2. Multiple routes for communicating nitrogen availability in plants. Nitrate activates a network of gene regulation to provide amino acids and uncleic acids, and also reguates cytokinin biosynthesis and translocation to drive the His-Asp phosphorelay in target cells. *Abbreviations in parentheses* represent genes; *NRT* nitrate transporter, *NR* nitrate reductase, *NiR* nitrite reductase, *GS2* plastid glutamine synthetase, *GOGAT MDH* malate dehydrogenase, *Fd* ferredoxin, *FNR* Fd-NADP+ oxidoreductase, *G6PDH* glucose-6-phosphate dehydrogenase, *6PGD* 6-phospogluconate dehydrogenase

both regulatory pathways seems to be crucially important for the integration of nitrogen signals at the whole plant level (Sakakibara 2003).

A major route of inorganic nitrogen signaling is mediated by nitrate

Nitrate is a substrate for nitrogen assimilation and also functions as a primary signal triggering gene expression. The uptake of nitrate ions and their subsequent distribution throughout the plant body are enabled by a series of nitrate transporters (Crawford and Glass 1998). Although nitrate receptors (sensors) have not yet been identified, it seems clear so far that a nitrate-specific signaling pathway activates genes related to nitrate assimilation and amino acid and nucleotide synthesis (Fig. 3). Nitrate-specific induction was demonstrated for enzymes functioning in nitrate uptake (Tsay et al. 1993), nitrate reduction (Vincentz et al. 1993), ammonia assimilation (Redinbaugh and Campbell 1993; Sakakibara et al. 1997), provision of reducing power (Matsumura et al. 1997; Redinbaugh and Campbell 1998; Ritchie et al. 1994), allocation of the carbon skeleton for organic acid synthesis (Scheible et al. 1997), and modulation of root architecture (Zhang and Forde 1998). In all these cases, the induction of expression did not require protein synthesis and therefore appeared to depend on signal transduction elements already present.

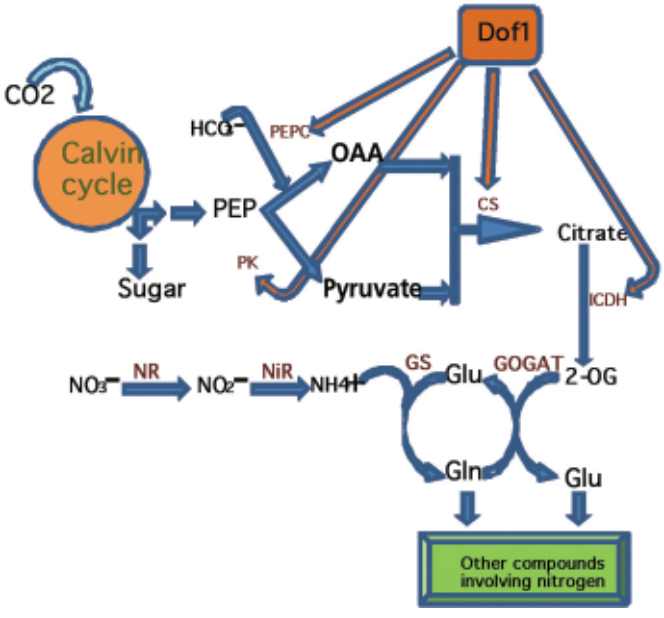

Fig. 3. The metabolic pathways for nitrigen assimilation in plants. Dof1 transcription factor acts as master regulator for the expression of photosynthetic enzymes and thereby improving nitrogen assimilation in plants. abbreviations in patenthesis represent genes: *PEPC* Phospho Enol Pyruvate Carboxylase, *PK* Pyruvate Kinase, *CS* Citrate synthase, *ICDH* Isocitrate Dehydrogenase

Transcriptional regulation of several hundreds of nitrate responsive genes by nitrate as a signal requires cis-acting regulatory sequences or nitrate response elements (NRE) (Raghuram et al. 2006). One such sequence, originally reported to be comprised of an A[G/C]TCA core sequence motif, preceded by a 7-bp AT rich region, based on promoter deletion analyses in nitrate and nitrite reductases from *Arabidopsis thaliana* and birch (Hwang et al. 1997; Warning and Hatchel 2000). However, a genome-wide computational analysis of all the known nitrate responsive genes in *Arabidopsis* and rice indicated that these motifs were present almost randomly throughout these genomes, and were neither specific nor common to nitrate responsive genes (Das et al. 2007). These findings demand a fresh search for candidate sequences that qualify to be NREs in plants. The identification of putative cis elements that are responsive to carbon and nitrogen signaling interactions (Palenchar et al. 2004) also necessitate a search for different cis-regulatory elements that might work in concert. Identification of such regulatory elements provides an end point for nitrate signaling and provides new avenues for characterizing/manipulating the rest of the signaling pathway to enhance NUE.

Hormones and nitrate signaling

Cytokinin metabolism and translocation could be modulated by the nitrogen nutrition status i.e. the changes in root pressure and root water permeability which occur in response to nitrate uptake (Hoarau et al. 1996). Thus, nitrate ions and cytokinins are concomitantly translocated from root to shoot. Nitrogen-dependent accumulation of cytokinin was also observed in roots of *Arabidopsis thaliana* (Takei et al. 2002) and barley (Samuelson and Larsson 1993), suggesting that nitrogen-dependent cytokinin accumulation is common among higher plants. After the nitrogen signal has been converted into a rise in cytokinin level, cytokinin is recognized by target cells and the signal is transmitted to target genes and/or proteins. Recent studies have revealed that His-Asp phosphorelay systems, also known as two-component regulatory systems, are involved in cytokinin perception and signaling (Haberer and Kieber 2002; Inoue et al. 2001; Suzuki et al. 2001).

Need of potential biomarkers relating NUE

In the majority of crop species, including cereal grasses, the plant life cycle can be roughly divided into two main phases. During the vegetative growth phase, young developing roots and leaves behave as sink organs that efficiently absorb and assimilate minerals such as inorganic nitrogen for amino acid and protein synthesis. During the remobilization phase leaves start to behave as source organs translocating carbon and organic molecules to ensure the formation of new developing tissues and/or storage tissues involved in plant survival such as seeds, tubers, bulbs, or trunks (Masclaux et al. 2000). A better understanding of the metabolic and genetic control of acquisition and recycling during these two phases of plant growth and development is therefore of particular importance not only to improve crop quality and productivity, but also to avoid excessive use of fertilizers. Until now, a number of studies have been undertaken by plant molecular physiologists to decipher the regulatory control mechanisms involved during the transition from sink to source organs (Harrison et al. 2000; Hellman et al. 2000; Lewis et al. 2000; Masclaux et al. 2000). However, these approaches that involve whole plant physiology and/or transgenic plants are lim-

ited in that they only allow the role of a single or limited number of enzymes or regulatory elements to be identified and do not account for the variation of complex traits such as nitrogen use efficiency (NUE) often found in agronomic applications. Conventional breeding procedures have been performed empirically over the last two decades by selecting the most appropriate traits in terms of yield or technological characteristics to improve plant productivity (Masclaux et al. 2000; Richards 2000). Although, these approaches have been successful in terms of yield enhancement, there have so far been no real attempts to understand in a more integrated manner the physiological and genetic basis of these improvements, especially in relation to NUE. At present, the use of quantitative genetic studies associated with the use of molecular markers may be a way to identify genes involved in the genetic variation of a complex character. Molecular markers have accelerated plant breeding in a number of areas including biotic (disease and insect) resistance and abiotic (drought, low nitrogen fertilization, and frost) tolerance. There are several types of molecular markers used in marker-assisted slection; these include restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified restriction fragment length polymorphism (AFLP), single sequence repeats (SSR), and single nucleotide polymorphisms (SNPs). Marker-based technology has already provided scientists with a powerful approach for identifying and mapping quantitative trait loci (QTL) and would lead to the development of a better understanding of genetic phenomena. The development of molecular markers has facilitated the evaluation of the inheritance of NUE using specific quantitative trait loci (QTLs) that could be identified. NUE is a complex polygenic trait that has been subjected to quantitative trait locus (QTL) analyses. The first QTL studies were focused on integrative traits such as NUE itself or its components on different crop species (e.g. Bertin and Gallais 2001 on maize; An et al. 2006 on wheat) The combination of agronomical analyses and physiological studies on N-metabolism enabled the identification of candidate genes putatively involved in both the control of NUE and yield (Habash et al. 2007; Hirel et al. 2001; Obara et al. 2001). Understanding the complexity of the N-metabolism network through QTL analysis could lead to the cloning of regulatory loci or factors interacting with them. This new approach of whole-plant N physiology has been performed on maize using field trials (Agrama et al. 1999; Bertin and Gallais 2000; Hirel et al. 2001). It often leads to a discussion of the concept of NUE which represents the quantity of N used to build up a certain amount of biomass (or yield). The study of well-chosen traits allows the discussion of the relationship between processes corresponding to different levels of organization, through the identified QTL (Lebreton et al. 1995; Prioul et al. 1997). For example, in a maize study, coincidences were detected between QTL for yield (and its components) and QTL for GS enzyme activity, both of which co-localize with genes encoding cytosolic GS (Hirel et al. 2001). There is also evidence that the glutamate dehydrogenase enzyme (NAD(H)-GDH, EC 1.4.1.2) enzyme may also be implicated in the control of crop productivity at least in maize as demonstrated by using a quantitative genetics approach (Dubois et al. 2003). GS- and GOGAT-related QTLs were also mapped in rice (Obara et al. 2001). The size of the maize (or even rice) genome, however, does not facilitate the fine-mapping of these QTL and the cloning of the corresponding genes.

QTLs for NUE have been identified in mapping populations of maize, rice, barley, and *Arabidopsis*, and their association with plant N status has been reviewed recently (Hirel et al. 2007). In maize, studies on different genotypes or populations of recombinant inbred lines based on NUE components, chromosomal regions, and putative candidate genes have hinted at some factors that might control yield and its components directly or indirectly, when the amount of N fertilizers provided to the plant is varied (Hirel et al. 2007). Marker-assisted-selection (MAS) should be able to offer significant advantages in cases where phenotypic screening is particularly expensive or difficult, including breeding projects involving multiple genes, recessive genes, late expression of the trait of interest, seasonal considerations, or geographical considerations. In addition to reducing costs of conventional breeding, MAS also has the potential to generate time savings. Possibly, the greatest contribution of QTL mapping to plant breeding will be the basic understanding of the genetic architecture of quantitative traits, thereby relating specific genetic loci with the biological mechanisms associated with desirable phenotypes. Intensively managed crop systems are normally dependent on nitrogen input to maximize yield potential. Improvements in NUE in crop plants may support the development of cropping systems that are more economically efficient and environment friendly.

Molecular approaches for identification of N-responsive genes and new biomarkers for NUE

Efforts to enhance NUE by individually overexpressing some of the proteins and enzymes responsible for the uptake and assimilation of nitrate in transgenic plants have failed. Since, the levels of carbon and nitrogen metabolites mutually influence each other, implying the intimate link between carbon and nitrogen metabolism (Yanagisawa et al. 2004). Therefore, modulation of carbon skeleton production might be an alternate approach to improve nitrogen assimilation in plants. However, because a number of enzymes are involved in carbon skeleton production, it is not practical to intensify the pathway supplying carbon skeletons by the transfer of individual genes for respective enzymes. The signal transduction pathways and the regulatory elements that function to regulate the genes involved in the N uptake and assimilation pathways are yet to be identified. The various modern molecular techniques to validate the cause and effect of candidate genes, i.e. genes involved in NUE include DDRT-PCR, substractive hybridization, differential display, microarrays, knock out, and RNAi. Several studies of plant N-responses based on microarray gene expression profiling has been done. Wang et al. (2000) studied the response of seedlings grown on ammonium to the addition of low or high levels of nitrate. They used the Arabidopsis GEM1 microarrays, which contained 7,942 cDNA clones corresponding to 5,524 unique genes, and identified 25 and 49 N-responsive genes to low or

high nitrate induction, respectively. Subsequently, Wang et al. 2003 used the Arabidopsis whole-genome Affymetrix ATH1 microarray containing 22,626 genes, to study the addition of the low level of nitrate to discover more N-responsive genes. Scheible et al. 1997 also used the ATH1 microarray combined with real-time RT-PCR of > 1,400 transcription factor genes to identify genes affected by N-deprivation or N-induction after 30 min or 3 h from N-starved seedlings. Since C and N metabolism are very closely linked and tightly regulated (Coruzzi and Bush 2001; Coruzzi and Zhou 2001). Price et al. (2004) used the ATH1 microarray to identify the individual contributions of nitrogen, sugar, and nitrogen plus sugar on global gene expression. Recently, Lian et al. 2006 reported expression profiles of 10,422 genes at an early stage of low N stress in rice seedling. So far, these studies have provided valuable insights into N response and its linkage to other biological pathways.

In terms of finding a global target for manipulation of nitrogen use efficiency (NUE), recent studies revealed that Dof (DNA binding with one finger only) transcription factor acts as master regulator in the expression of photosynthetic genes and thereby improving nitrogen assimilation of crop plants (Yanagisawa and Sheen 1998; Yanagisawa and Tetuya 2004). The potential of the master regulator has been visualized for enhancing the biomass and in turn yield parameter in cereal grains. Recent studies of wheat varieties grown under different nitrogen treatments showed that TaDof 1 expression was up-regulated in low nitrogen treatment (Kumar et al. 2009). Thus, it gives us some insight to relate the role of TaDof 1 transcription factor in controlling the nitrogen use efficiency through higher expression of Dof1.

The PBF DOF gene of *Eleusine coracana* first reported based on *in silico* studies (Kushwaha et al. 2008) in our lab is now under study for its temporal and spatial expression analysis so as to confirm their function related with regulation of seed storage protein genes. Therefore, the application of transcription factors that could selectively enhance whole steps of carbon and nitrogen metabolic pathway may be a powerful approach for metabolic engineering of crops having superior characteristics with improved nitrogen utilization efficiency and improved nutritional quality of grains.

Future prospects

In the recent years, combined molecular, physiological, and genetic approaches have facilitated significant progress in the understanding of plant N-economy in agronomic context. These approaches have also allowed the identification of key elements involved in the control of NUE molecular cascades, i.e. in relation to crop productivity and crop yield. The physiological and agronomical studies on N metabolism have enabled the identification of candidate genes putatively involved in both the control of NUE and yield. Several efforts have been made to enhance NUE by individually overexpressing genes involved in nitrogen uptake and assimilation; however such strategy could not enhance net nitrogen assimilation/amino acid biosynthesis. Therefore, utilization of transcription factors might be a powerful approach for modification of metabolism for generation of crops having superior characteristics because a single transcription factor frequently regulates coordinated expression of a set of key genes for metabolic pathways. It has been proposed that the combination of different cis-elements and trans-acting factors may produce the diversity and specificity required for the regulation of gene expression. Several such elements have already been identified and speculation is that some of these elements act as master switches in the regulation of genes involved in carbon and nitrogen metabolism. For identification of such candidate genes, several genomics and proteomics approaches are in progress these days. Hence, once such master regulator is identified and validated, their association with NUE trait could facilitate the screening of genotypes with different NUE, i.e. high nitrogen responsive and low nitrogen-responsive genotypes.

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