Agricultural Nitrogen Use & Its Environmental Implications

Agricultural Nitrogen Use & Its Environmental Implications provides a comprehensive, interdisciplinary description of problems related to the efficient use of nitrogen in agriculture, in the overall context of the nitrogen cycle, its environmental and human health implications, as well as various approaches to improve N use efficiency. The book has been divided into six sections and targets graduates, postgraduates, research scholars and policy makers in Agricultural and Environmental Sciences.

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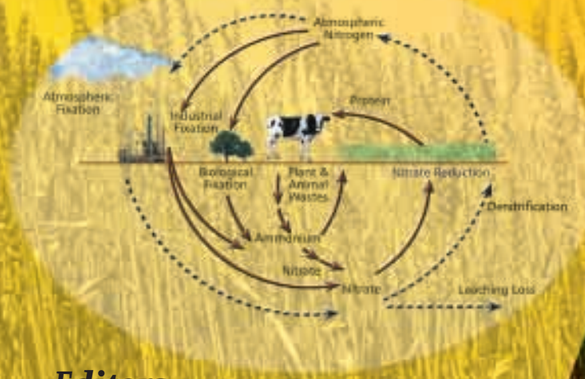

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AGRICULTURAL NITROGEN USE & ITS ENVIRONMENTAL IMPLICATIONS

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Molecular Approaches for Enhancement of Nitrogen Use Efficiency in Plants

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Summary: The form and amount of N available to the plant can be improved by manipulating fertilizer-soil-water-air interactions, but the inherent efficiency of the plant to utilize available N for higher productivity needs to be tackled biologically. There are many ways to determine N use efficiency (NUE) in terms of input and output, but the lack of a clear understanding of the molecular basis for the regulation of nitrate assimilation has made crop improvement difficult. This chapter describes how regulation of nitrate assimilation goes far beyond the enzymes of primary nitrate assimilation and is governed by a complex web of interactions between nitrogen and carbon metabolites, nitrate signaling and regulation by light, hormones and other factors, involving several hundreds of plant genes. This chapter also summarizes the outcomes of various efforts directed at genetic manipulation of nitrate transporters, genes of primary and secondary N assimilating enzymes, signaling intermediates, transcription factors and other regulatory proteins. The possible options and strategies for future interventions are also discussed.

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1. INTRODUCTION

Nitrogen (N) is the most important factor limiting crop productivity. Worldwide, nitrogen use efficiency (NUE) for the production of cereals such as wheat, rice, corn, barley, sorghum, millet, oat and rye is approximately 33%. The reasons for this are fertilizer N losses via denitrification and surface runoff, plant N losses as NH_3, and the inability to translate better N utilization into improvements in output. The unaccounted 67% represents a $15.9 billion annual loss of N fertilizer, assuming fertilizer-soil equilibrium (Raun and Johnson, 1999). Therefore, improvement of nitrogen uptake, translocation and assimilation towards a desirable outcome has been a long-term goal in agricultural research. 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 management and crop management strategies, the inherent efficiency of the plant to utilize available N for higher productivity needs to be tackled biologically (Abrol *et al*., 1999, Abdin *et al*., 2005, Raghuram *et al*., 2006). In terms of crop improvement, this includes identification of phenotypes, genotypes, molecular markers and target gene(s) of relevance to NUE for plant breeding and/or genetic manipulation of NUE. The failure of single gene transgenics involving primary N assimilatory pathway and the recent realization that plant N response involves coordinated expression of hundreds of genes and regulation of their enzymes compels a more integrated approach to understand/manipulate NUE. The aim of this chapter is to summarize the current state of our understanding of the molecular basis of plant N response and NUE, and the various attempts made so far to manipulate NUE at the plant level.

1.1 Defining NUE

There are various ways in which different scientists currently determine NUE based on different parameters of efficiency, as summarized in **Table 1.** NUE basically includes N uptake, utilization or acquisition efficiency, and N-use (physiological/internal) efficiency, and is expressed as a ratio of

Table 1: *Various criteria used to determine NUE*

Parameter	Nomenclature	Formula	Reference
Biomass index	NUE	NUE = Sw/N	Steenbjerg and Jakobsen, 1963
	Usage index	UI = Sw*(Sw/N)	Siddiqi and glass, 1981
Grain weight index	NUE grain	NUE = Gw/Ns	Moll *et al*., 1982
	Utilization efficiency	UtE = Gw/Nt	Moll *et al*., 1982
	Agronomic efficiency	AE = $(Gw_f\text{-}Gw_c)/N_f$	Crasswell and Godwin, 1984
	Physiological efficiency	PE = $(Gw_f\text{-}Gw_c)$/ (N_f uptake-N_c uptake)	Crasswell and Godwin, 1984
Capacity of N uptake	Uptake efficiency	UpE = Nt/Ns	Moll *et al*., 1982
	Apparent nitrogen recovery	AR = (N_f uptake-N_c uptake)/ N_f*100	Crasswell and Godwin, 1984

Sw: Shoot weight; N: Total nitrogen content of shoots; Gw: Grain weight; Ns: Nitrogen supplied in gram per plant; Nt: Total nitrogen in plant; Gw_f: Grain weight with fertilizer; Gw_c: Grain weight without fertilizer (control); N_f :nitrogen fertilizer applied; N_f uptake: Plant nitrogen with fertilizer; N_c uptake: Plant nitrogen unfertilized control; Physiological N-use efficiency (PE).

output (total plant nitrogen, biomass produced, grain yield, grain nitrogen, total plant biomass nitrogen) and input (total N, soil N, or N fertilizer supplied). Three NUE parameters have been widely used for quantification:

1. Agronomic
2. Apparent nitrogen recovery
3. Physiological (Craswell and Godwin, 1984)

Agronomic efficiency of nitrogen (AE), which, in economic terms, is also referred to as partial factor productivity (PFP), measures overall efficiency or an integrative index of total economic outputs relative to the use of all sources of N (indigenous soil N and applied fertilizer N). Apparent nitrogen recovery (AR) takes the efficiency of the plant to take up N into account. Physiological N-use efficiency (PE) considers the efficiency with which the plant uses N from acquired available N to produce grain or total plant dry matter. However, since none of these definitions are based on a clear understanding of the underlying mechanism of NUE, they do not offer any options for biological intervention to improve NUE in plants. Once the mechanism(s) of nitrate response and its regulation in plants is unraveled, it may be possible to adopt a new definition of NUE that provides better insights into the possible sites for intervention.

2. NITROGEN METABOLISM AND BEYOND

There are various forms of nitrogen available to the plant. Regardless of the form in which nitrogen is supplied viz., urea, ammonia or nitrate, the microbial process of denitrification ensures that nitrate is the most abundant form of N available to the plant. Nitrate is also a preferred source of N for most plants. Nitrate taken up through the nitrate transporters in the roots is subsequently converted to ammonia by the sequential action of nitrate reductase and nitrite reductase and then incorporated into amino acids through glutamine synthetase and glutamate synthase. The availability of organic acids is critical for the supply of carbon skeletons needed for amino acid synthesis, which, in turn, demands optimum partitioning of photosynthetic sugars between various metabolic pathways. This necessitates the coordinated regulation of multiple metabolic and regulatory pathways (including nitrogen and carbon) by nitrate as a signal. The presence of multiple isoforms of many of the nitrate-responsive enzymes and their differential regulation by internal metabolites and external signals constitute sophisticated regulatory controls, which are not yet fully understood. In addition, N responses of a plant also vary in a tissue-specific, organ-specific and developmental stage-specific manner. When viewed from this perspective, the internal factors that determine NUE in a plant go far beyond the levels of uptake and the enzymes of primary N metabolism. This also explains why making more N available to the plant by manipulating the soil-fertilizer-water interactions does not automatically guarantee concomitant improvements in plant NUE.

2.1. Nitrate Uptake and Reduction

Nitrate (NO_3^-) is acquired by higher plants from the soil through the combined activities of high- and low-affinity uptake systems in the roots. The low affinity transport system (LATS) is used preferentially when external nitrate concentration is high (above 1 mM). The high affinity transport system (HATS) is able to take-up nitrate at low concentrations (between 1 μM and 1 mM). So far, two gene families, *NRT1* and *NRT2*, have been identified on the basis of their deduced amino acid sequences (Orsel *et al*., 2002a). The individual families are represented by multiple genes that are

differentially regulated and code for transporters with different regulatory or kinetic properties (Forde, 2000; Orsel *et al*., 2002b; Glass *et al*., 2002). The nitrate taken up may be accumulated or reduced in root cells, transported via the xylem vessels to be assimilated or stored in the shoot, or released via root efflux systems.

The reduction of NO_3^- involves two enzymatic steps—reduction of NO_3^- to nitrite (NO_2^-) by nitrate reductase (NR) in the cytosol, and the transport of nitrite to the chloroplast where it is further reduced to NH_4^+ by nitrite reductase (NiR). NR exists in multiple copies as well as multiple (NADH or NADPH specific) isoforms with tissue specific distribution. Ammonia once formed is then incorporated into amino acids through the plastidic glutamine synthetase (GS2) and glutamate synthase (Fd-GOGAT). The cytosolic isoforms of GS (GS1) and GOGAT (NADH-GOGAT) are involved in secondary ammonia assimilation. These enzymes and their genes have been well characterized from several plants, and mutants, and transgenic plants are also available for *in vivo* studies (Crawford, 1995, Stitt and Sonnewald, 1995, Lam *et al*., 1996). *NRT1.1* and *NRT2.1*, *NIA1* and *NIA2*, are inducible by NO_3^- and up-regulated by sugars (Loque *et al*., 2003).

2.2. Ammonium Uptake and Assimilation

The pathway for ammonium assimilation in higher plants has been well-documented. Ammonium uptake also takes place in a biphasic manner, involving LATS and HATS. (Glass *et al*., 2001). Ammonium can also be generated from primary nitrate assimilation, reassimilation of internal metabolites or other secondary sources, and is then incorporated into amino acids in a reaction catalyzed by glutamine synthetase (GS) and then by glutamate synthase (GOGAT) (Miflin and Lea, 1980). There is now a good understanding of properties of GS/GOGAT enzymes, their subcellular location, and, in most cases, the genes that encode them (Ireland and Lea, 1999). Glutamine synthetase (GS) (EC 6.3.1.2) is the central enzyme in ammonium assimilation in plants (Lam *et al*, 1995, 1996), with a cytosolic isoform (GS1) and a plastidic isoform (GS2) that assimilate ammonium ions generated in the chloroplast (Hirel and Gadal, 1980; Hirel *et al*., 1993). In angiosperms, a complex multigene family encodes GS1, and a single nuclear gene encodes GS2 (Tingey and Coruzzi, 1987; Cock *et al*., 1991; Peterman and Goodman, 1991; Sakakibara *et al*., 1992; Li *et al*., 1993; Oliveira *et al*., 1997; Oliveira and Coruzzi, 1999). Similarly, GOGAT exists in ferredoxin-dependent plastidic isoform (Fd-GOGAT) and NADH-dependent cytosolic isoform (NADH-GOGAT), out of which the former enzyme is involved in primary ammonia assimilation together with GS2 in the chloroplast.

2.3. Secondary Ammonia Assimilation/Remobilization

In addition to the common pathway of nitrate assimilation for generating ammonia, the major part, up to 90% of the ammonia fed into the GS/GOGAT cycle, is derived from the mitochondrial glycine decarboxylase (GDC) reaction, which is an integral part of the photorespiratory carbon and nitrogen cycle (Bauwe and Kolukisaoglu, 2003; Hirel and Lea, 2001; Wingler *et al*., 2000). As nitrogen is a major limiting factor for plant growth, the efficient re-assimilation of metabolically generated ammonia is highly important for plant performance and prevents losses of ammonia to the atmosphere. Cytosolic GS (GS1) has been proposed as a key component of nitrogen utilization efficiency in plants (Miflin and Habash, 2002), and its metabolic role is particularly important for nitrogen remobilization and recycling in woody plants (Suarez *et al*., 2002; Gallardo *et al*., 2003). Cytosolic NADH-GOGAT has also been implicated in some plants this regard. The importance of glutamate dehydrogenase

(GDH) in higher plant N metabolism is still controversial, as it has never been clearly demonstrated that the enzyme plays a significant role either in ammonia assimilation or carbon recycling in plants (Dubois *et al*., 2003 and Terce-Laforgue *et al*., 2004b). Moreover, the role of GDH in N management and recycling has recently been reviewed in a number of whole-plant physiological studies performed on tobacco (Terce-Laforgue *et al*., 2004a) and maize (Hirel *et al*., 2005b). Although some important changes in leaf enzyme activity were observed, both during plant development and at the different leaf stages, no significant correlation was obtained between GDH activity and all the other metabolic markers. This finding reiterates the fact that GDH is probably not directly involved in the control of N management (Terce-Laforgue *et al*., 2004b).

2.4. Metabolite Partitioning and C:N Interactions

N and C metabolism are tightly linked at the cellular and whole plant level. This is enabled by the ability of the plant to sense the C/N balance and regulate it by complex signaling networks (Lam *et al*., 1994; Oliveira and Coruzzi, 1999). Nitrate acts as a signal to regulate a range of plant responses (Redinbaugh and Campbell, 1991, Crawford, 1995, Stitt, 1999a). The relative abundance of nitrogen pools in the plant plays a significant role in regulating C/N metabolism (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001; Foyer *et al*., 2003). Nitrate supply has been shown to result in the decrease of starch synthesis and diversion of carbon towards the conversion of organic acids into amino acids (Scheible *et al*., 1997; Stitt *et al*., 2002). It is marked by an increase in the transcript levels and enzyme activities of phosphoenolpyruvate carboxylase, pyruvate kinase, citrate synthase, and isocitrate dehydrogenase, and a consequent accumulation of malate and 2-oxoglutarate. On the other hand, nitrate deficiency results in the decrease of many amino and organic acids, along with an increase in the level of several carbohydrates, phosphoesters, and a handful of secondary metabolites (Fernie *et al.,* 2004a).

Recent studies on the genome-wide response to nitrate in *Arabidopsis* (Wang *et al*., 2000, 2003) and tomato (Wang *et al*., 2001) revealed several hundreds of nitrate-responsive genes. These include the enzymes of glycolysis, trehalose-6-phosphate metabolism, iron transport/metabolism, and sulphate uptake/reduction. Recent studies on global gene expression have revealed that a significant number of the previously reported nitrate-responsive genes actually required the presence of both nitrogen and sugar, suggesting significant interaction between C and N metabolites in regulating gene expression, with carbon modulating the effects of nitrogen and *vice versa* (Price *et al*., 2004). The response of key metabolic intermediates has been determined in many cases, wherein nitrate assimilation has been modified by genetic or environmental perturbation (Matt *et al*., 2001a, b; Muller *et al*., 2001; Masclaux-Daubresse *et al*., 2002). However, analyses of the entire range of metabolites involved in the plant's response to altered nitrogen nutrition by metabolite profiling studies are yet to be applied widely (Stitt and Fernie, 2003; Sumner *et al*., 2003, Fernie *et al*., 2004a, Urbanczyk-Wochniak and Fernie, 2005).

2.5. Nitrate Signaling

The role of nitrate as a signal, as mentioned above, has been known for a long time, but the mechanism of nitrate sensing and the signaling events associated with it have not yet been fully understood. While the nitrate sensing protein, proposed over a decade ago (Redinbaugh and Campbell, 1991), is yet to be identified, nitrate sensing by a cytokinin precursor followed by His-Asp

phosphorelay has been proposed recently (Sugiyama and Sakakibara, 2002). But it is not clear whether this constitutes nitrate signaling or a crosstalk with hormone signaling.

A few elements/events possibly associated with nitrate signaling have been characterized using pharmacological approaches. For example, Ca^{2+} and protein kinases/phosphatases have been implicated in mediating the nitrate signal for the expression of NR, NiR and GS2 mRNAs (Sakakibara *et al*., 1997, Sueyoshi *et al*., 1999). In addition to the kinases that post-translationally modulate NR, SPS or PEP carboxylase, Hartwell *et al*. (1999) described a Ca^{2+} independent PEPCase protein kinase, which is a novel member of the Ca^{2+} calmodulin-regulated group of protein kinases. Although a number of kinases/phosphatases involved in nitrate signaling have been described (Krapp *et al*., 2002), their specific roles in mediating nitrate and other interacting signals have not been clearly delineated. A better understanding of the nitrate signaling cascade might emerge from the detailed characterization of mutants related to the signal transfer cascade from nitrate to the NR gene (Ogawa *et al*., 2000), revealing more intermediates and potential sites for the manipulation of NUE.

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, Hachtel and Strater, 2000, 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*., submitted). 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 provide an end point for nitrate signaling and provide new avenues for characterizing/manipulating the rest of the signaling pathway to enhance NUE.

3. REGULATION OF NITROGEN FLUX BY OTHER FACTORS

The flux of N metabolites through various primary and secondary pathways is affected by a host of other factors that may include biotic and abiotic stresses, but only a few well studied factors that work in a healthy plant such as light, regulatory proteins, downstream N metabolites, and hormones are described here.

3.1. Role of Light

Light is an additional signal that regulates the expression of many nitrate responsive genes, though it has been studied in depth in only a few of them (Raghuram and Sopory, 1995a; Chandok *et al*., 1997; Lillo and Appenroth, 2001). Light regulation of nitrate reductase expression and activity operates differently in green plants and etiolated seedlings, and is mediated by different photoreceptors. The effects of light in green plants are probably mediated more indirectly through photosynthesis and sugars (Lillo and Appenroth, 2001). Using pharmacological approaches, the phytochrome-mediated regulation of NR gene expression in etiolated maize seedlings was shown to be mediated through

G-protein (Raghuram *et al*., 1999), PI cycle and protein kinase C (Raghuram and Sopory, 1995b). Similar effects of cholera toxin and lithium ions were also found recently on NR mRNA and activity in light-grown-dark-adapted rice seedlings, but both PMA and okadaic acid had similar inhibitory effects on NR mRNA and activity, indicating different responses in maize and rice, either due to etiolated/green plant or C3/C4 differences (Ali *et al*., 2006).

3.2. Role of 14-3-3 Proteins

At the post-translational level, light acts by modulating the phosphorylation status of the NR enzyme in conjunction with regulatory binding proteins known as 14-3-3 proteins. NR is inactivated by 14-3-3 following phosphorylation by protein kinases responding to light-dark transitions and changes in cellular energy status. It is now becoming increasingly evident that 14-3-3 proteins could play a crucial role in bringing about metabolic coordination between multiple enzymes of the nitrogen and carbon metabolism. The plant cytosolic enzymes—nitrate reductase, glutamine synthetase, sucrose-phosphate synthase, trehalose-phosphate synthase, glutamyl-tRNA synthetase, and an enzyme of folate metabolism have all been found to bind to 14-3-3 proteins in a phosphorylation dependent manner (Moorehead *et al*., 1999; Cotelle *et al*., 2000). Recent experiments in transgenic potato plants indicate that repression of 14-3-3 proteins leads to significant increase in NR and SPS activities, and even higher levels of starch accumulation in the tuber (Zuk *et al*., 2003). In addition to the direct regulation of metabolism by 14-3-3 proteins via enzyme activation/inactivation, 14-3-3 proteins have been shown to interact with components of plant signalling pathways. For example, they interact with RGS3—a negative regulator of the G-alpha subunits of heterotrimeric G proteins (Niu *et al*., 2002)—suggesting a possible role in the regulation of G-protein signalling pathways, which, in turn, have been shown to be involved in mediating light regulation of NR (Raghuram *et al*., 1999).

3.3. Role of Downstream Nitrogen Metabolites

The downstream products of nitrate assimilation such as nitrite, ammonium ions, and amino acids are generally considered to be inhibitory to nitrate uptake and/or nitrate reductase activity, though the specific effect might depend on the metabolite, tissue and the plant type under study. For example, ammonium ions were found to have a stimulatory effect on NR activity in excised leaves of etiolated maize seedlings (Raghuram and Sopory, 1999). Nitrogen metabolites also have wider roles in facilitating carbon metabolism to support the demands of increasing N flux. The addition of ammonium or nitrate to N-limited whole plants or plant cells induces (at the transcript and/or activity level) enzymes of glycolysis and Krebs cycle (e.g., pyruvate kinase, PEP carboxylase, citrate synthase, and aconitase) that are required for the synthesis of 2-OG (Lancien *et al*., 1999; Larsen *et al*., 1981; Paul *et al*., 1978; Scheible *et al*., 1997). In concert, respiratory chain activity is enhanced *in vivo* as evidenced by enhanced O_2 consumption (Barneix *et al*., 1984; Bloom *et al*., 1992; Rigano *et al*., 1996; Scheible *et al*., 2004). Recently, Escobar *et al.* (2006) indicated that nitrogen promotes bypass pathways of the mitochondrial respiratory chain to accommodate the increase in redox load accompanying 2-OG synthesis. Overall, these studies suggest that the manipulation of N nutrition leads to dynamic alterations in plant respiratory metabolism in response to changes in cellular energetic demands. By implication, optimizing N response and NUE might also require manipulating/modulating these dynamic alterations.

3.4. Role of Hormones

The role of hormones in mimicking, mediating or modulating the nitrate response has received considerable attention in the recent years. For example, cytokinin accumulation and translocation occurred after sensing a change in nitrogen availability. (Samuelson and Larsson, 1993; Takei *et al*., 2001, 2002). Application of cytokinin can mimic the nitrogen-dependent regulation of gene expression in photosynthesis, cell cycling, and translational machinery (Takei *et al*., 2002). In maize and *A. thaliana*, some response regulators of the His-Asp phosphorelay system have been found to be upregulated by both cytokinins and nitrate (Sakakibara *et al*., 1998, 1999; Taniguchi *et al*., 1998; Imamura *et al*., 1999). While cytokinins may not account for all the known responses of plants to nitrate, these findings indicate a possible interaction between the two, and a role for cytokinins in communicating the availability of nitrogen from roots to leaves (Sugiyama and Sakakibara, 2002). Nitrogen sensing and response also seems to be affected by the cross talk between various plant hormones. Auxin synergistically affects cytokinin activity on cell division and organogenesis (Soni *et al*., 1995), while ABA antagonises the cytokinin-mediated nitrogen signalling by negatively regulating cytokinin-inducible response regulator genes. Unlike cytokinins, which are positively regulated by nitrate, ABA biosynthesis is down regulated by nitrogen-sufficiency (Gawronska *et al*., 2003). Gibberellins do not seem to play any role in the control of nitrate assimilation, at least in the vegetative stages of *Arabidopsis* (Bouton *et al*., 2002). Despite these findings, establishing the role of hormones in nitrogen signalling needs further characterization of the complete signalling pathway.

4. GENETIC MANIPULATION OF NITROGEN USE EFFICIENCY

Several attempts were made during the last decade by trial and error. While some studies were specifically directed to genetically manipulate nitrate response or N-use efficiency, others used genetic manipulation as a tool to test the wider role of the target gene in plant metabolism and growth. The following paragraphs summarize these studies by categorizing them into various aspects of nitrate uptake and assimilation:

4.1. Manipulation of Nitrogen Transporters

Optimum uptake of N is the first step to enhance N-use in any plant. While the differential regulations of various nitrate and ammonium transporters in plants are well-known (Forde, 2000 and Wiren *et al*., 2000), very few studies have analyzed the effect of over expression of the genes of these

Table 2: *Transgenic studies on nitrate transporters (modified after good et al., 2004)*

Gene	Gene product and gene source	Promoter	Target plant	Phenotype observed	Reference
Nrt 1.1	High affinity nitrate transporter (*Arabidopsis*)	CaMV35S	*Arabidopsis*	Increase in constitutive nitrate uptake but not in induced.	Liu *et al*., 1999
Nrt 2.1	High affinity nitrate transporter (*Nicotiana plumbaginifolia*)	CaMV35S, rol D	*Nicotiana tabaccum*	Increased nitrate influx under low N conditions	Fraisier *et al*., 2000

transporters **(Table 2)**. Studies involving transgenic overexpression of a *CHL1* cDNA (representing the constitutive high-affinity nitrate transporter) driven by the cauliflower mosaic virus 35S promoter in a *chl1* mutant revealed effective recovery of the nitrate uptake defect for the constitutive phase. This was, however, not reflected in case of the induced phase, which was consistent with the constitutive level of *CHL1* expression in the transgenic plant. In another experiment involving transgenic tobacco plants expressing the *NpNRT2.1* gene (encoding high affinity nitrate transporter), Fraisier *et al*. (2000) reported steady state increase in its mRNA levels accompanied by an increase in the NO_3^- influx, but the NO_3^- contents were found to be remarkably similar in wild-type and transgenic plants. These findings indicate that increasing the uptake of nitrate by genetic manipulation may not necessarily lead to concomitant improvement in nitrate utilization or NUE, though it remains to be seen whether different plants respond differently to the overexpression of different transporters. Ammonium transporters are very well characterized in plant systems (Glass *et al*., 2001 and Loque and Wiren, 2004), but the effect of their overexpression on plant growth and development are yet to be elucidated.

4.2. Manipulation of the Genes of Primary Nitrate Assimilation (NR and NiR)

Nitrate reductase has long been considered to be the rate limiting step in nitrate assimilation, but transgenic manipulation of NR expression in *Nicotiana* spp. indicated the importance of other steps (Stitt *et al.,* 1999b). Constitutive NR expression led to a 2-fold increase in NR activity and a 20% decrease in foliar nitrate content along with an increase in total amino acid contents, but without any changes in total N, soluble sugars, starch, and productivity parameters (Foyer *et al*., and Quillere *et al*., 1994), while the NR double mutant *Nia30* did not show detectable NR activity. When transformed with the *Nia2*-cDNA, it showed decreased NR activity with higher levels of nitrate accumulation. (Hansch *et al*., 2001). Transformed *Nicotiana plumbaginifolia* plants constitutively expressing nitrate reductase (NR) show a temporarily delayed drought-induced losses in NR activity, thereby allowing more rapid recovery of N assimilation following short-term water deficit. Deregulation of NR gene expression by constitutive expression in transgenic plants resulted in reduced nitrate levels in the tissues of tobacco (Quillere *et al*., 1994) and potato (Djannane *et al*., 2002a). Although other factors such as NO_3^- availability regulate flux through the pathway of N assimilation, the NR transformants were better equipped in terms of available NR protein, which rapidly restores N assimilation. While no tangible effects on biomass accumulation could be attributed in the short term, under field conditions of fluctuating water availability, constitutive NR expression was able to confer a physiological advantage by preventing slowly reversible losses in N-assimilation capacity. (Ferrario-Mery *et al*., 1998). Since nitrate reductase (NR) is posttranslationally regulated by phosphorylation and binding of 14-3-3 proteins, various attempts have been made to counter this inhibitory effect on NR regulation. Deletion of 56 amino acids in the amino-terminal domain of NR was previously shown to impair this type of regulation in *Nicotiana plumbaginifolia* (Provan *et al*., 2000). Additional reports of overexpression of *NR* genes from various plants have been accumulating over the last decade (Lillo *et al*., 2003; Provan *et al*., 2000; Ferrario *et al*., 2001 and Djannane *et al*., 2002b), but none of these have had any major implications for improving nitrogen use efficiency **(Table 3)**.

Table 3: *Transgenic studies on primary N assimilating genes (modified after Good et. al., 2004)*

Gene	Gene product and gene source	Promoter	Target plant	Phenotype observed	Reference
NR	Nitrate reductase (*Nicotiana plumbaginifolia*)	CaMV35S	*Nicotiana tabacum*	3-4 fold drop in NR activity, no change in NR transcript	Vincentz and Caboche, 1991
	Nitrate reductase (*Nicotiana plumbaginifolia*)	CaMV35S	*Nicotiana tabacum*	Increased NR activity, biomass, drought stress	Ferrario-Mery *et al*., 1998
Nia	Nitrate reductase (*Nicotiana tabacum*)	CaMV35S	*Lactuca sativa*	Reduced nitrate content, chlorate sensitivity	Curtis *et al*., 1999
Nia2	Nitrate reductase (*Nicotiana tabacum*)	CaMV35S	*Solanum tuberosum*	Reduced nitrate levels	Dejennane *et al*., 2002, a,b
Nia	Nitrate reductase (*Nicotiana tabacum*)	CaMV35S	*Nicotiana plumbaginifolia*	No phenotypic difference in optimum condition, nitrate accumulation in high nitrate supply	Lillo *et al*., 2003
NiR	Nitrite reductase (*Nicotiana tabacum*)	CaMV35S	*Nicotiana tabacum, Arabidopsis*	NiR activity, no phenotypic difference	Crete *et al*., 1997
NiR	Nitrite reductase (*Spinacea oleracea*)	CaMV35S	*Arabidopsis*	Higher NiR activity, higher nitrite accumulation	Takahashi *et al*., 2001
GS2	Chloroplastic glutamine (*Oryza sativa synthetase*)	CaMV35S	*N. tabacum*	Improved photorespiration capacity, and increased resistance to photooxidation	Kozaki and Tabaka *et al*., 1996
	Chloroplastic glutamine synthetase (*Oryza sativa*)	CaMV35S	*Oryza sativa*	Enhanced photorespiration, salt tolerance	Hoshida *et al*., 2000
	Chloroplastic glutamine synthetase (*N. tabacum*)	Rubisco small subunit	*N. tabacum*	Enhanced growth rate	Migge *et al*., 2000
Fd-GOGAT	Ferredoxin dependent glutamate synthase (*N. tabacum*)	CaMV35S	*N. tabacum*	Diurnal changes in NH_3 assimilation	Ferrario-Mery *et al.,* 2002

Overexpression of NiR genes in *Arabidopsis* and tobacco resulted in increased NiR transcript levels but decreased enzyme activity levels, which were attributed to posttranslational modifications (Crete *et al*., 1997; Takahashi *et al*., 2001). There is no evidence yet of any benefit of NIR overexpression in terms of plant NUE.

4.3. Manipulation of the Genes of Primary Ammonia Assimilation (GS2 and Fd-GOGAT)

Transgenic tobacco plants enriched or reduced in plastidic glutamine synthetase (GS2, a key enzyme in photorespiration) were constructed (Kozaki and Takeba, 1996). Those transgenic plants having twice the normal amount of GS2 had an improved capacity for photorespiration and an increased tolerance to high-intensity light, whereas those with a reduced amount of GS2 had a diminished capacity for photorespiration and were photoinhibited more severely by high-intensity light compared to control plants. Reassimilation of ammonia in transformed tobacco was also studied by Ferrario-Mery *et al*. (2002). Further studies containing constructs for the overexpression of GS2 in rice plants (Hoshida *et al*, 2000) and tobacco Migge *et al*., 2000) have been reported. However, these have been inconclusive so far due to lack of physiological and agronomic data. Barley mutants with reduced Fd: GOGAT revealed changes in various nitrogenous metabolites, decreased leaf protein, rubisco activity, and nitrate contents (Hausler *et al*., 1994).

4.4. Manipulation of the Genes of Secondary Ammonia Assimilation (GSI and NADH-GOGAT)

Ectopic expression of GS1 has been shown to alter plant growth (Gallardo *et al*., 1999; Fuentes *et al*., 2001; Oliveira *et al*., 2002), and the overexpression of GS1 in transgenic plants **(Table 4)** could affect the enhancement of photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (Fuentes *et al*., 2001; Fu *et al*., 2003; El-Khatib *et al*., 2004). The over expression of soybean cytosolic GS1 in the shoots of *Lotus corniculatus* was reported to accelerate plant development, leading to early senescence and premature flowering, particularly when plants were grown under conditions of high ammonium (Vincent *et al*., 1997). Ectopic expression of pea GS1 in tobacco leaves was suggested to provide an additional or alternative route for the reassimilation of photorespiratory ammonium, resulting in an increase in the efficiency of N assimilation and enhanced plant growth (Oliveira *et al*., 2002). Man (2005) provided additional empirical evidence for enhanced nitrogen-assimilation efficiency in GS1 transgenic lines. However, differences in the degree of ectopic GS1 expression have been reported (Fuentes *et al*., 2001) and attributed to positional effects, effectiveness of chimeric constructs, or differences in growth conditions. These differences could account for the lack of correlation between the enhanced expression of GS1 and concomitant growth (Eckes *et al*., 1989; Hemon *et al*., 1990; Hirel *et al*., 1992; Temple *et al*., 1993; Vincent *et al*., 1997; Ortega *et al*., 2001). Transgenic overexpression and antisense technology have been employed recently to modulate the expression of NADH-GOGAT in alfalfa and rice plants (Schoenbeck *et al*., 2000, Yamaya *et al*., 2002). The studies on transgenic rice plants expressing antisense RNA for either GS1 or NADH-GOGAT point towards the possible involvement of GS1 in the export of N via phloem in senescing leaves. On the other hand, in case of developing leaf blades and spikelets, NADH-GOGAT was implicated in the utilization of glutamine transported from senescing organs (Yamaya *et al*., 2003). While these genes appear to be good candidates for improving NUE in the short run, the degree of improvement may vary with the crop and cropping conditions.

Table 4: *Transgenic studies on secondary ammonia assimilating genes (modified after Good et. al., 2004)*

Gene	Gene product and gene source	Promoter	Target plant	Phenotype observed	Reference
GS1	Cytosolic glutamine synthetase (*Glycine max*)	CaMV 35S	*Lotus corniculaus*	Accelerated senescence	Vincent *et al*., 1997
	Cytosolic glutamine synthetase (*Glycine max*)	rol D	*Lotus japonicus*	Decrease in biomass	Limami *et al*., 1999
	Cytosolic glutamine synthetase (*Phaseolus vulgaris*)	Rubisco small unit	*Triticum aestivum*	Enhanced capacity to accumulate nitrogen	Habash *et al*., 2001
	Cytosolic glutamine synthetase (*Medicago sativa*)	CaMV 35S	*N. tabacum*	Enhanced growth under N starvation	Fuentes *et al*., 2001
	Cytosolic glutamine synthetase (*Glycine max*)	CaMV 35S	*Medicago sativa*	No increase in GS activity	Ortega *et al*., 2001
	Cytosolic glutamine synthetase (*Pea*)	CaMV 35S	*N. tabacum*	Enhanced growth, leaf-soluble protein, ammonia levels	Oliviera *et al*., 2002
	Cytosolic glutamine synthetase (*Pinus sylvestris*)	CaMV 35S	*Hybrid poplar*	Enhanced growth rate, leaf chlorophyll, total soluble protein	Gallardo *et al*., 1999; Fu *et al*., 2003
	Cytosolic glutamine synthetase (*Glycine max*)	CaMV 35S	*Pisum sativum*	No change in whole plant N	Fei *et al*., 2003
	Cytosolic glutamine synthetase (Alfalfa)	CaMV 35S	*Lotus japonicus*	Higher biomass and leaf proteins	Ortega *et al*., 2004
NADH-GOGAT	NADH-dependent glutamate synthase (*Oryza sativa*)	*O. sativa* NADH-GOGAT	*Oryza sativa*	Enhanced grain filling, increased grain weight	Yamaya *et al*., 2002
	NADH-dependent glutamate synthase (*Medicago sativa*)	CaMV 35S	*N. tabacum*	Higher total C and N content, increased dry wt.	Chichkova *et al*. 2001
GDH	Glutamate dehydrogenase (*E. coli*)	CaMV 35S	*N. tabacum*	Increased biomass and dry weight	Ameziane *et al*., 2000
GDH	Glutamate dehydrogenase (*E. coli*)	CaMV 35S	*N. tabacum*	Increased ammonium assimilation and sugar content	Mungur *et al*., 2005
ASN1	Glutamine dependent Asparagine synthetase (*A. thaliana*)	CaMV 35S	*A. thaliana*	Enhanced seed protein	Lam *et al*., 2003
ASNI	Asparagine synthetase (*Pisum sativum*)	CaMV 35S	*N. tabacum*	Reduced biomass and increased level of free asparagine	Brears *et al*., 1993
AspAT	Mitochondrial aspartate aminotransferase (*prosomillet*)	CaMV 35S	*N. tabacum*	Increased, AspAT, PEPC activity	Sentoku *et al*., 2000

4.5. Manipulation of Source-sink Relationships and Nutritional Quality

An enhanced source and effective sink are critical for the improvement of amino acid contents and composition of seeds. Enhancing the nutritional value, of grains, seeds or other plant products for consumption is of high agricultural and economic value and of particular interest is the increase in both the quality and quantity of seed proteins in crop plants. Molecular manipulation provides an attractive mean to achieve these dual objectives. One way to optimize N utilization is to allocate more N resources to the organ of interest, such as grains. The efficiency of protein synthesis has been shown to be dependent on the light/dark regulation of asparagine synthetase (AS) activities (Dembinski *et al*., 1996), with elevations of leaf AS activities and Asn levels being used as parameters to screen for high grain protein cultivars in maize (Dembinski *et al*., 1995) and rye, *Secale cereale* (Dembinski and Bany, 1991). Alternatively, controlling the expression of the *ASN1* gene to manipulate the relationship between Asn and seed N status is an another way to enhance nutritional quality, which needs to be tested. Various studies showed that AS can be one of the major controlling forces for nitrogen flux, when GS is limiting in plants (Harrison, 2003). There are several reports of transgenic overexpression of AS genes (Brears *et al*, 1993; Lam, 2003; Wong *et al*, 2004), resulting in enhanced seed protein content and total protein content. Among other approaches, based on previous studies on microorganisms, two potentially important N-regulation systems have been identified in plants: P11 (Hsieh *et al*., 1998) and GCN 9 (Zhang *et al*., 2003), though detailed analysis of the effect of their transgenic overexpression is not reported and needs attention.

4.6. Manipulation of Signaling Targets

While identification of global nitrate-induced transcription factors in higher plants has not been successful, a recent study suggests that targeting other transcription factors may help to improve N assimilation and NUE. Yanagisawa *et al*. (2004) generated transgenic *Arabidopsis* lines overexpressing Dof1, a maize protein that belongs to the Dof family of plant-specific transcription factors known to activate the expression of several C-metabolizing genes associated with organic acid metabolism. The transformants showed upto 30% higher N content, higher levels of amino acids, better growth under low-nitrogen conditions, and higher levels of mRNAs and enzyme activities for PEP carboxylase and pyruvate kinase, without any reduction of NR, GS, and GOGAT RNAs. The genes up-regulated

Table 5: *Transgenic studies on signaling proteins involved in the regulation of N metabolism*

Gene	Gene product and gene source	Promoter	Target plant	Phenotype observed	Reference
ANR1	MADS transcription factor (*Arabidopsis*)	CaMV35S	*Arabidopsis*	Lateral root induction and elongation	Zhange and Forde., 1998
GLB1	PII regulatory protein (*Arabidopsis*)	CaMV35S	*Arabidopsis*	Growth rate, increased anthocyanin production in low N	Hseih *et al*., 1998
Dof1	Transcription factor and activator associated with C metabolism (*Zea mays*)	35S C4PDK	*Arabidopsis*	Enhanced growth rate under N limited conditions, increase in amino acid content	Yangisawa *et al*., 2004

by Dof1 overexpression clearly belong to the list of known nitrate-responsive genes, though it is not clear whether Dof1 is inducible by nitrate. If Dof1 is not nitrate-inducible, it means that multiple transcription factors may be involved in the coordinated expression of N and C metabolizing genes. A few other attempts to manipulate signaling/regulatory proteins have been shown in **Table 5**, but they did not yield any significant advantage in terms of NUE.

5. QTL MAPPING TO FIND NEW TARGETS FOR MANIPULATION

Nitrogen use efficiency in plants is a complex quantitative trait that involves many genes and depends on a number of internal and external factors in addition to soil nitrogen availability, such as photosynthetic carbon fixation to provide precursors required for amino acid biosynthesis or respiration to provide energy. Although this trait is controlled by a large number of genetic *loci* acting individually or together, depending on nutritional, environmental and plant developmental conditions, it is possible to find enough phenotypic and genotypic variability to partially understand the genetic basis of NUE and thus identify some of the key components of yield for marker assisted breeding. A number of QTL analyses using interspecific crosses have been conducted to identify the loci controlling physiological traits in various crop plants. In rice, QTL analysis with DNA markers, based on a well-saturated genetic linkage map, has been employed to detect genomic regions associated with several traits exhibiting complex inheritance (Yano and Sasaki, 1997).

QTL mapping was also shown more recently to be a very powerful tool for the analysis of complex physiological traits such as NUE (Prioul *et al*., 1997; Agrama *et al*., 1999, Hirel *et al*., 2001; Limami and de Vienne, 2001). Use of this approach in model species such as maize (Hirel *et al*., 2001), rice (Obara *et al*., 2001), and *Arabidopsis* (Rauh *et al*., 2002, Loudet *et al*., 2003) resulted in the identification of putative candidate genes encoding enzymes involved in either N assimilation or recycling that may exert a major control on either plant productivity or plant growth. Among these candidate genes, those encoding GS1 were found to be always associated with agronomic or developmental traits, outlining the central role of the enzyme in the control of plant growth and development (Limami *et al*., 1999; Miflin and Habash, 2002).

Recently, the regulation of N uptake, N assimilation, and N recycling, and their progression during the growth and development of maize plants grown in the field have been approached in an integrated manner by combining whole-plant molecular physiology (Hirel *et al*., 2005) and quantitative genetic studies (Hirel *et al*., 2001). Notably, a number of biochemical markers and putative candidate genes representative of the biological events occurring during the transition between primary N assimilation and N remobilization were identified, making available the link between the physiological function of these genes and the expression of yield and its components (Hirel *et al*., 2005b). These studies have also provided a better understanding of why some genotypes differ in their mode of N management in order to achieve similar yield. In turn, a more integrated view can be obtained of the regulation of N management within the plant or within a particular organ, thus partly filling the gap that existed between traditional physiological and agronomic studies.

6. CONCLUSION AND FUTURE PROSPECTS

All available evidence from the literature indicates that optimising the response of plants to N nutrition goes far beyond the processes of N uptake or primary nitrate assimilation, and involves wider adjustments such as metabolite partitioning, root-shoot allocation and remobilization of resources

during the productive phases of plant growth. It is becoming increasingly evident that any effort to find biological routes for improving NUE has to account for the complexities of metabolic coordination and the multigenic responses involved. Efforts to enhance NUE by individually over expressing some of the individual proteins and enzymes responsible for the uptake and assimilation of nitrate in transgenic plants have failed, indicating that the earlier notions of single-point rate-limiting regulation were too simplistic (Stitt, 1999b, Andrews *et al*., 2004). This is particularly true for the enzymes of primary nitrate assimilation, which do not seem to be suitable targets for metabolic engineering to improve NUE, at least in the plants in which they have been tested. On the other hand, QTL studies indicate that enzymes of secondary ammonium assimilation (cytosolic GS1, NADH-GOGAT and GDH) seem to hold more promise, especially in cereal crops (Miflin and Habash, 2002, Andrews *et al*., 2004). These and other leads must be pursued towards:

- enhancing overall N assimilation and amino acid production in source tissues;
- manipulating the metabolic pathways to produce essential amino acids in sink tissues;
- trapping the free essential amino acids into appropriate seed storage proteins; and
- identifying and manipulating global regulators in nitrogen metabolism.

While no such global regulator has been characterized beyond doubt, the recent success in improving nitrate assimilation by overexpressing Dof1 transcription factor in *Arabidopsis* (Yanagisawa *et al*., 2004) and efforts in other plants to manipulate regulatory proteins indicate the possibility of alterative routes for metabolic engineering. Thus, future manipulations of NUE may also involve the manipulation of regulatory switches outside the metabolic pathways.

Quantitative studies for genetic variability in NUE need to be conducted on Indian crop cultivars using a combination of molecular, agronomic and physiological approaches to identify new opportunities for intervention in the short and medium term. However, long term benefits will accrue from a deeper understanding of the genome-wide N response, wider regulation of N metabolic pathways and their interconnection with amino acids, carbon assimilation and recycling, metabolite partitioning and source-sink relationships. Such an understanding allows the development of integrated models for managing N flux in the whole plant context (Hammer *et al*. 2004). These modeling approaches will allow the integration of physiology and molecular genetics in a crop with the aim to optimize yield as a function of N nutrition in different genotypes and under different environmental conditions. As genetic manipulations can only be done on one or few genes at a time, these efforts should help in the identification of the most suitable target genes for manipulation in each crop for a given agricultural context.

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