Molecular physiology of plant nitrogen use efficiency and biotechnological options for its enhancement

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Nitrogen use efficiency (NUE) in plants is a complex phenomenon that depends on a number of internal and external factors, which include soil nitrogen availability, its uptake and assimilation, photosynthetic carbon and reductant supply, carbon–nitrogen flux, nitrate signalling and regulation by light and hormones, to name a few. The molecular basis for organism-wide regulation of nitrate assimilation is not yet fully understood, and biotechnological interventions to improve crop NUE have met with limited success so far. This article summarizes the physiological, biochemical and molecular aspects of NUE, QTL mapping studies as well as transgenic efforts to improve it in crop plants and model plants. It encompasses primary and secondary N-assimilatory pathways and their interplay with carbon metabolism, as well as signalling and regulatory components outside the metabolic cascade. The article highlights the need for an integrated approach combining fertilizer management techniques with biotechnological interventions to improve N flux and NUE for Indian crop cultivars.

Keywords: Biotechnological interventions, molecular physiology, nitrogen flux, nitrogen use efficiency.

NITROGEN (N) is one of the most critical inputs that define crop productivity and yield under field conditions, and must be supplemented to meet the food production demands of an ever-increasing population. Efficient utilization of fertilizer N is essential to ensure better value for investment as well as to minimize the adverse impacts of the accumulation of reactive N species in the environment. The current average nitrogen use efficiency (NUE) in the field[1] is approximately 33% and a substantial proportion of the remaining 67% is lost into the environment, especially in the intensively cropped areas[2]. This concern was reflected in the recent Nanjing Declaration of the International Nitrogen Initiative (http://www.initrogen.org/nanjing_declaration.0.html), which called for immediate development of a comprehensive approach to optimize N management in every sphere of life.

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Though the form and amount of N available to the plant can be improved by managing fertilizer–soil–water–air interactions, the innate efficiency of the plant to utilize this available N has to be tackled biologically. The biological processes involved include nitrogen uptake, translocation and assimilation, and their optimal contribution towards a desirable agricultural outcome, such as biomass growth and/or increased grain/leaf/flower/fruit/seed output, depending on the plant/crop involved. Identification of appropriate phenotypes, genotypes, molecular markers and target candidates for improvement of NUE poses a formidable challenge. The purpose of this article is to summarize the current state of our understanding of the physiological and molecular aspects of plant N response and NUE, with a brief overview of the attempts made so far towards manipulating it and the possible options and strategies for future interventions.

Concept and definition of NUE

As a concept, NUE includes N uptake, utilization or acquisition efficiency, expressed as a ratio of output (total plant N, grain N, biomass yield, grain yield) and input (total N, soil N or N-fertilizer applied). NUE is quantified based on apparent nitrogen recovery using physiological and agronomic parameters[3]. Agronomic efficiency is an integrative index of total economic outputs relative to the available soil N (native and applied). Apparent nitrogen recovery is related to the efficiency of N uptake; physiological NUE deals with N utilization to produce grain or total plant dry matter. The most suitable way to estimate NUE depends on the crop, its harvest product and the processes involved in it.

Molecular physiology of nitrogen uptake and assimilation

Among the various forms of N available to the plant, nitrate (NO_3^-) is the most preferred source for most plants. It is taken up by active transport through the roots, distributed through the xylem and assimilated by the sequential action of the enzymes nitrate reductase (NR) and

nitrite reductase (NiR). The end-product, ammonium (NH_4^+), is incorporated into amino acids via the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle[4]. 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 and the coordinated operation of multiple metabolic and regulatory pathways.

Nitrate uptake

Optimum uptake of nitrate is the first step to enhance N use in any plant. It has been established from a number of physiological studies that plants acquire their nitrate from the soil through the combined activities of a set of high- and low-affinity transporter systems[5], with the influx of NO_3^- being driven by the H^+ gradient across the plasma membrane. Some of these transporters are constitutively expressed, while others are nitrate-inducible and subject to negative feedback regulation by the products of nitrate assimilation. The low affinity transport system (LATS) is used preferentially at high external nitrate concentrations above 1 mM, while the high affinity transport system (HATS) works at low concentrations (1 μM–1 mM). LATS is constitutive in nature and possibly has a signalling role to induce the expression of HATS and nitrate assimilatory genes, presumably playing a nutritional role only above a certain threshold. The identification of two gene families, *NRT1* and *NRT2*, on the basis of their deduced amino acid sequences[5] has contributed towards unravelling the mechanisms of nitrate uptake in higher plants.

Physiology of nitrate reduction in crops

A portion of the nitrate taken up is utilized/stored in the root cells, while the rest is transported to other parts of the plant. Due to the abundant availability of photosynthetic reductants, leaf mesophyll cells are the main sites of nitrate reduction. This is initiated by the NAD/NADP-dependent NR enzyme, which catalyses the two-electron reduction of nitrate to nitrite in the cytosol. Nitrite is transported into the chloroplast, where it is further reduced into ammonium ion by a ferredoxin-dependent NiR. Being the first, irreversible and often rate-determining step of the N-assimilatory pathway, nitrate reduction has been a favourite step for physiological and biochemical approaches to optimize fertilizer N use (Table 1). The transgenic approaches have been dealt with separately later in the article.

Pattern of NR activity

NR activity in leaf blades, expressed either as seasonal average or converted into seasonal input of reduced N, has been related to total reduced N, grain N and grain yield of cereals[6]. The pattern of nitrate assimilation from different plant parts, viz. the main shoot of wheat[7], developing ear of wheat plants grown at different soil N levels[8] and in the leaf blades at different stages of growth[9] has revealed a direct positive correlation between increasing NR activity and increasing rates of nitrogenous fertilization. Most plant tissues have the capacity to assimilate nitrate, though their NR activity varies widely[9–12].

Analysis of the shoot components revealed that leaf blades are the main sites in cereals like wheat[8], corn[13] and barley[10]. Detectable level of NR activity has also been observed in the developing ear components of wheat, barley and pod covers of chickpea[14]. Among the ear components, the *in vivo* activity was highest in awns. It was also observed that the ontogenetical pattern of NR activity corresponds to the nitrate content, with which it is significantly correlated[15]. The light/dark conditions also affect NR activity; heterotrophic nitrate assimilation in darkness is closely linked to the oxidative pentose phosphate pathway and the supply of glucose-6-phosphate. Under photoautotrophic conditions, glucose-6-phosphate dehydrogenase is inhibited by reduction with thioredoxin in light, thus replacing the heterotrophic dark nitrate assimilatory pathway with regulatory reactions functioning in light[16]. These studies as well as bioenergetic calculations[17] have indicated that both yield and N harvest or protein can be increased to some extent with adequate nitrogen supply by altered management practices, thus improving the fertilizer NUE.

Genotypic differences in NR activity

Genotypic differences in the NR levels have been reported in corn, wheat, sorghum and barley. In sorghum, a positive relationship between decline in the height of the plant and enhancement of NR activity was observed[18], though no such relationship was evident in tall and dwarf cultivars of wheat, *T. aestivum*[19]. Wheat genotypes revealed over twofold variability in NR activity[20], which supports genetic findings that the enzyme level is highly heritable, its differences are reflected in N harvest and that hybrids could be bred with predictable NR levels by selecting parents appropriately. In the high NR genotypes, higher levels of NR activity were found under low N levels, often with significantly higher N concentration in the grains[21]. They also have sustained activity at later stages of growth, such as flag leaf emergence and anthesis[20]. The reasons for these genetic differences are not fully understood, except that the regulation operated at the level of gene expression[16] and that low levels of NADH might limit NR activity in low NR genotypes[22].

Ammonium assimilation

Ammonium is taken up directly through the roots, though uptake can also occur in a biphasic manner, involving

Table 1. Transgenic studies on N transport, primary and secondary N assimilating genes

Gene product and gene source		Promoter	Target plant	Phenotype observed
Nrt1.1 – High affinity nitrate transporter (*Arabidopsis*)		CaMV 35S	*Arabidopsis*	Increase in constitutive nitrate uptake but not in induced[57]
Nrt2.1 – High affinity nitrate transporter (*N. plumbaginifolia*)		CaMV 35S, rol D	*N. tabacum*	Increased nitrate influx under low N conditions[58]
NR – Nitrate reductase	*N. plumbaginifolia*	CaMV 35S	*N. tabacum*	3–4 fold drop in NR protein and activity, no change in NR transcript[92]
	N. plumbaginifolia	CaMV 35S	*N. tabacum*	Increased NR activity, biomass, drought stress[61]
Nia – Nitrate reductase	*N. tabacum*	CaMV 35S	*L. sativa*	Reduced nitrate content, chlorate sensitivity[93]
	N. tabacum	CaMV 35S	*N. plumbaginifolia*	Nitrite accumulation in high nitrate supply[94]
Nia2 – Nitrate reductase	*N. tabacum*	CaMV 35S	*S. tuberosum*	Reduced nitrate levels[60]
NiR – Nitrite reductase	*N. tabacum*	CaMV 35S	*N. plumbaginifolia*, *Arabidopsis*	NiR activity, no phenotypic difference[62]
	S. oleracea	CaMV 35S	*Arabidopsis*	Higher NiR activity, higher nitrite accumulation[63]
GS2 – Chloroplastic glutamine synthetase	*O. sativa*	CaMV 35S	*N. tabacum*	Improved photorespiration capacity, and increased resistance to photo-oxidation[64]
	O. sativa	CaMV 35S	*O. sativa*	Enhanced photorespiration, salt tolerance[65]
	N. tabacum	Rubisco small subunit	*N. tabacum*	Enhanced growth rate[67]
Fd-GOGAT – Fd dependent glutamate synthase (*N. tabacum*)		CaMV 35S	*N. tabacum*	Diurnal changes in NH_3 assimilation[66]
GS1 – Cytosolic glutamine synthetase	*G. max*	CaMV 35S	*L. corniculatus*	Accelerated senescence[70]
	G. max	rol D	*L. japonicus*	Decrease in biomass[71]
	P. vulgaris	Rubisco small unit	*T. aestivum*	Enhanced capacity to accumulate nitrogen[72]
	M. sativa	CaMV 35S	*N. tabacum*	Enhanced growth under N starvation[69]
	G. max	CaMV 35S	*M. sativa*	No increase in GS activity[73]
	Pea	CaMV 35S	*N. tabacum*	Enhanced growth, leaf-soluble protein, ammonia levels[95]
	P. sylvestris	CaMV 35S	*Hybrid poplar*	Enhanced growth rate, leaf chlorophyll, total soluble protein[74]
	G. max	CaMV 35S	*P. sativum*	No change in whole plant N[75]
	Alfalfa	CaMV 35S	*L. japonicus*	Higher biomass and leaf proreins[96]
NADH-GOGAT– NADH-dependent glutamate synthase	*O. sativa*	*O. sativa*	*O. sativa*	Enhanced grain filling, increased grain weight[79]
	M. sativa	CaMV 35S	*N. tabacum*	Higher total C and N content, increased dry weight[67]
GDH – Glutamate dehydrogenase	*E. coli*	CaMV 35S	*N. tabacum*	Increased biomass and dry weight[97]
	E. coli	CaMV 35S	*N. tabacum*	Increased ammonium assimilation and sugar content[98]
	L. esculentum	CaMV 35S	*L. esculentum*	Twice GDH activity, higher mRNA levels and twice glutamate concentration[99]
ASN1 – Glutamine dependent asparagine synthetase (*A. thaliana*)		CaMV 35S	*A. thaliana*	Enhanced seed protein[86]
ASNI – Asparagine synthetase (*P. sativum*)		CaMV 35S	*N. tabacum*	Reduced biomass and increased level of free asparagine[85]
*Asp*AT – Mitochondrial aspartate aminotransferase (prosomillet)		CaMV 35S	*N. tabacum*	Increased AspAT, PEPCase activity[100]
AlaAT – Alanine aminotransferase (barley)		btg26	*Brassica napus*	Good yields even with 50% less N fertilizer[101]
ANR1 – MADS transcription factor (*Arabidopsis*)		CaMV 35S	*Arabidopsis*	Lateral root induction and elongation[81]
GLB1 – PII regulatory protein (*Arabidopsis*)		CaMV 35S	*Arabidopsis*	Growth rate, increased anthocyanin production in low N[89]
Dof1 – Transcription factor (*Zea mays*)		35S C4PDK	*Arabidopsis*	Enhanced growth rate under N limited conditions, increase in amino acid content[80]

LATS and HATS[23]. Ammonium generated from primary nitrate assimilation, re-assimilation of internal metabolites or other secondary sources, is incorporated into amino acids in a reaction catalysed by GS and then by GOGAT[24]. GS is the central enzyme in ammonium assimilation in plants with a cytosolic (GS1) and a plastidic (GS2) isoform. Similarly, GOGAT has two isoforms, a ferredoxin-dependent plastidic isoform (Fd-GOGAT) and a NADH-dependent cytosolic isoform (NADH-GOGAT). The plastidic isoforms of both the enzymes (GS2 and Fd-GOGAT)[24] are involved in primary ammonia assimilation, while their cytosolic isoforms are involved in secondary assimilation.

Secondary ammonium assimilation/remobilization

As nitrogen is a major limiting factor for plant growth, the efficient re-assimilation of metabolically generated ammonium is highly important for NUE, plant performance and to prevent loss of ammonia to the atmosphere. Cytosolic GS1 and NADH-GOGAT are of critical importance in this regard. GS1 has been proposed as a key

component of NUE in plants[24] and its metabolic role is particularly important for nitrogen remobilization and recycling in woody plants[25,26]. Ammonium ions are also derived from the mitochondrial glycine decarboxylase reaction, which is an integral part of the photorespiratory carbon and nitrogen pathways. The importance of glutamate dehydrogenase (GDH) in higher plant N metabolism is still controversial, as it has never been clearly demonstrated that it plays a significant role either in ammonium assimilation or carbon recycling in plants. The role of GDH in N management and recycling has been recently reviewed in a number of whole-plant physiological studies performed on tobacco[27] and maize[28].

Signalling and regulation of nitrogen assimilation

Since N demand and its actual availability tend to vary in time, space and environmental conditions, the regulation of plant-N metabolism must be responsive to nutritional, metabolic and environmental cues. The following sections deal with the recent advances in our knowledge of the complex web of interactions in the regulation of nitrate assimilation by internal and external signals and its coordination with the overall metabolism of the plant.

Role of nitrate

Nitrate is not only a nutrient but also a signal for the regulation of hundreds of nitrate-responsive genes, which include N and C metabolizing enzymes, redox enzymes and a whole range of signalling proteins and transcription factors. However, the mechanism of nitrate signalling is not well understood, though calcium and protein kinases have been implicated, as reviewed reccently[29]. The transcriptional regulation of nitrate responsive genes could involve *cis*-acting regulatory sequences or nitrate response elements (NRE)[29]. Our recent genome-wide computational analysis of a previously reported NRE comprising the consensus sequence [$(a/t)_7$Ac/gTCA] based on NR and NiR turned out to be neither unique nor common to all nitrate responsive genes in *Arabidopsis* and rice, necessitating a fresh search for newer candidate NRE sequences[30]. However, the identification of putative *cis* elements that are responsive to C/N signalling interactions indicates the possible combinatorial role of different *cis*-regulatory elements[31]. Identification of such regulatory elements might provide an end-point for nitrate signalling and open up avenues for characterizing/manipulating the rest of the signalling pathway to enhance NUE.

Role of light

Light is an additional signal that regulates the expression of many nitrate responsive genes[32,33]. Light regulation of NR 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[33]. Using pharmacological approaches, the phytochrome-mediated regulation of NR gene expression in etiolated maize seedlings was suggested to be mediated through G-protein, PI cycle and protein kinase C (refs 34, 35). 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 inhibitory effects on NR-mRNA and activity, indicating different responses in maize and rice, either due to etiolated/green plant or C_3/C_4 differences[36].

Role of 14-3-3 proteins

It is becoming increasingly evident that 14-3-3 proteins play a crucial role in bringing about metabolic coordination between enzymes of C and N metabolism, modulating their activity by binding them in a phosphorylation-dependent manner. NR, GS, sucrose-phosphate synthase (SPS), trehalose-phosphate synthase, glutamyl-tRNA synthetase, and an enzyme of folate metabolism have been found to bind to 14-3-3s in a phosphorylation-dependent manner[37]. Experiments in transgenic potato plants indicate that repression of 14-3-3 proteins led to significant increases in NR and SPS activities[38]. More recently, the effect of repression of 14-3-3 genes on actual activity of NR in *Nicotiana benthamiana* leaves, was studied by silencing the *Nb14-3-3a* and *Nb14-3-3b* genes using virus-induced gene silencing method, which implicated Nb14-3-3a and/or Nb14-3-3b proteins in the inactivation of NR activity under darkness in *N. benthamiana*[39]. The 14-3-3 proteins also interact with components of plant signalling pathways as observed in their interaction with RGS3, a negative regulator of the G-alpha subunits of heterotrimeric G proteins[40], suggesting a possible role in the regulation of G-protein signalling pathways, which in turn have been implicated in mediating light regulation of NR[34].

Role of downstream N metabolites

Nitrite accumulation is toxic to the plant and is also inhibitory to nitrate induction, whereas the effects of ammonium and glutamine vary depending on the tissue and plant type as well as conditions of the study[41,42]. 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 the Krebs cycle, which are required for the synthesis of 2-OG[43]. Glutamate and 2-oxoglutarate have recently been shown to stimulate nitrate induction of NR and NiR in rice seedlings[42]. The role of glutamate as a signalling molecule in plant nitrogen metabolism has been reviewed recently[24], indicating that

the manipulation of N nutrition leads to dynamic alterations in plant respiratory metabolism in response to changes in cellular energetic demands. Therefore, the roles and interactions of downstream N metabolites may have to be factored into strategies for optimizing N response and NUE.

Role of hormones

Plant hormones like cytokinin have been shown to mimic the N-dependent regulation of gene expression in photosynthesis, cell cycling and translational machinery[44]; hinting at a possible role in communicating the availability of nitrogen from roots to leaves[45]. Additionally, N sensing and response also seem to be affected by the crosstalk between various plant hormones. Auxin synergistically affects cytokinin activity on cell division and organogenesis[46], while ABA antagonizes 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[47]. Although gibberellins do not seem to play any role in the control of nitrate assimilation, at least in the vegetative stages of *Arabidopsis*[48], benzyladenine in combination with nitrate was shown to enhance NR-specific mRNA[21]. Despite these findings, establishing the role of hormones in nitrogen signalling needs further characterization of the complete signalling pathway.

Interaction of nitrogen and carbon metabolism

The tight regulation of C/N metabolism has been revealed through numerous studies, which have indicated that the net photosynthesis rate and amount of photosynthetic components are correlated with the leaf-N content. The relative abundance of N pools in the plant plays a significant role in regulating the C/N metabolism[49]. 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[43]. 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[50]. 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[51]. Recent evidences of post-transcriptional control of C/N regulation by microRNAs have revealed globally coordinated regulation of specific sets of molecular machines in the plant cell[52].

Given the strong relationship between N and photosynthetic rates, plants maximize photosynthesis by optimizing partioning of N, which further depends on other environmental factors such as irradiance, nutrients, CO_2 concentration, etc. Consequently, the photosynthetic nitrogen use efficiency (PNUE) is determined by the rate of carbon assimilation per unit leaf nitrogen[53]. Plants possessing C_4 photosynthesis have a greater PNUE than C_3 plants, owing to the C_4 concentrating mechanism that leads to CO_2 saturation of Rubisco. Higher CO_2 concentrations both compensate for the poor affinity of Rubisco for CO_2 and suppress oxygenase activity, consequently increasing the PNUE at elevated concentrations. Further evaluation of the key components of photosynthesis and interactions of C/N metabolites might offer avenues for improving N uilization by optmizing N content in accordance with photosynthetic demand.

Interaction of nitrogen and sulphur metabolism

The importance of sulphur as a nutrient and its management vis-à-vis other nutrients like nitrate have been reviewed recently[54]. Under sulphur-deficit conditions, reduced protein synthesis is accompanied by accumulation of organic and inorganic nitrogenous compounds, leading to reduced NUE[55], indicating the need to achieve optimum N/S balance for improved NUE.

Options for improvement in NUE

NUE can be improved to some extent by optimizing fertilizer–soil–water interactions, though the biological aspects of crop improvement form the main purpose of this article. Regardless of the approach adopted, the challenges in improving NUE include optimization of N supply and demand, maximization of crop N uptake and assimilation, minimization of N losses and ultimately, specific improvements in the yields of biomass, leaves, fruits or grains, as the case may be. The current section deals with some of these approaches and their impact on crop NUE.

Fertilizer-N application management (repeated N fertilization in split doses)

Prevalent fertilizer management practices result in high nitrate content and NR activity in the first-formed leaf blades, which decline in the subsequently formed ones[20]. The pattern was paralleled by soil nitrate concentration and its total content. Incubation of excised leaf blades in a nutrient solution containing 15 mM NO_3^- resulted in slight increase in the NR activity of the lower leaf blade of wheat, while the activity of the upper ones was enhanced manifold; the level of enhancement being higher in 'high NR' cultivars than in 'low NR' cultivars[20]. It has

been demonstrated successfully that application of the same amount of nitrogen fertilizer in more than two splits under field conditions clearly increases the nitrogen availability at later stages of growth, exploiting the sub-optimal activity of the upper laminae in wheat[20]. Studies on Indian mustard genotypes with contrasting NUE showed that plants with high N uptake efficiency (UE) and high physiological N utilization efficiency (PUE) are able to not only take up N efficiently, but also utilize N efficiently. Such plants are highly desirable because they can be grown with limited N supply for environment-friendly farming systems. Genotypes with high UE accumulated higher N content than those with low UE under limited N conditions. High PUE is essential for optimum seed yield, because these genotypes absorbed N efficiently. Although the genotype with high UE and low PUE takes up N efficiently from the soil, it remains unutilized in the form of non-protein-N, as UE showed significant positive correlation with the free amino acid pool[56]. Thus, development of such N-efficient genotypes, which can grow and yield well at low N levels further enhance options for better management of the applied N fertilizer.

Transgenic efforts to manipulate NUE

The speed and precision of the transgenic approach enables one not only to test the candidate genes considered to be critical for NUE by overexpressing them, but also to identify such genes by knock-out mutations. The following sections describe various transgenic studies involving different categories of nitrate responsive genes.

Manipulation of transporters

Studies in the last decade have shown that enhancing the uptake of N by overexpressing transporters may not necessarily improve NUE. For example, transgenic overexpression of a *CHL1* cDNA (representing the constitutive HATS) driven by the cauliflower mosaic virus 35S promoter in a *chl1* mutant, recovered the phenotype for the constitutive phase but not for the induced phase[57]. Similarly, the NO_3^- contents in transgenic tobacco plants overexpressing the *NpNRT2.1* gene (encoding HATS), were remarkably similar to their wild-type levels, despite an increase in the NO_3^- influx[58]. These findings indicate that genetic manipulation of nitrate uptake may not necessarily lead to concomitant improvement in nitrate retention, utilization or NUE, though it remains to be seen whether different plants respond differently to the overexpression of different transporters.

Manipulation NR and NiR genes

NR has long been considered to be the rate-limiting step in nitrate assimilation. Efforts to improve NUE by manipulating *NR* and *NiR* genes have yielded mixed results, with transformed *Nicotiana plumbaginifolia* plants constitutively expressing NR, showing a temporarily delayed drought-induced loss in NR activity, thereby allowing more rapid recovery of N assimilation following short-term water-deficit (Table 1). At the transcriptional level, de-regulation of *NR* gene expression by constitutive expression in transgenic plants caused a reduction in nitrate levels in tissues of tobacco[59] and potato[60]. While 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 restored N assimilation. Though 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[61]. Similarly, overexpressing *NiR* genes in *Arabidopsis* and tobacco resulted in increased NiR transcript levels but decreased enzyme activity levels, which were attributed to post-translational modifications[62,63]. Therefore, the utility of transgenic overexpression of NR/NiR for major improvements of NUE remains uncertain, though the possibility that different crops respond differently cannot be ruled out yet.

Manipulation of GS2 and Fd-GOGAT genes

Improvement in NUE via manipulation of plastidic GS2 and Fd-GOGAT genes has met with limited success. Transgenic tobacco plants with twofold overexpression of GS2 were shown to have an improved capacity for photorespiration and an increased tolerance to high-intensity light. On the other hand, transgenics with reduced amount of GS2 had a diminished capacity for photorespiration and were photoinhibited more severely by high-intensity light compared to control plants[64]. Overexpression of GS2 has also been reported in rice[65] and tobacco[66], with improved reassimilation of ammonia in tobacco[67]. Studies on barley mutants with reduced Fd-GOGAT revealed changes in various nitrogenous metabolites, decreased leaf protein, Rubisco activity and nitrate content[68]. While these studies hint at the potential of such transgenic attempts, most of them have been inconclusive regarding NUE so far, due to lack of physiological and agronomic data.

Manipulation of GS1 and NADH-GOGAT genes

Ectopic expression of pea GS1 in tobacco leaves was suggested to provide an additional or an alternative route for the reassimilation of photorespiratory ammonium, resulting in an increase in the efficiency of N assimilation and enhanced plant growth[69]. Efforts to raise more efficient GS1 transgenic lines have met with varying degrees

of success[70–76], with Man *et al.*[77] providing additional empirical evidence for enhanced nitrogen-assimilation efficiency in GS1 transgenic lines. Transgenic overexpression and underexpression studies to modulate the expression of NADH-GOGAT in alfalfa and rice plants[78,79] have implicated the 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. Though these genes of secondary ammonia assimilation appear to be more viable candidates for improving NUE, the degree of success needs to be tested across crops and cropping conditions.

Manipulation of signalling targets

Yanagisawa *et al.*[80] 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 up to 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 upregulated by Dof1 overexpression clearly belong to the list of known nitrate responsive genes, opening up attractive possibilities of improving NUE through coordinated expression of N and C metabolizing genes. A few other attempts to manipulate signalling/regulatory proteins have been made[81], without significant advantage in terms of NUE. Other attempts, such as the one to manipulate a MADS box protein that controls nitrate-induced changes in root architecture, have not been assessed for their impact on NUE[81].

Manipulation of source–sink relationships and nutritional quality

Molecular manipulation of certain key enzymes of N metabolism provides an attractive means to enhance the nutritional value of plant products along with increasing the quality and quantity of seed proteins in crop plants. The enzyme asparagine synthetase (AS) catalyses asparagine, one major function of which is to transport and store nitrogen according to the plant's need. It can also reallocate nitrogen during specific developmental stages and environmental changes. The efficiency of protein synthesis has been shown to be dependent on the light/dark regulation of AS activities[82], with elevation of leaf AS activities and Asn levels being used as parameters to screen for high-grain protein cultivars in maize[83] and rye[83]. On the other hand, regulating the expression of the *ASN1* gene to manipulate the relationship between Asn and seed N status might enhance nutritional quality. Studies have implicated AS as one of the major controlling forces for nitrogen flux when GS is limiting in plants[84], and several studies on transgenic overexpression of *AS* genes have revealed enhanced seed protein content and total protein content[85–87]. Recent genetic modification of rice and wheat using barley alanine amino transferase (*AAT*) gene have also yielded encouraging results, including increased biomass and seed yield compared to their wild-type counterparts. Recently, Arcadia Biosciences claimed to have improved NUE by transgenic overexpression of *AAT* in canola, *Arabidopsis*, tobacco and rice, though their actual field performance is yet to be ascertained (http://www.arcadiabio.com/nutrient.htm). In other studies, two potentially important N regulation systems of *Arabidopsis*, PII (ref. 88) and GCN2 (ref. 89) have been targetted; though detailed analyses of the effect of their transgenic overexpression on NUE are yet to be reported[90].

QTL mapping to find new targets for manipulation

The development of molecular markers has facilitated the evaluation of the inheritance of NUE using specific quantitative trait loci (QTLs) that could be identified. 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[91]. 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[91].

Future perspectives

It is clearly evident that optimizing the plants NUE goes beyond the primary process of uptake and reduction of nitrate, involving a paraphernalia of events, including metabolite partitioning, secondary remobilization, C–N interactions, as well as signalling pathways and regulatory controls outside the metabolic cascades. Despite the various attempts to manipulate each of the above steps in some plant or the other, we are far from finding a universal switch that controls NUE in all plants. However, transgenic studies and QTL approaches seem to increasingly suggest that the enzymes of secondary ammonia remobilization are better targets for manipulation, followed by regulatory processes that control N–C flux, rather than the individual genes/enzymes of primary nitrate assimilation. However, it is possible that different plants respond differently to various targets of manipulating NUE, especially since field-level improvements in NUE result from many more complex interactions. The

need of the hour is integration of physiology and molecular genetics involving Indian crop cultivars to optimize yields in different genotypes and environmental conditions.

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