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BIOTECHNOLOGICAL APPROACHES TO IMPROVE NITROGEN USE EFFICIENCY IN PLANTS

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Chapter 2

SIGNALLING AND THE MOLECULAR ASPECTS OF N-USE-EFFICIENCY IN HIGHER PLANTS

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Nitrogen (N) use efficiency is critical for plant growth, biomass, crop yield and protein content, as well as for optimal utilisation of fertilisers. This requires coordinated expression of the genes involved in nitrate reduction as well as carbon partitioning for amino acid synthesis. This coordination between C and N metabolism is brought about by nitrate, which acts as a nutrient signal. Genome-wide analysis of nitrate responsive genes in various plants revealed that hundreds of genes are induced (or repressed) by nitrate in some tissue or the other. The 'nitrate response elements' that were originally identified for nitrate

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reductase are likely to be relevant for other nitrate-responsive genes as well, providing a common end-point for nitrate signal transduction. The search for nitrate-specific transcription factors has not been very successful, though other transcription factors (eg. Dof1) that regulate the genes of organic acid metabolism have been shown to be useful targets for the improvement of N utilisation and N content in Arabidopsis. Nitrate uptake is a highly regulated process that responds to nitrate, downstream metabolites, hormones etc., and the activity of high affinity nitrate transporters is critical for plant growth and productivity under N-limiting conditions. The precise mechanism of nitrate sensing and signalling is not yet fully understood, but hormones, calcium and protein kinases have been implicated in the transcriptional regulation of nitrate-responsive genes. Post-translational regulation of some of the nitrate-responsive enzymes is brought about by 14-3-3 proteins, though they mainly mediate the effect of light and other signals, rather than nitrate. The failure to improve N-use efficiency in transgenic plants by overexpressing individual enzymes of nitrate and ammonia assimilation has strengthened the view that metabolic flux through these pathways may be controlled by regulatory switches outside these pathways. The searches for quantitative trait loci (QTL) associated with N-use efficiency have shown that manipulating the genes of secondary ammonium assimilation (cytosolic GS1, NADH-GOGAT and GDH) may also be a useful strategy, at least in some crop plants. In terms of finding a global target for manipulation of NUE, the successful manipulation of N content by overexpression of the Dof1 transcription factor indicates that unravelling the signalling mechanisms that bring about their coordinated expression of nitrate-responsive genes by N and C metabolites could reveal new targets and approaches for future metabolic engineering efforts.

1. INTRODUCTION

Nitrogen use efficiency (NUE) at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity. In terms of agriculture, it is the optimal utilisation of nitrogenous manures or fertilisers for plant growth, yield and protein content, as atmospheric nitrogen gas is not utilised by most higher plants, except symbiotic legumes. 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). This includes nitrogen uptake and assimilatory processes, redistribution within the cell and balance between storage and current use at the cellular and whole plant level. Moreover, since N demand and its actual availability tend to vary in time, space and environmental conditions, the regulation of plant nitrogen metabolism must be responsive to nutritional, metabolic and environmental cues. This article deals 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.

2. NITRATE AS A NUTRIENT AND SIGNAL

Regardless of the form in which N is supplied viz., urea, ammonia or nitrate, the microbial process of nitrification in most aerobic soils ensure that nitrate is the most abundant form in which N is available to the plant. Nitrate is also a preferred source of N for most plants. The ability of plants to respond to nitrate is known for well over three centuries now. $KN0_3$ was shown to improve plant growth by Glauber in 1665. However, it was not until 1957 that the induction of nitrate reductase (NR) by nitrate was shown, making it the first substrate-induction system in plants (Tang and Wu, 1957). As a nutrient, nitrate is taken up from the soil with the help of nitrate transporters and converted into ammonium by the sequential action of the enzymes nitrate reductase (NR), nitrite reductase (NiR), and then incorporated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle. These enzymes and their genes have been well characterized from several plants and mutants and transgenic plants are also available for *in-vivo* studies (Campbell, 2002; Crawford, 1995; Stitt and Sonnewald, 1995; Lam *et al*., 1996).

The larger role of nitrate as a signal was first articulated by Redinbaugh and Campbell (1991), who demonstrated that the induction of nitrate reductase gene expression is one of the first responses of the plant to nitrate, and that the biochemical apparatus needed for nitrate response is constitutively expressed. Subsequent experiments by Tischner *et al.* (1993) on the response of NR gene to short nitrate pulses in intact barley seedlings revealed that: a) permanent presence of external nitrate is not necessary for initiation of NR gene expression; b) there is no lag in nitrate uptake and c) nitrate pulses cause an acceleration of nitrate uptake rate (positive feedback). Based on these observations, they suggested that nitrate acts more as a signal than as a N-source and that the signal transduction apparatus may be constitutively expressed. The identification of a nitrate-induced nitrate transporter confirmed the positive feedback loop (Tsay *et al.*, 1993).

Subsequent investigations from several laboratories soon revealed that nitrate response is not limited only to the uptake and expression of NR, but also induction of genes involved in ammonia assimilation (Redinbaugh and Campbell, 1993), reductant supply (Ritchie *et al*, 1994), cofactor biosynthesis (Sakakibara *et al.*, 1996), as well as enzymes of carbon assimilation such as PEP carboxylase and sucrose phosphate synthase (Champigny and Foyer, 1992), cytosolic pyruvate kinase and isocitrate dehydrogenase, mitochondrial citrate synthase etc, and downregulation of ADP-glucose pyrophosphorylase (Scheible *et al.*, 1997). This made sense, as utilisation of reduced nitrogen (ammonium) towards amino acid synthesis through the GS-GOGAT cycle depends on the availability of carbon (C) skeletons derived from the utilisation of photosynthetic sugars. This involves the regulation of carbon traffic between starch/sucrose synthesis and amino acid synthesis. Thus, nitrate acts as a signal for the regulation of metabolite partitioning, organic acid and amino acid synthesis, starch synthesis and redox metabolism (Stitt, 1999a). On the other hand, ever since sugars were found to stimulate nitrate reductase transcription (Cheng *et al.*, 1992) and the role of sugars in modulating plant gene expression became established (Koch, 1996), the criss-cross regulation of C and N metabolism by N and C metabolites became a dominant theme of research (Coruzzi *et al.*, 2001; Stitt *et al.*, 2002; Stitt and Fernie, 2003; Foyer *et al.*, 2003).

2.1. Nitrate-Response Spans Over a Thousand Genes

The advent of high-throughput methods in the recent years began to reveal the true extent of the influence of nitrate in plant gene

expression, metabolism, growth and development. Using, Incyte cDNA microarrays containing 5,524 unique genes / clusters representing about a quarter of the *Arabidopsis* transcriptome, Wang *et al.* (2000) identified over 15 new nitrate-responsive genes, including metabolic enzymes such as transaldolase and transketolase of the nonoxidative pentose pathway, malate dehydrogenase, asparagine synthetase and histidine decarboxylase, regulatory genes such as a MYB transcription factor, a calcium antiporter, putative protein kinases and some proteins with unknown functions, such as nonsymbiotic hemoglobin, a senescence associated protein and two methyl transferases. The expression patterns of these genes were analysed in terms of response to low (0.25 mM) or high nitrate (5-10 mM) concentrations, the degree of induction as well as transient or sustained change in the RNA levels. Most of the known nitrate-regulated genes including the enzymes of nitrate transport (NRT1) and assimilation appeared in the 40 most strongly nitrate-induced genes. In general, low concentrations of nitrate produced only transient induction, whereas higher concentrations of nitrate yielded sustained induction of the responsive mRNAs. However, this study was conducted using whole seedlings of *Arabidopsis*, and could not differentiate spatial changes in gene expression.

In a subsequent study, Wang *et al*. (2003) used Affymetrix microarrays containing probe sets for 22,500 genes of *Arabidopsis*, and analysed them for response of roots and shoots to low nitrate (0.25 mM) provided for 20 min. This study revealed that the overall response was much stronger in the roots, involving 1,176 genes, as compared to the shoots, in which only 183 genes responded to nitrate in terms of significant increase or decrease in their RNA levels. In addition to confirming many known genes, this study revealed many novel nitrate-responsive genes, including enzymes of the glycolytic pathway (glucose-6-phosphate isomerase and phosphoglycerate mutase), trehalose6-P metabolism (trehalose-6-P synthase and trehalose-6-P phosphatase), iron transport / metabolism (nicotinamine synthase) and in sulfate uptake / reduction. Many potential regulatory genes were also found to be nitrate responsive, including several transcription factors, protein kinases / phosphatases, zinc finger proteins and response regulators. This study showed that nitrate response spans a vast array of over a thousand genes, spanning up to 10 % of the detectable *Arabidopsis* transcriptome, though the number was revised subsequently (Wang *et al*., 2004, see below). It also showed that only a small number of members of multi-gene families or

metabolic pathways are nitrate-responsive. This means that barring the genes of nitrate transport and assimilation, nitrate response does not control entire gene clusters, families or metabolic pathways, but only few members spanning a large number of families/clusters/pathways.

Other investigators have used suppression subtractive hybridisation (SSH) approach to identify nitrate-responsive genes and characterise their expression in tomato (Wang *et al*., 2001) and rice (Wang *et al*., 2002). In tomato roots, the SSH approach allowed the identification of 1,280 cDNAs related to mineral nutrition, which were then spotted on nylon microarrays and tested for nitrate response using RNA preparations from control and nitrate-treated roots. This approach revealed 115 nitrate responsive genes in tomato, most of which coincided with those reported in *Arabidopsis*, and included water channels, potassium and phosphate transporters, ribosomal proteins, stress response proteins, regulatory proteins and signalling proteins. An interesting finding of this study was that both high affinity and low affinity transporters were also induced by deficiencies of other nutrients such as phosphate, potassium and iron, indicating new relationships between nitrate and other mineral nutrients at the level of transport regulation. In rice, Wang *et al* (2002) used SSH method in split root-experiments to identify 92 nitrate-responsive genes and differentiate the changes in gene expression in nitrate-supplied side roots and nitrate-deficient side roots. Among other things, this study revealed that a fast cycling of assimilated N metabolites occurred between nitrate-replete and nitrate-deficient side roots, as well as between roots and shoots, For example, some nitrate-regulated genes such as GOGAT were also up-regulated in nitrate-deficient side roots. This study also linked nitrate-induced lateral root growth to the enhancement of carbon source partition, and expression of genes for signal transduction and transcription regulation, auxin transport and ethylene synthesis and cyclin-dependent kinases.

An important limitation in studying nitrate responsive gene expression using prolonged nitrate treatments is that it is difficult to separate the effect of nitrate as a signal from the effects of the downstream metabolites of nitrate reduction. This limitation was overcome recently by using *Arabidopsis* knock-out mutants deficient in both NR1 and NR2 for repeating the microarray analysis of nitrate-responsive genes (Wang *et al*., 2004). This study revealed a total of 595 genes that respond to nitrate either positively or negatively in some tissue or other. This study also showed that shoots are as

responsive to nitrate as roots, in terms of the number of genes and degree of induction, provided the duration of nitrate treatment was adequate (20 min for roots and 2 hrs for shoots). This is a significant improvement from their earlier study using wild type plants (Wang et al., 2003), in which roots showed higher response than shoots, owing to shorter duration of nitrate treatment. An important revelation from the above genome-wide analyses is that some of the nitrate-responsive genes are among those that are known to respond to other signals such as stress, hormones or light. In other words, the effect of nitrate on genome wide changes in plant gene expression is extensive rather than intensive, and nitrate-use-efficiency is probably a result of this mega-coordination.

2.2. Nitrate Response at the Gene Level Requires *Cis*-Elements

It is a well known concept in signal transduction that whenever multiple genes are subject to transcriptional regulation by a common signal, it is mediated through a regulatory sequence that exists in all the genes that respond to the signal. These signature sequences, commonly known as response elements, are identified by mutations that abolish their function, and their conserved nature as revealed by homology comparisons. Early experiments in transgenic *Nicotiana* plants using GUS gene fused to NR and NiR promoter sequences clearly demonstrated for the first time that nitrate induction of gene expression requires some sequence(s) associated with the NR and NiR promoters (Vaucheret *et al.*, 1992; Rastogi *et al.*, 1993). Subsequent studies in transgenic tobacco incorporating the 5' flanking regions of the two *Arabidopsis thaliana* nitrate reductase genes *NR1* and *NR2* (designated *NP1* and *NP2*) demonstrated that 238 and 330 bp of *NP1* and *NP2* respectively are sufficient for nitrate-dependent transcription (Lin *et al.*, 1994). Using linker-scanning analysis, the same group eventually identified the *cis*-acting elements within NP1 and NP2 that are essential for nitrate-dependent transcription of nitrate reductase genes in *Arabidopsis thaliana* (Hwang *et al.*, 1997).

These nitrate-responsive elements (NREs) are composed of several copies of a core A[G/C]TCA sequence motif preceded by an ~7-bp AT-rich sequence present in the 5'flanking regions of nitrate reductase (*NR1* and *NR2*) genes. This particular sequence motif was also found to be very well conserved in the 5' flanking regions of NR and NiR genes from eight other plants. Using gel-shift assays, it was also demonstrated that the NRE sequence motif is essential for binding to proteins in the nuclear extracts of *Arabidopsis* (Hwang *et al.*, 1997).

Similarly, deletion analyses of birch promoter regions revealed the presence the same NRE sequence motifs in NR (Hachtel, and Strater, 2000) and NiR (Warning, and Hachtel, 2000). However, experimental analyses of NREs so far were restricted to NR from *Arabidopsis* and birch, and NiR from birch alone. Even the theoretical sequence comparisons with NR and NiR promoters from other plants were restricted to very few plant species (Hwang *et al.*, 1997).

In an attempt to verify whether the proposed NREs are equally relevant to the expanding list of nitrate responsive genes, Sarkar (2003) compared the flanking sequences of all available plant nitrate responsive genes and found that the NRE core sequence (A[C/G]TCA) was present in multiple copies on both strands in all the known nitrate-responsive genes in many dicots, monocots and cyanobacteria. Though most of the NREs examined contained both the core sequence and a proceeding AT rich sequence, there were some cases which had GC rich regions or did not reveal any AT/GC bias. A more detailed bioinformatic analysis of the entire *Arabidopsis* genome in our lab revealed that the proposed NREs are randomly distributed, with no difference between nitrate responsive genes and the presumably non-responsive genes and intergenic regions in the rest of the genome (unpublished data). These findings raise doubts on the validity of the proposed NRE as comprising of (A[C/G]TCA) elements preceded by AT-rich sequence. Further work in this area will need a combination of bioinformatic and experimental approaches to redefine the NREs that mediate the expression of all nitrate responsive genes in all plants. The discovery of NREs is important, as it provides an end point for nitrate signal transduction.

2.3. *Trans*-Acting Factors in Nitrate-Responsive Gene Expression

The role of trans-acting factors in higher plant nitrate response is poorly understood. In fungi such as *Aspergillus nidulans* and *Neurospora crassa*, nitrate inducibility of nitrate transport, NR and NiR activities is controlled by transcriptional regulatory proteins, NIRA (*A. nidulans*) and NIT4 (*N. crassa*). Their effect on NR transcription is counteracted by AREA and NIT2 respectively, which mediate ammonium-repression (Marzluf *et al,* 1997). They belong to the GATA family of transcription factors. However, attempts to find their homologs in higher plants have not been very successful. Even though their binding motifs have been found in the upstream regions of some nitrate-responsive genes, no GATA boxes were found in the sequences essential for nitrate response (Rastogi *et al.*, 1997). On the other hand, ANR1, a putative

transcription factor homologous to the MADS box family has been reported in *A. thaliana* (Zhang and Forde, 1998). ANR1 is nitrate inducible and root-specific, and has been shown to be involved in nitrate-dependent stimulation of lateral root proliferation in transgenic plants (Forde, 2002). However, this root-specific transcription factor does not account for the transcription of all the known nitrate responses even in the root, besides being irrelevant for nitrate-responsive gene expression in the shoots.

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 improve nitrogen assimilation and NUE. Yanagisawa *et al.* (2004) raised transgenic *Arabidopsis* lines overexpressing Dof1, a maize transcription factor that belongs to the Dof family of plant-specific transcription factors known to activate the expression of several C-metabolising genes associated with organic acid metabolism. The transgenic lines showed improved nitrogen content (by 30 %), 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 transcripts. It is not clear whether Dof1 is inducible by nitrate, but the genes upregulated by Dof1 overexpression clearly belong to the list of known nitrate-responsive genes. If Dof1 is not nitrate-inducible, it means that multiple transcription factors may be involved in the coordinated expression of N and C metabolising genes.

3. NITRATE UPTAKE IS REGULATED

Plants have evolved an active, regulated and multiphasic transport system making their NO_3^- uptake scheme efficient enough to transport sufficient NO_3^- to satisfy total nitrogen demand of the plant in face of varying external NO_3^- concentrations. Plants can also take up other forms of nitrogen, such as amino acids and ammonium ions. Root NH_4^+ uptake is carried out by both high affinity and low affinity NH_4^+ transporters that are encoded by a multigene family (Glass *et al.*, 2002). However, nitrate is the most abundant form of nitrogen available to the plant roots in aerated soils. Nitrate influx is an active process driven by the H^+ gradient and can work against an electrochemical potential gradient (Vidmar *et al.*, 2000). The uptake involves high and low affinity transport systems, also known as HATS and LATS respectively (Forde, 2000, Tischner, 2000, Touraine *et al.*, 2001). One of the high affinity systems is strongly induced in presence

of NO_3^- and is known as inducible high affinity transport system (or iHATS,), while the second high affinity system (the cHATS) and LATS are constitutively expressed (Aslam *et al*., 1993; Glass *et al*., 1995, Forde, 2002). The K_m values of iHATS, cHATS and LATS for nitrate are in the ranges of 13-79 µM, 6-20 µM and >1mM respectively.

The iHATS is a multicomponent system encoded partly by genes of the NRT2 family or nitrate – nitrite porter family of transporters. Recently, two dual affinity transporters have been identified in *Arabidopsis*, AtKUP1 and CHL1 or AtNRT1.1, of which the latter is induced as HATS by phosphorylation at threonine residue 101. Upon dephosphorylation it functions as a low affinity nitrate transporter. This mode of regulation and function may be critical when the plant is competing for limited nitrogen (Liu and Tsay, 2003). AtNRT1.1 is the only higher plant member of the PTR or POT family to be cloned on the basis of chlorate resistance. Members of this family are widely distributed in prokaryotes and eukaryotes and function as H+/ oligopeptide cotransporter in the plasma membrane (Steiner *et al*., 1995). This family of transporters is recognized as being exceptional in both the variety of different substrates which its members can mobilise (oligopeptides, amino acids, NO_3^-, chlorate) and in the ability of individual transporters to handle substrates of very different sizes and charges.

Nitrate acts as a regulator for its own uptake, a specific property which is not seen in other ion transport systems such as phosphate, sulfate etc. On exposure of the cells to external NO_3^- the uptake capacity increases after a lag period of 0.5 to 1.5 hours and reaches a new steady state after 4 to 6 hrs. Use of RNA and protein synthesis inhibitors provided early evidence that induction of the iHATS involves gene expression and the synthesis of new transporter protein (Hole *et al*., 1990; Aslam *et al*., 1993). The evidence that the inducer of iHATS is indeed nitrate ion and not its downstream metabolite came from NR-deficient mutants of *Arabidopsis* and *N. plumbaginifolia* (Krapp *et al*., 1998, Lejay *et al*., 1999). Studies in NR-deficient mutants have also revealed that nitrate reductase activity is not required for nitrate transport in higher plants, unlike in lower organisms (Unkles *et al*., 2004).

Nitrate transporters are often subject to feedback inhibition by downstream metabolites. Ammonium strongly inhibits NO_3^- uptake and has both short term and long term effects. Inhibition of NO_3^- uptake is also observed when amino acids are used as the only source of nitrogen (Muller and Touraine, 1992, Krapp *et al*., 2002). Recent

studies revealed that feed back inhibition of nitrate transport by reduced nitrogen metabolites involves transcriptional as well as post-translational controls (Fraisier *et al*., 2000, Vidmar *et al*., 2000, Glass *et al*., 2002, Orsel *et al*., 2002), though earlier studies in NR mutants did not indicate any post-translational regulation (Lejay *et al*, 1999). Light is known to enhance NO_3^- uptake in a number of plant species (Le Bot and Kirkby, 1992; Delhon *et al*., 1995; Cárdenas-Navarro *et al*., 1998), and diurnal changes in nitrate uptake have been observed. These changes seem to be linked to the imbalance between nitrate uptake and reduction due to the light regime (Matt *et al*., 2001) and as well as to the rate of photosynthesis in shoots (Delhon *et al*., 1996). Reduced nitrate uptake during darkness could be reversed by exogenous supply of sugars (Sehtiya and Goyal, 2000). Recent evidence on the up-regulation of AtNRT1.1 gene expression by auxin (Guo *et al*, 2002) suggests that nitrate transporters may also be regulated by hormones.

4. HORMONES AND NITRATE SIGNALLING

Several studies during the last decade point to the role of hormones in mimicking, mediating or modulating the nitrate response. For example, cytokinin metabolism and translocation could be modulated by the nitrogen nutrition status; in other words, 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). These findings strongly suggest a role for cytokinins in communicating the availability of nitrogen from roots to leaves (Sugiyama and Sakakibara, 2002).

The cross talk between various plant hormones also has implications for nitrogen sensing and response. For example, auxin synergistically affects cytokinin activity on cell division and organ development. (Soni *et al*., 1995). On the other hand, ABA antagonises the cytokinin-mediated nitrogen signalling by means of negative regulation of cytokinin-inducible response regulator genes. Unlike cytokinins, which are positively regulated by nitrate as a signal, ABA biosynthesis is down regulated by nitrogen-sufficiency (Gawronska

et al, 2003). These findings regarding the role of hormones in nitrogen signalling await further characterization of the complete signalling pathway. 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).

5. NITRATE SENSING AND LIGHT SIGNALLING

While the role of nitrate as a signal and the range of responses it elicits have been well characterized, the mechanism of nitrate sensing and the exact signalling events that bring about signal-response coupling have not yet been understood. While the nitrate sensing protein proposed by Campbell (Redinbaugh and Campbell, 1991) over a decade ago is yet to be identified, nitrate sensing by a cytokinin precursor followed by His-Asp phosphorelay has been proposed recently as discussed above (Sugiyama and Sakakibara, 2002). However, some other elements of the signalling cascade have been suggested 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). Other kinases that post-translationally modulate NR, SPS or PEP carboxylase have been purified and partially characterized. 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. Other kinases / phosphatases have been described as well (reviewed by Krapp *et al*., 2002), but their specific roles in mediating nitrate and other interacting signals have not been clearly delineated. Mutants related to the signal transfer cascade from nitrate to the NR gene have been reported (Ogawa *et al*., 2000), and their detailed characterization may reveal more intermediates and potential sites for manipulation.

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. The role of light in regulation of NR gene expression has often been reviewed (Raghuram and Sopory, 1995a, Chandok *et al*., 1997, Lillo and Appenroth, 2001). At the transcriptional level, the expression of NR is regulated differently in green plants and etiolated seedlings and is mediated by different photoreceptors. Using pharmacological approaches, the phytochrome-mediated regulation of NR gene expression in maize was shown to be mediated through G-protein (Raghuram *et al*., 1999), PI cycle and protein kinase C (Raghuram and Sopory 1995b). The effects of light in green plants are

probably mediated more indirectly, through photosynthesis and sugars (Lillo and Appenroth, 2001). At the post-translational level, light acts by modulating the phosphorylation status of the enzyme, in conjunction with 14-3-3 proteins.

6. 14-3-3 PROTEINS AND METABOLIC REGULATION

Efficient utilisation of available nitrate requires coordinated gene expression and/or post-translational regulation of the proteins/enzymes involved in nitrate transport and reduction, as well as those involved in carbon partitioning for amino acid synthesis. This is brought about in part by the criss-cross regulation of C-metabolising enzymes by nitrate (Stitt 1999b) and N metabolising enzymes by sugars (Koch 1996) at the transcriptional level. At the post-translational level, it is becoming increasingly evident that regulatory binding proteins known as 14-3-3 proteins bring about this metabolic coordination (Huber *et al.*, 2002, Comparot *et al.*, 2003). 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 14-3-3s in a phosphorylation dependent manner. (Moorehead *et al.*, 1999; Cotelle at al. , 2000). NR, for example, is inactivated by 14-3-3 following phosphorylation by protein kinases responding to light–dark transitions and changes in cellular energy status (Huber *et al.*, 2002, Comparot *et al.*, 2003).

Recent experiments in transgenic potato plants indicate that repression of 14-3-3 proteins lead to significant increases in NR and SPS activities, and even higher levels of starch accumulation in the tuber (Zuk *et al.*, 2003). This indicates 14-3-3 regulation at the endpoint of signalling pathways, but 14-3-3 proteins are also implicated at earlier points in the same pathways. The 14-3-3 binding site in NR is known to be phosphorylated by at least two protein kinases; a calcium-dependent protein kinase (CDPK) and an SNF1-related kinase (SnRK1). It is striking that 14-3-3 proteins have been found to interact with both of these classes of kinase, including the *Arabidopsis* CPK1 isoform (Camoni *et al.*, 1998), and more significantly, the wheat SnRK1 homologue, WPK4 (Ikeda *et al.*, 2000). Apart from interaction with protein kinases, 14-3-3 proteins also interact with other components of signalling pathways, for example with RGS3, a negative regulator of the G-alpha subunits of heterotrimeric G proteins (Niu *et al.*, 2002). Thus, it would be interesting to examine whether 14-3-3 proteins form

a link between G-protein signalling pathways and metabolic regulation in plants at the post translational level.

7. QTL APPROACH TO NUE

Nitrogen use efficiency in plants is a complex quantitative trait that 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 *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. In maize, Hirel *et al* (2001) and Masclaux *et al*. (2001) analyzed recombinant inbred lines for physiological traits such as nitrate content, NR and GS activities. When the variation in these traits and yield components were compared, it was found that there was a positive correlation between nitrate content, GS activity and yield. When the loci that govern quantitative traits were determined on the map of the maize genome, the positions of QTLs for yield components and the locations of the genes for cytosolic GS (GS1) coincided. Similar results were obtained in rice by Obara *et al* (2001), confirming the earlier indications that the GS1 enzymatic activity in the leaf cytosol is one of the major steps controlling organic matter reallocation from source to sink organs during senescence and for grain-filling in cereals.

Previous studies have already demonstrated that when GS1 is over expressed in *Lotus*, nitrogen remobilization was prematurely induced leading to early senescence of the plant (Vincent *et al*., 1997). In rice (Yamaya, 2002) and wheat (Habash *et al*., 2001), preliminary investigations with enhanced or decreased GS1 activity indicated that grain yield and grain nitrogen content were modified. In other species such as tobacco (Migge *et al*., 2000) or poplar (Gallardo *et al*., 1999), overexpression of GS2 or GS1 significantly increased plant biomass production at early stages of plant development. Prioul *et al*. (1999) reported that QTLs for the activities of sucrose-phosphate-synthase and acid-soluble invertase were detected in the regions where each structural gene was mapped in maize. In these experiments, two out of seven QTLs for GS1 protein content and three out of six QTLs for NADH-GOGAT protein content were detected in different regions from other biological and physiological traits. Thus, quantitative studies

of genetic variability for NUE using molecular markers and combining agronomic and physiological studies will be increasingly used in the future to identify new genes or *loci* involved in the regulation of these metabolic pathways and their interconnection with carbon assimilation and recycling and to select genotypes that assimilate or remobilize nitrogen more efficiently.

8. CONCLUSIONS AND FUTURE PROSPECTS

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, indicating that the earlier notions of single-point rate-limiting regulation were too simplistic (Stitt, 1999b, Andrews *et al.*, 2004). For example, reducing NR activity several fold by mutation or overexpressing it in transgenic plants did not have much effect on the growth and protein content of the plant. Similarly, both overexpression of plastidic GS as well as blocking it with antisense RNA in transgenic tobacco did not alter the amino acid content, composition or protein levels significantly (Temple *et al.*, 1993). This was also true of Fd-GOGAT. On the other hand, greenhouse conditions (high carbon dioxide) which enhance photosynthesis and therefore C yield have been found to cause N limitation, reducing the N:C ratio (Ferrario *et al.*, 1997).

In general, transgenic studies from several plants (reviewed by Andrews *et al.*, 2004) clearly indicate that these enzymes of primary nitrate assimilation are not suitable targets for metabolic engineering to improve NUE. On the other hand, 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). However, this is not to underestimate the importance of primary nitrate assimilation in cereals such as rice. Recent evidence suggests that *indica* rice utilises nitrate better than ammonia (the form in which N is usually available to the plant in water-logged fields), and that it could have a higher NUE and yield potential with nitrate fertilisation (Kronzucker *et al*, 2000). However, at the plant level, tapping the opportunities for enhancing NUE in rice (Ladha *et al*, 1998), may necessitate targeting the enzymes of secondary ammonia assimilation.

In terms of finding a global target for manipulation of NUE, the recent success in improving nitrate assimilation by overexpressing Dof1 transcription factor (Yanagisawa *et al.*, 2004) indicates that signalling processes provide an attractive route for metabolic

engineering. Unravelling the details of nitrate signal transduction may provide new avenues in this regard.

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