Nitrogen Use Efficiency in Plants

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Nitrogen fertilizers are necessary to enhance agricultural production and to sustain food security. However, their inefficient use accrues from inherent limitations of the crop plants as well as the manner in which N fertilizers are formulated, applied and managed. The main aim of the book is to assess the various aspects of the fate of fertilizer N in context of the overall N inputs to agricultural systems, with a view to enhance the efficiency of nitrogen use and reduce the negative impacts on environment. The cross cutting issues relate to improvement in nitrogen use by emerging technologies (genetic enhancement, QTL mapping), meeting N needs by understanding its interactions with other nutrients, and mitigation of nitrogen losses caused by environmental factors and management practices.

Nitrogen Use Efficiency in Plants develops links between basic and applied research and practical crop production by addressing a wide range of topics relating to nitrogen use efficiency, and plant and crop responses to applications of nitrogen via fertilizers, including nitrogen acquisition and reduction, molecular approaches, nitrate induction and signaling; and nitrogen use under abiotic stresses.
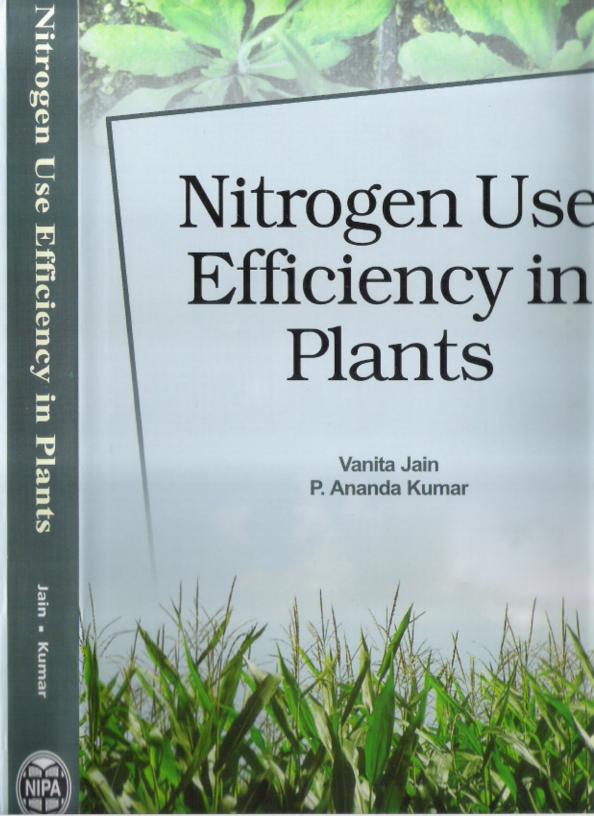
Nitrogen Use Efficiency in Plants is an invaluable classroom aid for academics working in plant physiology, biochemistry, biotechnology, molecular breeding and agronomy, and an essential professional resource for researchers working in plant and crop systems as it provides a comprehensive, interdisciplinary description of problems related to the efficient use of nitrogen in agriculture.

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Chapter **3**

Nitrate Sensing and Signaling in Genomewide Plant N Response

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ABSTRACT

Nitrogen is the most important element in terms of nutrition for plants, nitrate being the most preferred form. Nitrate not only acts as nutritional element but also as a signal to modulate metabolism and plant architecture. Nitrate sensing mostly happens in the root tip through a hitherto unknown mechanism, but its response can be demonstrated on an organism-wide scale. Functional genomic studies revealed over a thousand nitrate-responsive genes, involved not only in N and C metabolism, but also in various other physio-logical processes. Identification of nitrate response element/s common to all these genes could pave the way to unravel the mechanism of nitrate signaling, but findings in this direction have remained inconclusive so far. Several trans-acting factors have been implicated in N signaling and response, but none of these have been convincingly demonstrated to be specific to this response. Nitrate uptake, which is sometimes associated with nitrate sensing, is also highly regulated process and involves multiple transport systems like HATS, LATS and dual affinity transporters. Nitrate signaling also exerts its effect in co-ordination

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with various other signals such as hormones and light. A complete understanding of the nitrate signaling and response, as well as its interaction with other factors that regulate plant growth and productivity, requires the integration of physiological, genomic, proteomic and metabolic engineering approaches.

1. Introduction

As one of the important macronutrient nitrogen (N) modulates plant growth, plant architecture and inter-organ allocation of resources. Plants grown on low nitrogen not only exhibit less biomass accumulation, but also show decreased shoot/root ratios in comparison to plants grown on high nitrogen conditions (Kruse *et al.* 2002). Plants respond to even small variation in the supply of nutrients, especially nitrogen (Forde and Lorenzo 2001, Robinson 1994). Regardless of the form in which N is supplied, whether urea, ammonia or nitrate, the microbial process of nitrification in most aerobic soils ensures that nitrate is the most abundant form and is therefore the main source of N for plants. It is known since mid-sixteenth century that KNO_3 affects plant growth (Glass and Siddiqi 1995). 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.

Nitrate is not only a nutrient but also acts as a signal to reprogram plant metabolism and architecture (Stitt 1999, Raghuram *et al.* 2006). Nitrogen status of the plant regulates the expression of genes related to N and C metabolism (Vincentz *et al.* 1993, Stitt *et al.* 2002, Takei *et al.* 2002, Wang *et al.* 2000), photosynthesis (Sugiharto and Sugiyama 1992), mRNA translation (Suzuki *et al.* 1994) and cell cycle (Soni *et al.* 1995).

2. Nitrate Sensing

The ability to sense nitrate and distinguish it from other N metabolites is an important prerequisite for nitrate signaling. Substantial evidence has accumulated regarding the existence of nitrate sensing and signaling, as well as their interactive effects with other N and C metabolites, though their mechanisms are far from understood (Corruzi and Zhou 2001). Most of the plant's sensing for nutrients, water, etc. happens in the root cap (Barlow 2002). Root tip activity is also responsive to a set of long-range endogenous signals, which include phytohormones, sugars and probably other less well-characterized signal molecules such as peptides (Takayama and Sagakami 2002, Beveridge *et al.* 2003). Growth of the primary root is almost completely insensitive to the NO_3^- supply.

The NO_3^- assimilatory pathway is NO_3^--inducible and the first evidence that the NO_3^- ion is perceived as a signal by plants came from studies showing that the rapid induction of NR genes occurred even in the absence of NR activity. Studies using alternative N sources (NH_4^+ or glutamine) and an NR-deficient mutant showed that the lateral root tips were stimulated by the NO_3^- ion itself rather than a product of NO_3^- metabolism (Zhang *et al.* 1999, Zhang and Forde 1998). These experiments led to a model in which the lateral root has a NO_3^- sensory mechanism that enables it to modulate meristematic activity in response to local changes in the external NO_3^- concentration (Deng *et al.* 1989, Pouteau *et al.* 1989). Based on the observations on the response of NR gene to short nitrate pulses in intact barley seedlings, Tischner *et al.* (1993) suggested that nitrate acts more as a signal than as a mere 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). Further investigations revealed that nitrate response is also involved in 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 (Champigny and Foyer 1992), such as cytosolic pyruvate kinase and isocitrate dehydrogenase, mitochondrial citrate synthase etc, and downregulation of ADP-glucose pyrophosphorylase (Scheible *et al.* 1997).

The utilization of reduced N (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, which involves the regulation of carbon traffic between starch/sugars, which involves the regulation of carbon traffic between starch/sugars and amino acid synthesis. Thus, nitrate acts as a 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 1999). Co-regulation of C and N metabolism became a major focus for researchers, ever since it was found that sugars stimulate nitrate reductase transcription (Cheng *et al.* 1992, Coruzzi and Zhou 2001, Stitt *et al.* 2002, Stitt and Fernie 2003, Foyer *et al.* 2003).

3. Genomewide Nitrate Response

Studies over the last decade have shown that nitrate responsive gene expression is far more extensive than the range of responses discussed above. Using microarray studies in *Arabidopsis*, Crawford and coworkers (Wang *et al.* 2000, 2003, 2004) revealed thousands of nitrate responsive genes spanning up to 10% of the detectable *Arabidopsis* transcriptome. Many of them were previously unknown to be nitrate responsive, 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.

Other groups used subtractive hybridization (SSH) approach to identify nitrate-responsive genes and characterize their expression in tomato (Wang *et al.* 2001) and rice (Wang *et al.* 2002). Their studies not only reported the previously known nitrate responsive genes which included water channels, potassium and phosphate transporters, ribosomal proteins, stress response proteins, regulatory proteins and signalling proteins but also high affinity and low affinity nitrate transporters.

Most, if not all nitrate responsive genes also respond to other signals, such as light, hormones etc. Recent microarray studies in our lab have revealed the full list of nitrate and/or light responsive genes in rice, making it possible to segregate light and nitrate effects for the first time (Pathak 2010). 1157 genes were found to be differentially regulated by nitrate in presence of light, whereas 1015 genes were differentially regulated by nitrate in etiolated plants. A venn selection of both lists revealed 159 genes, suggesting these are influenced by nitrate only and not by light.

4. Nitrate Response Elements

Even as the list of nitrate responsive genes in plants has grown over the years, the mechanism of nitrate-regulation of gene expression remains largely unknown. Nitrate signaling may culminate in nitrate response elements (NREs) and trans-acting factors that interact with them. Therefore, identification of common nitrate response elements (NREs) would help in unraveling the nitrate signaling pathway (Raghuram *et al.* 2006). There were several attempts throughout the 1990s to identify the NRE for NR and NiR, but it was Hwang *et al.*

(1997) who first claimed that *cis*-acting elements comprising [(a/t)$_7$Ag/cTCA] motif mediate nitrate dependent transcription of NR in *Arabidopsis*. This was later corroborated in birch for NR (Hachtel and Strater 2000) and NiR (Warning and Hachtel 2000).

However, our detailed *in silico* analysis of the entire *Arabidopsis* and rice genomes revealed that the [(a/t)$_7$Ag/cTCA] motif is randomly distributed throughout these genomes with no difference between nitrate responsive genes and the presumably nonresponsive genes and intergenic regions (Das *et al.* 2007), questioning its validity as a consensus sequence. Similar results were obtained in our genomewide bioinformatic analysis of other candidate motifs like GATA and Dof binding elements implicated in nitrate response in *Arabidopsis*, and our own bioinformatic search for new candidate NRE motif has remained inconclusive due to inconsistencies in motif samplers (Pathak *et al.* 2009). Therefore, the identification of nitrate response elements remains an open area for future investigation.

4.1. Trans-acting factors

The role of trans-acting factors in the nitrate response of higher plants has remained poorly understood. In fungi such as *Aspergillus nidulans* and *Neurospora crassa*, induction of nitrate transport by nitrate, as well as the activities of NR and NiR enzymes, are 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 which belong to the GATA family of transcription factors (Marzluf *et al.* 1997). GATA factors have also been implicated in the regulation of N assimilation, apart from their many other roles known in plants. GATA motifs have been identified in the regulatory regions of many genes involved in nitrate assimilation such as nitrate reductase (NIA), nitrite reductase (NiR) and glutamine synthetase (Jarai *et al.* 1992, Oliveira and Coruzzi 1999, Rastogi *et al.* 1997).

The fungal transcription factors, NIT2 of *Neurospora crassa* (Tao and Marzluf 1999) and AREA of *Aspergillus nidulans* (Caddick *et al.* 1986) are GATA factors that globally regulate genes in N metabolism. In yeast, four global N regulatory factors, namely GLN3, NIL1, NIL2 and DAL80, are GATA factors with a single GATA zinc finger (Hofman-Bang 1999). GLN3 and NIL1 are transcriptional activators, whereas DAL80 and NIL2 act as negative regulators of multiple N catabolic genes (Hofman-Bang 1999).

Several features of the regulation of nitrate assimilation are common between fungi and higher plants. Previous experiments have shown that NIT2 binds specifically to two fragments of the NIA gene of tomato *in vitro*, whereas mutant NIT2 proteins failed to bind to the same fragments, which suggests that there might be a NIT2-like homolog regulating the expression of the nitrate assimilation pathway in higher plants (Jarai *et al.* 1992). The spinach NiR promoter has been reported to contain some regions that are involved in N regulation (Back *et al.* 1991, Rastogi *et al.* 1993, 1997) and footprinting results suggested that GATA factors play a role in NiR gene regulation (Rastogi *et al.* 1997). Bi *et al.* (2005) reported that one member of the GATA transcription factor family, GNC is induced by nitrate and plays an essential role in chlorophyll synthesis and glucose signaling.

NLA (Nitrogen Limitation Adaptation), a RING-type ubiquitin ligase from *Arabidopsis*, was found to be a positive regulator of plant adaptation to N limitation (Peng *et al.* 2007). DOF transcription factor, known for its role in light-regulation of gene expression and other plant responses including regulating the genes of organic acid metabolism, has also been implicated in N metabolism and N use efficiency. Overexpression of a maize DOF-factor in transgenic *Arabidopsis* improved nitrogen content (by 30%) and growth in the plants under low-nitrogen supply, accompanied by up-regulation of multiple genes involved in carbon-skeleton production without any reduction of NR, GS and GOGAT transcripts (Yanagisawa *et al.* 2004).

The localized proliferation of lateral roots in nitrate-rich soil patches is under the control of the MADS box transcription factor ANR1 (Zhang and Forde 1998). Moreover, the nitrate transporter NRT1.1 has been proposed to be a nitrate sensor that acts upstream of ANR1 in this signalling pathway (Remans *et al.* 2006b). Castaings *et al.* (2009) reported that in *Arabidopsis*, NIN-like protein 7 (NLP7), a putative transcription factor, is involved in nitrate signal transduction pathway and also regulates nitrogen assimilation in non-nodulating plants.

4.2. Transporters

Nitrate uptake is a fundamental aspect of plant nutrition, several families of transporters have been identified (Orsel *et al.* 2007). Physiological measurements of nitrate uptake by roots have defined two systems of high and low affinity uptake. The first higher plant nitrate transporter gene was isolated from *Arabidopsis* (Tsay *et al.* 1993). It had 12 putative trans-membrane domains similar to the first eukaryotic nitrate transporter isolated from the fungus *Aspergillus* (Unkles *et al.* 1991) but these transporters were phylogenetically unrelated. Physiological investigations of NO_3^- uptake by the roots of many different types of plants have led to the identification of multiple nitrate transporters that differ in terms of nitrate affinity and inducibility, which presumably enable the plant to cope with the variations in NO_3^- concentrations in cultivated soils (Crawford and Glass 1998). Two saturable high affinity transport systems (HATS) are able to take up NO_3^- at low external concentrations (1 μM to 1 mM). The constitutive system (cHATS) is available even when plants have not been previously supplied with NO_3^-. The inducible system (iHATS) is stimulated by NO_3^- in the external medium. The low affinity transport system (LATS) contributes to NO_3^- uptake at external high NO_3^- concentrations above 1 mM (Crawford and Glass 1998) (Please refer to chapter 1 in this volume).

The iHATS is a multicomponent system encoded partly by the genes of the NRT2 family or nitrate–nitrite porter family of transporters. Several different regulatory mechanisms have been identified for AtNRT2.1 (HATS), which include feedback regulation and phosphorylation. These various changes in the protein may be important for its second function in sensing NO_3^- availability at the surface of the root. Another transporter protein, AtNRT1.1 also has a role in NO_3^- sensing that is independent of its transport function, like AtNRT2.1. 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).

The regulation of NRT2.1 expression has been thoroughly investigated at the mRNA level. NRT2.1 transcript accumulation mainly occurs in epidermis and cortex of the mature root regions (Nazoa *et al.* 2003), and is strongly influenced by a range of different environmental factors. Expression of NRT2.1 is induced by NO_3^-, repressed by high N status through a negative feedback regulation involving reduced N metabolites such as NH_4^+ or amino acids (Zhou *et al.* 1999, Nazoa *et al.* 2003), and stimulated by light and sugars (Lejay *et al.* 2003).

The initiation and elongation of lateral root (LR) development is stimulated by local availability of NO_3^- and it has been proposed in

Arabidopsis roots that NRT2.1 may itself be a NO_3^- signal transducer or sensor (Little *et al.* 2005). This function of the transporter is reckoned to be independent from NO_3^- influx (Little *et al.* 2005, Remans *et al.* 2006a). Furthermore, *AtNRT1.1* has been implicated in the signalling pathway triggering root colonization of NO_3^--rich patches and this has been linked to changes in the expression of a putative transcription factor MADS box gene (Remans *et al.* 2006b). In *Arabidopsis*, a role for *AtNRT1.1* has been specifically implicated in breaking seed dormancy (Alboresi *et al.* 2005) which can provide another useful model system for studying nitrate signalling in plants like root development.

5. Nitrate and Hormone Signaling

5.1. Cytokinins

Several lines of evidence indicate that cytokinin function as long-distance signals that control nitrogen assimilation and status in plants (reviewed by Sakakibara *et al.* 2006). It has been known that increasing nitrate supply through the roots, but not the shoot, induces expression of genes regulating nitrate and carbon metabolism in leaves, a response which is also mimicked by addition of cytokinin to plants (Brenner *et al.* 2005, Scheible *et al.* 2004) suggesting that cytokinin may act as long-range messenger, travelling from root to shoot, to control nitrate responses. The facts that nitrate application increases cytokinin biosynthesis and that these hormones can be transported through the vascular vessels support this hypothesis (Rahayu *et al.* 2005, Sakakibara 2006). Besides their proposed function as long-distance signals, cytokinin may also control local responses to nitrogen supply. The evidence comes from the fact that cytokinin inhibit accumulation of nitrate and ammonia transporters in roots of nitrate-supplied *Arabidopsis* plants, which may represent a negative feedback regulatory process that slows down nitrogen uptake under non-limiting conditions (Brenner *et al.* 2005, Kiba *et al.* 2005).

Brenner *et al.* (2005) measured immediate early and delayed cytokinin responses through genome-wide expression profiling using Affymetrix ATH1 full genome array. They found that after 2 h of cytokinin treatment, a large number of genes coding for transcriptional regulators, signaling proteins, developmental and hormonal regulators, primary and secondary metabolism, energy generation and stress reactions were differentially regulated. It was also found that several genes of nitrogen metabolism and transport were cytokinin regulated, including genes encoding a glutamine-dependent asparagine synthetase and a glutamate dehydrogenase which showed 24- and 15-fold upregulation, respectively. The NIA gene transcript abundance was increased 2.6-fold after 120 min cytokinin treatment indicating an increased need for NH_4^+. It is also noteworthy that three high-affinity nitrate transporter genes, NRT2.1, NRT2.3 and NRT2.6, were found to be strongly downregulated after 120 min. Similarly, the ammonium transporter genes AMT1.1, AMT1.2 and AMT1.3 were repressed up to one-third of their original levels. Collectively, these data support the earlier notion that cytokinin plays an important role in regulating N utilization and that early responses to cytokinin and changed nitrogen availability overlap in part (Kiba *et al.* 1999, Sakakibara, 2003, Wang *et al.* 2003).

5.2. Abscisic acid (ABA)

Interrelationship between ABA and nitrate homeostasis has been uncovered by analyzing the effect of altering ABA signaling in the response of plants to nitrate resupply and, conversely, by studying nitrate control of ABA biosynthesis. Zhang *et al.* (2007) showed that ABA and nitrate signaling share common regulatory elements using *Arabidopsis* mutants displaying ABA-insensitive lateral root initiation (labi mutants), which showed reduced sensitivity in their root responses to nitrate resupply.

5.3. Auxins

There have been studies to show a possible relationship between nitrate supply and auxins, but the potential physiological implications are not well understood. It was shown that nitrate supply conditions altered the rate of auxins biosynthesis or that of shoot-to-root transport (Caba *et al.* 2000, Walch-Liu *et al.* 2006). Soybean and *Arabidopsis* plants grown under low-nitrate conditions accumulated higher levels of auxins in the root compared with plants grown under high-nitrate conditions. On the contrary, auxin levels in the shoot of nitrate deprived plants were lower than those of plants grown in a nitrate-rich media (Walch-Liu *et al.* 2006). This altered pattern of auxin accumulation indicated that these hormones play a role in suppressing the effect of high nitrate supply in root development, such as arrest of lateral root proliferation. However, this hypothesis needs to be further investigated, since application of auxins did not reduce repression of lateral root development in plants grown under high-nitrate conditions, suggesting that these hormones do not directly control nitrate signaling (Zhang *et al.* 2007).

6. Nitrate and Light Signaling

Light is a signal that regulates the expression of many of the nitrate responsive genes, though it has been studied in depth in only a few of them. Light has been shown to play an important role as an external signal for regulation of the expression and activity of NR (Lillo 1994, Mohr et al. 1992, Sivasankar and Oaks 1996, Pattanayak and Chatterjee 1998) and has often been reviewed (Raghuram et al. 1999, Chandok et al. 1997, Lillo and Appenroth 2001). NR has been shown to be positively regulated by light at two levels: a coarse regulation at the level of gene expression in the time scale of hours and a fine regulation at the post-translational level in the time scale of minutes. In etiolated plants, phytochrome is the main photoreceptor involved and its low fluence response (LFR) is the common response mode. The effect of the very low fluence response (VLFR) has been reported for NIA2 isoform of NR in Arabidopsis thaliana (Pilgrim et al. 1993). The fast post-translational regulation of NR by light is based on the phosphorylation/dephosphorylation of a serine residue in the hinge 1 region and the subsequent Mg^{2+}/polyamine-dependent binding of the phosphorylated form to a 14-3-3 protein (Please refer to chapter 2 in this book).

At the transcriptional level, light regulation of NR is considered to work differently in green plants and etiolated seedlings, involving different photoreceptors. Using pharmacological approaches, the phytochrome-mediated regulation of NR gene expression in maize was linked to signaling events such as G-protein (Raghuram et al. 1999), phospho inositol (PI) cycle and protein kinase C (Raghuram and Sopory 1995). Light regulation is also known for other N-metabolic genes like Fd-GOGAT (Hecht and Mohr 1990, Elmlinger and Mohr 1991, Becker et al. 1993, Teller et al. 1996 and Suzuki et al. 2001), though the role of specific photoreceptors in it needs to be elucidated further.

7. Conclusions and Perspectives

Despite the tremendous progress made over the last two decades in exploring nitrate sensing, signaling and response, a complete understanding of the universal mechanism(s) for any of these aspects remain elusive. However, we do know the extent of genomewide nitrate response and the need for common nitrate response elements and transcription factors for coordinated expression of hundreds of nitrate responsive genes. We also know the existence of nitrate sensing and signaling pathways that culminate in gene regulation and a few

possible events/intermediates in these pathways. While further work in this direction may help in understanding gene regulation, proteomic approaches need to be initiated to establish its correspondence with protein levels and post-translational regulation. Only through a better understanding of the molecular mechanisms of nitrate sensing, signaling and response, we will be able to find newer targets for improving N-use efficiency in plants.

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Chapter 4

Molecular Approaches to Improve Nitrogen Use Efficiency

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ABSTRACT

Nitrogen is the most essential nutrient for all plants and is also the major limiting factor in plant productivity. High yielding varieties of all crops are responsive to nitrogen but its ever-increasing use has shown detrimental impact on the environment along with extremely low use efficiency. Nitrogen use efficiency (NUE) in crop plants depends on various internal and external factors, which are dealt in detail. There has been a significant interest in genetic engineering of the crops to improve NUE. The use of biotechnological interventions by manipulating genes of the nitrogen utilization pathway to improve NUE has not been very successful. But transgenics/mutants with modified capacities for nitrate uptake, assimilation and remobilization have enhanced our understanding of the genetic control of NUE. In both cellular, and at whole plant level the mechanisms involved in N remobilization from the senescing organs towards the grain have recently gained importance but their understanding is still preliminary. Recent evidences have shown that grain filling during later stages of crop growth is supported by N recycling. The genome wide regulation of the various genes and interaction between the nitrogen and carbon metabolism is being

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