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REVIEW PAPER

Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency

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Abstract

Development of genetic varieties with improved nitrogen use efficiency (NUE) is essential for sustainable agriculture. Generally, NUE can be divided into two parts. First, assimilation efficiency involves nitrogen (N) uptake and assimilation and second utilization efficiency involves N remobilization. Understanding the mechanisms regulating these processes is crucial for the improvement of NUE in crop plants. One important approach is to develop an understanding of the plant response to different N regimes, especially to N limitation, using various methods including transcription profiling, analysing mutants defective in their normal response to N limitation, and studying plants that show better growth under N-limiting conditions. One can then attempt to improve NUE in crop plants using the knowledge gained from these studies. There are several potential genetic and molecular approaches for the improvement of crop NUE discussed in this review. Increased knowledge of how plants respond to different N levels as well as to other environmental conditions is required to achieve this.

Key words: Assimilation, genetic improvement, nitrogen limitation, nitrogen use efficiency, remobilization.

Need for genetic improvement of nitrogen use efficiency (NUE)

In the past 50 years there has been a marked increase in food productivity allowing for a significant decrease in world hunger, despite a doubling of the population (Godfray *et al.*, 2010). However, it will be challenging over the next 50 years to increase crop productivity further in order to meet a growing population, due to a range of issues such as decreasing arable land, increasing water scarcity, rapid global climate change, changing food habits, and the use of biomass for the production of biofuels. In addition, the use of agricultural inputs, particularly fertilizers, are costly to farmers and the environment. The addition of nitrogen (N) fertilizer is typically the single highest input cost for many crops and, since its production is energy-intensive, this cost is dependent on the price of energy (Rothstein, 2007). With the introduction of chemical fertilizers, the primary goal has been to increase the yield output per unit of land area and to achieve this, N fertilizers were applied close to the economic optimum levels (Firbank, 2005). However, the corollary of these efforts was a decrease in the percentage of N fertilizer actually used by the crop. N compounds used are typically present in the form of nitrate and ammonium and are very mobile in the soil and crop plants are able to utilize only 30–40% of the applied N (Raun and Johnson, 1999). Thus, more than 60% of the soil N is lost through a combination of leaching, surface run-off, denitrification, volatilization, and microbial consumption. It is estimated that a 1% increase in NUE could save ~$1.1 billion annually. Therefore, to minimize the loss of N, reduce environmental pollution, and decrease input cost, it is crucial to develop crop varieties with a higher NUE.

The increased crop productivity has been associated with a 20-fold increase in the global use of N fertilizer applications during the past five decades (Glass, 2003) and this is expected to increase at least 3-fold by 2050 (Good *et al.*, 2004). Conventional breeding efforts in the past few decades have significantly increased crop yield and, as a corollary to this, also improved NUE. For example, a comparison between maize hybrids from the 1970s and 1990s has shown that hybrids from the 1990s exhibited a higher yield response to increasing N supply (O'Neill *et al.*, 2004).

Genetic differences in N uptake and/or grain yield per unit of N applied has also been reported in different crops including wheat, rice, maize, sorghum, and barley (OrtizMonasterio *et al.*, 1997; Muchow, 1998; Le Gouis *et al.*, 2000; Presterl *et al.*, 2003; Anbessa *et al.*, 2009; Namai *et al.*, 2009). However, the molecular knowledge governing genetic variation among crop varieties and hybrids for NUE is poorly understood. In order to use molecular breeding and genetic engineering approaches to improve crop plants for complex traits like NUE, it is necessary to have a comprehensive knowledge of the regulatory mechanisms controlling N use, particularly when N is limited in the environment. In this review, several approaches that are being taken to meet this goal are discussed.

Physiological and molecular components governing NUE

NUE has been defined in various ways (Good *et al.*, 2004), but the simplest is the yield (grain, fruit or forage) per unit of N available in the soil. There are two general stages for N use in the plant life cycle. First, during biomass formation there is the amount of N uptake, storage, and assimilation into amino acids and other important nitrogenous compounds. The second stage is the proportion of N that is partitioned to the seed, resulting in final yield. During the vegetative stage, young developing leaves and roots behave as sinks for inorganic N uptake and synthesis and the storage of amino acids via the nitrate assimilation pathway. These amino acids are further utilized in the synthesis of proteins and enzymes involved in different biochemical pathways and the photosynthetic machinery governing plant growth, architecture, and development. Later on, during the reproductive stage the increased supply of nitrogenous compounds is necessary for optimum flowering and grain filling. At this stage, both N assimilation and remobilization become critical and the leaves and shoot act as the source providing amino acids to the reproductive and storage organs. Therefore, understanding the mechanisms for N uptake, assimilation, and remobilization during the plant life cycle is important for increasing NUE.

Regulatory mechanisms for N uptake and assimilation

Plants take up N primarily as nitrate and ammonium, with nitrate being the predominant form in most agricultural soils (Crawford and Forde, 2002). The function of several structural genes involved in N uptake and assimilation have been studied extensively in the past decade. In *Arabidopsis*, there are three families of nitrate transporters NRT1, NRT2, and CLC with 53 *NRT1*, 7 *NRT2*, and 7 *CLC* genes identified. The *NRT2* are high-affinity nitrate transporters while most of the *NRT1* family members characterized so far are low-affinity nitrate transporters, except *NRT1.1* which is a dual-affinity nitrate transporter. *NRT1.1*, *NRT1.2*, *NRT2.1*, and *NRT2.2* are involved primarily in nitrate uptake from the external environment (Miller *et al.*, 2007; Tsay *et al.*, 2007; Ho *et al.*, 2009). Amongst the *CLC* family members, *CLCa* is known to mediate nitrate accumulation in the plant vacuole (De Angeli *et al.*, 2006). Nitrate, after entering the plant cell, is reduced to nitrite by nitrate reductase and further to ammonium by nitrite reductase (Crawford and Forde, 2002). The ammonium derived from nitrate or from direct ammonium uptake by AMT transporters (Crawford and Forde, 2002) is further assimilated into amino acids via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. Previously, several attempts have been made to manipulate the expression of different nitrate transporters and assimilatory genes mostly in *Arabidopsis* and tobacco although also, in some instances, in crop plants (Good *et al.*, 2004). Recently, it has been reported that overexpression of *GS1.3* in maize led to an increase of 30% in kernel number (Martin *et al.*, 2006) and the enhanced N accumulation capacity by overexpression of a cytosolic GS1 in shoots and grains in transgenic wheat (Habash *et al.*, 2001). However, up to this point, there are no successful transgenic commercial lines reported where this gene has been over-expressed.

Nitrate is not only the predominant source of N supply to plants, but also acts as an important signal for several developmental processes. This regulation includes a rapid change in expression pattern of genes involved in carbon (C) and N metabolism and other metabolic pathways. Further, its concentration affects root development, root architecture, and the root-to-shoot ratio. Interestingly, not much is known about the molecular mechanisms and regulatory genes that govern these nitrate responses, although some transcription factors and kinases have been linked to these processes. Involvement of a *DOF1* transcription factor in improved *Arabidopsis* growth and higher N assimilation under low N conditions (Yanagisawa *et al.*, 2004) and the regulation of the *GS1* gene by *PpDOF5* in maritime pine (Rueda-Lopez *et al.*, 2008) has been reported. A MADS box transcription factor *ANR1* regulates localized lateral root proliferation in response to nitrate (Zhang and Forde, 1998). Later on, it was proposed that a key nitrate transporter *NRT1.1* acts upstream of *ANR1* (Remans *et al.*, 2006*a*). *NRT1.1* also functions as a nitrate sensor and is both a high and low affinity nitrate transporter depending upon phosphorylation and dephosphorylation mediated by a protein kinase CIPK23 which can phosphorylate *NRT1.1* under low nitrate conditions (Ho *et al.*, 2009). A NIN-like transcription factor *NLP7* functions in nitrate-mediated induction of several nitrate assimilatory genes (Castaings *et al.*, 2009). A calcineurin B-like (CBL)-interaction protein kinase gene, *CIPK8* positively regulates nitrate induced genes (Hu *et al.*, 2009). A putative regulatory role of different microRNAs for the plant adaptation to varying N conditions has been proposed (Pant *et al.*, 2009).

Regulatory mechanisms for N remobilization

The leaves are a sink for N during the vegetative stage and, afterwards, this N is remobilized for use in the developing seeds. The majority of this remobilization occurs during senescence whereto N is transported mainly via amino acids. Up to 80% of grain N contents are derived from

leaves in rice and wheat (Kichey *et al.*, 2007; Tabuchi *et al.*, 2007). Plants have developed efficient methods and mechanisms that release tied-up N entities from source tissues via protease activities during leaf senescence. The protein degradation occurs mainly by three pathways: the chloroplast degradation pathway; the vacuolar and autophagic pathway; and the ubiquitin-26S proteasome pathway (Liu *et al.*, 2008). Approximately 80% of total leaf N is located in the chloroplasts mainly in the form of proteins and this is an important N pool for remobilization (Adam *et al.*, 2001). Among chloroplastic proteins, Rubisco (~50% of total cellular proteins in C_3 and ~20% in C_4 plants) seems to serve as the major protein subjected to proteolysis and responsible for most N remobilized during leaf senescence for grain-filling (Mae *et al.*, 1993). During senescence, it has been proposed that this degradation of Rubisco is through the involvement of reactive oxygen species (Zimmermann and Zentgraf, 2005) or by a nuclear encoded protease CND41 (Kato *et al.*, 2004). Autophagy involves double-membrane-bound vesicles known as autophagosomes which transfer cytosolic proteins and protein complexes, as well as the mobilization of Rubisco from chloroplast to vacuole, by forming autophagic bodies. These are hydrolysed by vacuole localized exo- and endopeptidases (Ishida *et al.*, 2008) to release amino acids for subsequent recycling/remobilization. All plant species harbour *ATG* (AuTophaGy) genes destined to carry out this important recycling process and several *ATG* genes have been identified in plants. Among these, ATG8s participate in tagging proteins for degradation (Slavikova *et al.*, 2005).

The ubiquitin-26S proteasome mechanism is used for the removal of short-lived and abnormal proteins which is necessary for the maintenance of normal growth and development. This pathway involves attachment of the small 76-amino acid ubiquitin (Ub) polypeptide to the protein destined for degradation, which is then recognized and catabolized by the 26S proteasome. Precise tagging of proteins with ubiquitin, for their efficient removal is accomplished by the orchestrated functioning of three enzymes, namely ubiquitin-activating enzyme (El), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). The protein degradation pathway is initiated soon after E1 interacts with the C-terminal glycine of ubiquitin through a conserved cysteine residue by forming thioester bonds. Ubiquitin is subsequently accepted by E2 through a thioester linkage via a conserved UBC domain with a cysteinyl sulphhydryl group (Smalle and Vierstra, 2004). The last step for the attachment of ubiquitin to target protein is catalysed by E3 ligases. The *Arabidopsis* genome contains two E1, 37 E2, and ~1300 E3 genes (Smalle and Vierstra, 2004). Identification of an extremely large number of E3 ligases, and their ability to 'ubiquitinate' target genes, has led to the assumption that E3 ligases determine substrate specificity (Zhang and Xie, 2007). One of the E3 ligases, a RING-type ubiquitin E3 ligase named the nitrogen limitation adaptation (*NLA*) gene was recently characterized in our laboratory showing its role in *Arabidopsis* adaptation under N limitation (Peng *et al.*, 2007*b*).

After protein degradation during senescence, the amino acids released from roots and leaves are loaded into the phloem. The amino acids are the major form for N transport required for the grain development. While all the amino acids are remobilized, glutamine, asparagine followed by glutamate, aspartate, serine, and alanine are predominant in phloem sieve tubes (Sanders *et al.*, 2009). A large number of amino acid transporters have been identified in plants by genome analysis and sequence homology. Still, little is known about their function, although their use could have a large potential to improve NUE. The prominent gene family which might be involved in the phloem-loading process are the amino acid permeases (AAP). In *Arabidopsis*, eight AAP genes are present (Liu and Bush, 2006). AAP1, AAP2, AAP6, and AAP8 have been characterized for their varied roles in amino acids transport (Okumoto *et al.*, 2004; Tilsner *et al.*, 2005; Schmidt *et al.*, 2007; Sanders *et al.*, 2009). In addition, members from other transport families have been identified based on sequence information including a lysine/histidine transporter (LHT), cationic amino acid transporters (CAT), proline transporters (ProT), and aromatic and neutral amino acid transporters (ANT), and oligopeptide transporters (OPT) (Rentsch *et al.*, 1996; Chen *et al.*, 2001; Stacey *et al.*, 2002; Scheible *et al.*, 2004; Liu and Bush, 2006). Among them, only a few genes have been functionally characterized for their role in amino acid transport. *LHT1* is involved in root amino acid uptake and the supply of leaf mesophyll cells via xylem-derived amino acids (Hirner *et al.*, 1998) and a mutation in *AtOPT3* was lethal to embryos due to its critical role for peptide and amino acid transport in early embryo development (Stacey *et al.*, 2002). Recently, an early nodulin gene, *OsENOD93-1*, has been characterized in rice which has a potential role in amino acid accumulation in roots and transport towards the shoot (Bi *et al.*, 2009).

The degradation of proteins during senescence also releases ammonia and its accumulation could be lethal to plant cells, since the activity of *GS1* is induced during leaf senescence. Much evidence supports the role of cytosolic *GS1* in the efficient remobilization of amino acids for senescing leaves towards grain-filling. This includes the localization of *GS1* in the vascular tissues (Kichey *et al.*, 2005; Martin *et al.*, 2006), the positive correlation between grain number and a locus for *GS1* in rice (Obara *et al.*, 2004), the significant correlation between GS activity and grain N content in wheat (Martin *et al.*, 2006), the fact that a knockout-mutation in rice *GS1.1* resulted in reduced plant growth, fewer spikelets, and lower grain weight (Tabuchi *et al.*, 2005), and that mutations in maize *Gln1.3* and *Gln1.4* resulted in reduced kernel size and kernel number, respectively (Martin *et al.*, 2006).

Morphological response of root system to N supply

There are several genetic and environmental factors which might limit NUE such as root architecture, C/N ratio in the

soil and within the plant, soil type, rainfall, and temperature. A low N supply generally leads to decreased root growth, suppression of lateral root initiation, increase in the C/N ratio within the plant, reduction in photosynthesis, and early leaf senescence (Paul and Driscoll, 1997; Malamy and Ryan, 2001; Martin *et al.*, 2002; Malamy, 2005; Wingler *et al.*, 2006; Zhang, 2007). When *Arabidopsis* seedlings were grown in media containing a high sucrose-to-nitrate ratio lateral root proliferation was reduced and shoots were smaller (Malamy and Ryan, 2001; Kant *et al.*, 2008). Lateral root elongation by localized supply of high nitrate patches is a classic example of the stimulatory effects of external nitrate availability. *ANR1*, a MADS box transcription factor, was identified as a component governing this response in *Arabidopsis* (Zhang and Forde, 1998). In another report, it was suggested that a nitrate transporter *NRT1.1* may act upstream of *ANR1* in the localized stimulatory response of nitrate (Remans *et al.*, 2006*a*).

Contrary to local induction of lateral roots by high nitrate patches, a high nitrate concentration in the environment has inhibitory effects on lateral root elongation. The dual regulation of lateral roots by nitrate is developmental stage dependent. The induction by localized high nitrate promotes the elongation of existing lateral roots. Inhibition by overall high nitrate supply occurs immediately after the emergence of lateral root primordia, but before meristematic activation, resulting in shorter lateral roots. In addition, the signal responsible for the inhibition of lateral root elongation by high nitrate is mainly due to the accumulation of nitrate and N metabolites inside the plants (Zhang and Forde, 1998, 2000; Zhang *et al.*, 1999). Remans *et al.* (2006*b*) reported that *NRT2.1*, which is a high affinity nitrate transporter, acts as a positive regulator for lateral root initiation under N limitation conditions in *Arabidopsis*. By contrast, Little *et al.* (2005) reported a repressive role of *NRT2.1* in lateral root initiation. It is not clear how the same transporter could exert two different effects, with the possible explanation being the different N-limiting conditions used in these studies.

Understanding plant response to N limitation

Plants in natural field conditions face changing environmental conditions where N concentrations vary and frequently are limiting for growth due to many factors including surface run-off/soil erosion, rainwater leaching, gaseous losses by volatilization, and microbial consumption. Therefore, adaptation to limiting N conditions is an important survival strategy for plants successfully to complete their life cycle. For crops grown in developed countries, the use of large amounts of N fertilizer for many crops helps prevent fluctuating levels of N from impacting yield and, as a consequence, much is wasted to the environment. In developing countries, many farmers cannot afford to use much N fertilizer. Therefore, in either case developing crops that have improved genetics for yielding well under limiting N conditions would be very advantageous. In our laboratory, research has been focused on understanding the response of plants to limiting N and to try to use this knowledge to improve NUE either by increasing yield under existing levels of N supply or by maintaining yield by decreasing N levels.

Understanding plant response to N limitation by transcriptional profiling

To understand the signalling pathways of plants responding to N limitation, one approach is to use microarray or sequence-based transcription profiling technology to analyse genome-scale gene expression. Several studies have investigated transient changes in gene expression in *Arabidopsis* plants when nitrate is added to nitrate-starved seedlings (Wang *et al.*, 2000, 2003; Palenchar *et al.*, 2004; Price *et al.*, 2004; Scheible *et al.*, 2004; Gutierrez *et al.*, 2007*a*). Growth systems have been developed where N was the limiting factor for plants grown either hydroponically or using nutrient-free soil (Peng *et al.*, 2007*b*) to study the transcriptional changes of genes that were most affected by different levels of N limitation. Differentially expressed genes under mild or severe chronic N stress were identified, as well as some putative N regulatory elements to provide additional insights into the co-ordination of the complex N responses of plants and to reveal possible new components of the regulatory network for plant N responses (Bi *et al.*, 2007). The transcriptional difference in the *Arabidopsis* nitrogen limitation adaptation (*nla*) mutant, which is defective in developing the normal N limitation adaptive responses (Peng *et al.*, 2007*b*), was also investigated and it was found that the absence of the functional *NLA* in the *nla* mutant extensively altered its responsive transcriptome to N limitation (Peng *et al.*, 2007*a*).

Many transcriptional profiling studies are now focusing on crop plants, including rice which is a staple food for almost half the world's population. Expression profiles for 10 422 genes in rice seedlings at an early stage of low N stress was studied by Lian *et al.*, (2006). In our laboratory, a semi-hydroponic growth system for rice was developed wherein N was the growth-limiting factor. The N-responsive genes were identified by a whole genome transcriptional profiling experiment and some of these genes were evaluated for their functionality in NUE by a transgenic approach. One successful example was the identification of an early noduline (*ENOD93*) gene (Bi *et al.*, 2009) discussed below. Similar work is being carried out for corn, and the similarity and uniqueness of the N response in different species such as the *Arabidopsis* model plant and important crops such as rice and corn are being studied (S Kant, unpublished results).

In *Arabidopsis*, a systems approach has been adopted to identify N-responsive gene networks (Gifford *et al.*, 2008; Gutierrez *et al.*, 2007*b*, 2008), and to understand the signalling pathways that respond to a combination of N, C, and light signals (Krouk *et al.*, 2009), or a combination of N and hormone signals (Nero *et al.*, 2009). This type of analysis will be used for crop plants. Also, with the advance of new technology, transcriptome analysis using next-generation sequencing approaches is expected to give the dynamic range

of the expression differences, as well as the ability to detect low-level transcripts. Deep sequencing data can also be used to test predicted transcripts and to identify novel gene transcripts, splice variants, near-identical paralogs, and allelic variants in non-isogenic lines. These approaches will help to develop the understanding of the N signalling pathways.

Dissecting the plant response to N limitation using reverse genetics

Several physiological and biochemical changes occur in plants as adaptive responses to N limitation, including an increase in N uptake by high affinity transporters, remobilization of N from older to younger leaves and reproductive parts, retardation of growth and photosynthesis, and increased anthocyanin accumulation (Ono *et al.*, 1996; Chalker-Scott, 1999; Ding *et al.*, 2005; Diaz *et al.*, 2006). Several T-DNA insertion mutants in *Arabidopsis* were screened to isolate mutants with an altered adaptive response to N limitation. In this screen, one mutant line was found with an altered phenotype under these conditions (although not linked to the original T-DNA insertion screened) and called the nitrogen limitation adaptation (*NLA)* gene. This mutant grows normally under high-N conditions but was unable to show several adaptive responses under low-N conditions, such as failing to accumulate anthocyanin, having abrupt senescence, and not being able to remobilize N metabolites from rosette leaves towards developing seeds. The mutation was linked to a RING-type ubiquitin E3 ligase (Peng *et al.*, 2007*b*). The *nla* phenotype was specific only to growth under a low-N condition, whereas at optimum N and when grown with other stresses both *nla* and wild-type plants looked similar phenotypically as well as at the transcript and biochemical levels (Peng *et al.*, 2007*a*, *b*). While comparing *nla* with the wild type by whole genome transcript profiling, at 10 mM N (optimum N) none of the genes were differentially expressed, whereas, at 3 mM N (limiting N) 1272 genes were differentially regulated, with 807 genes up-regulated and 465 genes down-regulated in *nla* compared with wild-type plants. Several of these genes are involved in the degradation of proteins and amino acids, the synthesis of anthocyanin and phenylpropanoids, transcription factors and genes involved in signal transduction or are senescence related (Peng *et al.*, 2007*a*). Further, it was revealed that in *nla* mutant plants, the phenylpropanoid pathway was disrupted, with substrates from this pathway channelled towards lignin production and thereby anthocyanin synthesis was suppressed (Peng *et al.*, 2008). It is known that anthocyanin accumulation is an important plant-adaptive response under abiotic and biotic stresses as well as under low-N conditions (Ono *et al.*, 1996; Chalker-Scott, 1999; Diaz *et al.*, 2006). From the characterization of the *NLA* gene, it is evident that *NLA* is a positive regulator of *Arabidopsis* adaptation to low-N conditions. In another study, it has been shown that the *NLA* gene also has a role in immune responses but as a negative regulator for salicylic acid production (Yaeno and Iba, 2008). Recently, Pant *et al.* (2009) has proposed that *NLA* might be controlled by a micro-RNA (miR827) and is an important component for the integration of phosphorus- and N-limitation responses. These studies suggest that *NLA* has regulatory roles in N and salicylic acid-mediated biotic stress. However, we are still quite interested in dissecting the molecular mechanism for *NLA* functionality, and for this we are working on finding its interacting proteins by yeast two-hybrid assays and mapping the suppressors of the *nla* mutation and the recovery response of *nla* mutants to different hormones.

Dissecting the plant response to N limitation using forward genetics

Similar to the reverse genetics approach, forward genetics using reporter gene and activation tagged lines could be an interesting way to isolate components for the N-limitation response. For example, two regulatory genes for phosphate homeostasis, a MYB transcription factor, *PHR1* (Rubio *et al.*, 2001) and a SEC-12 related gene, *PHF1* (Gonzalez *et al.*, 2005) were isolated by screening an EMS-mutagenized population of *Arabidopsis* transgenic lines harbouring a reporter gene β-*glucuronidase* (GUS) driven by the IPS1 (induction by Pi starvation 1) promoter. Nitrate-inducible promoters have been reported, including studies showing a 130 bp fragment in the promoter of the spinach nitrite reductase gene conferring the nitrate inducibility (Rastogi *et al.*, 1993). Subsequently, a 238 bp and a 330 bp fragment from the *Arabidopsis* nitrate and nitrite reductase genes required for nitrate-induced transcription was reported (Lin *et al.*, 1994). Similarly, a 150 bp fragment located in the promoter region of the *NRT2.1* gene has been shown to be induced in response to low nitrate. Multiple motifs potentially involved in regulation by N were identified, which act as *cis*-acting elements (Girin *et al.*, 2007). However, use of these promoter fragments for screening of transgenic lines to identify novel N regulatory genes has not been reported yet. In an approach to find such regulatory genes Wang *et al.* (2009) used an unknown nitrate-inducible promoter fused to the yellow fluorescent protein marker gene in an *Arabidopsis* transgenic line. In the screening of an EMS-mutagenized population of these transgenic lines, they identified two mutants that were impaired for nitrate induction. Both these genes, *NLP7* and *NRT1.1* have been previously characterized for their nitrate regulatory roles, confirming that the screen using this nitrate-inducible promoter is valid and can hopefully be used to capture unique N regulatory genes. Similarly, Girin *et al.* (2010) used transgenic *Arabidopsis* plants harbouring a *NRT2.1* promoter::*LUC* reporter gene to screen EMS mutagenized plants and have identified three mutants that appear to be altered in their regulation of nitrate uptake.

Understanding the mechanism of plant adaptation to N limitation using plants that show better growth under N-limiting conditions

Most of the N transporters, assimilatory, and regulatory genes in plants have been first identified in the model plant *Arabidopsis*. However, *Arabidopsis* is not adaptive to low-N

conditions, since its growth is retarded sharply under changing N availability (Martin *et al.*, 2002; Peng *et al.*, 2007*a*, *b*; Kant *et al.*, 2008). A complementary approach would be to unravel the molecular mechanisms for identifying unique N regulatory genes and promoter elements in plant species that are naturally adapted to N limitation. However, it has so far not been easy to work with plants that show better growth under N-limiting conditions because of their genetic complexity and the lack of molecular and genetic tools available in these plants. For example, modern maize hybrids can grow better and produce higher yield than older lines under limited-N conditions and variations among rice, wheat, barley, and sorghum cultivars for NUE are known (OrtizMonasterio *et al.*, 1997; Muchow, 1998; Le Gouis *et al.*, 2000; Presterl *et al.*, 2003; O'Neill *et al.*, 2004; Anbessa *et al.*, 2009; Namai *et al.*, 2009). However, little is known about the molecular mechanisms leading to higher NUE in these newer hybrids and cultivars. Therefore, working with a model plant that grows better under N stress conditions would facilitate investigation of the molecular mechanisms regulating adaptation to low N that may confer an improved NUE.

Thellungiella halophila has emerged as a new model plant for the molecular elucidation of abiotic stress tolerance. *Thellungiella* shares similar morphology and sequence identity with *Arabidopsis* thus allowing for the utilization of *Arabidopsis* genetic information to investigate *Thellungiella* responses to stress. Also it has all the attributes of a model plant system including a short life cycle, a relatively small genome, copious seed production, and ease of transformation (Inan *et al.*, 2004; Kant *et al.*, 2006). It has been shown that *Thellungiella* can grow better than *Arabidopsis* under low-N conditions. *Thellungiella* plants could maintain higher N content, total amino acids, and total soluble protein by efficiently acquiring and utilizing nitrate compared to *Arabidopsis* under limiting-N availability. This was attributed to *Thellungiella* having differential expression of various nitrate transporter genes and N assimilatory enzymes compared with *Arabidopsis* under both N-sufficient and N-deficient conditions (Kant *et al.*, 2008). The molecular mechanism for better adaptability in *Thellungiella* to withstand low N stress could be due to various mechanisms. These include variations in gene regulation (transcriptional, post-transcriptional, and post-translational), divergent promoter structures, evolution of more active forms of gene products, and the presence of unique regulatory genes. The ongoing sequencing of the *Thellungiella* genome, the production of BAC and cDNA libraries, and the generation of EST and T-DNA insertion collections will further enhance the power of the *Thellungiella* system for identifying key components governing N utilization under low N conditions.

Candidate gene approach for improving NUE

Previously, attempts have been made to improve NUE in transgenic plants by ectopic regulation of key enzymes involved in N metabolism (Good *et al.*, 2004; Lea and Azevedo, 2007; Shrawat *et al.*, 2008). We were interested in genes potentially involved in the regulation of certain N metabolic pathway by selecting candidate genes based on existing knowledge. As an example, GATA transcription factor genes have been implicated in the regulation of N assimilation in plants (Jarai *et al.*, 1992; Rastogi *et al.*, 1997; Oliveira and Coruzzi, 1999). In fungi, *Neurospora crassa* NIT2 (Tao and Marzluf, 1999) and *Aspergillus nidulans* AREA (Caddick *et al.*, 1986) are GATA transcription factors that globally regulate genes in N metabolism. In yeast, four N regulatory genes, *GLN3*, *NIL1*, *NIL2*, and *DAL80* are GATA factors with a single GATA zinc finger (Hofman-Bang, 1999). The GATA binding domain is highly conserved throughout this protein family (Lowry and Atchley, 2000). Several features of the regulation of nitrate assimilation are common between fungi and higher plants. Regions of the spinach nitrite reductase (NiR) promoter that are involved in N regulation were identified (Back *et al.*, 1991; Rastogi *et al.*, 1993, 1997) and footprinting results suggest that GATA factors play a role in NiR gene regulation (Rastogi *et al.*, 1997). When the entire family of GATA transcription factor genes in the *Arabidopsis* genome was screened further, one member in this family, the *GNC* gene was identified as a factor playing an essential role in chlorophyll synthesis and in the regulation of many C metabolic genes (Bi *et al.*, 2005). Over-expression of this *GNC* gene and its paralogue *CGA1* (Naito *et al.*, 2007) in *Arabidopsis* resulted in some positive effects on plant growth (S Kant, unpublished results). The orthologues of the *Arabidopsis GNC* and *CGA1* genes in rice have been identified (Reyes *et al.*, 2004). Through the analysis of transgenic rice plants over-expressing these genes, it was found that they have some conserved functions, but also cause some negative pleiotropic effects (S Kant, unpublished results), necessitating the use of other promoters, or the overexpression of potential target genes to ensure an enhanced phenotype while avoiding negative effects.

The work done by Schofield *et al.* (2009) was an attempt to use a potential target gene of GNC to achieve an enhanced phenotype while avoiding negative effects. *STP13* is one of the C metabolic genes whose expression levels were tightly influenced by *GNC* (Bi *et al.*, 2005). In *Arabidopsis*, the Sugar Transport Protein (STP) subfamily is comprised of 14 monosaccharide/H^+ symporters, and is part of the monosaccharide transporter (MST) gene family comprising more than 50 members (Johnson *et al.*, 2006). Constitutive over-expression of *STP13* in *Arabidopsis* resulted in seedlings with increased biomass when grown on media supplemented with sugar. The *STP13* transgenic seedlings had increased rates of glucose uptake, higher internal sugar levels, and more total C per plant. The *STP13* overexpressor seedlings also displayed improved N use, with the induction of a nitrate transporter, *NRT2.2* and higher total N per plant (Schofield *et al.*, 2009). However, no obvious phenotypic change was observed when *STP13* overexpressor plants were grown on soil, indicating that C availability is limited by the rates of photosynthesis under the conditions

utilized and were not likely to have been altered by the *STP13* transgene (Schofield *et al.*, 2009).

Another approach used to select candidate genes is by using whole genome transcriptional profiling (Bi *et al.*, 2009). A small number of genes were selected from the significant gene lists as NUE candidate genes. Some of these function in the transport of ammonium or nitrate, whereas others probably have a regulatory function (e.g. some transcription factor genes), and still others, such as an early nodulin gene, have a completely unknown function. While the change in the expression for most of these genes was around 2–4-fold, the early nodulin gene was up-regulated over 7-fold, and responded to transient changes of N conditions. The transgenic plants over-expressing this early nodulin gene (*OsENOD93-1*) had a significant 10–20% increase in the number of spikes and spikelets, and seed yield under both limiting-N and optimum-N conditions, and a significantly higher shoot dry biomass than wild-type plants under limiting-N conditions (Bi *et al.*, 2009). The *OsENOD93-1* gene would not have been selected for testing in transgenic rice plants if it had not been identified through its transcriptional response to N levels. Additional transgenic rice plants over-expressing other genes identified through this approach are also showing some interesting phenotypes (S Kant, unpublished data). Thus, transcriptional profiling is an effective approach for the discovery of genes that may one day contribute to improved crop genetics for this important trait.

Future prospects

In order to use gene knowledge to improve NUE, it is important to develop a more complete understanding of how different plants respond to growth under different N conditions. We have only begun to understand the regulatory mechanisms that underpin these responses. Considerable work still needs to be done using model systems like *Arabidopsis* and *Thellungiella* to improve this understanding. However, to improve this trait in important crops it is necessary to use this knowledge to improve breeding selection or through the development of transgenic lines. Many important crop traits like NUE are traditionally considered to be complex multi-gene traits, with no single gene contributing more than a small percentage to the phenotype. Therefore, it was unclear whether a single transgene could have a significant enough effect on phenotype to make commercialization worthwhile. However, there are several examples where a single transgene has had a significant phenotypic effect and although none have yet been commercialized it gives hope that this approach will be worthwhile. Certainly this approach will require the testing of a large number of promoter–gene combinations and wisely choosing the best. In addition, it might require a combination of two or more genes in multiple gene cascades to add their cumulative potential benefit. This might, for example, involve increasing N influx, increased flux from roots towards shoots, metabolic flux for assimilation into amino acids, and flux towards sequestration (e.g. storage into the vacuole). In addition to improving NUE through better N acquisition, focus on improving N remobilization would be quite fruitful through the efficient degradation of proteins and other organic N forms and transport during grain-filling.

In addition, the components of NUE interact in multiple and complex ways with other metabolic pathways. Thereby, improving NUE is also dependent on other factors which affect N uptake, assimilation, and remobilization efficiency, such as C status, water, light, temperature, and soil type. Maintenance of optimum N and C homeostasis is required for optimum root and shoot development, photosynthetic rate, synthesis and mobilization of amino acids, organic acids, and lipids. Also, drought stress and N availability are linked for optimum yield, in that water use efficiency (yield per unit of transpiration) decreases when plant growth decreases with reduced N availability as photosynthesis is decreased more than transpiration (Cabrera-Bosquet *et al.*, 2007). Combining multiple interlinked agronomic traits particularly growth under low N and drought tolerance is also important. In conclusion, there are many potential genetic approaches for the improvement of crop NUE and these require increased knowledge of how plants respond to different N regimes as well as to other environmental conditions.

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