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REVIEW ARTICLE

Physiological approaches for increasing nitrogen use efficiency in rice

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Abstract Nitrogen (N) plays an important role in plant growth, development and also one of the major factor for developing a high-yielding rice cultivars. Nitrogen use efficiency (NUE) in plants is a complex phenomenon that depends on a number of internal and external factors, which include soil N availability, its uptake and assimilation of carbon and nitrogen. An increased awareness of the regulatory mechanisms controlling Nitrogen economy is imperative to enhance nitrogen uptake and use efficiency so as to reduce excessive input of fertilizers, while maintaining an acceptable yield. The physiological, biochemical, molecular aspects like QTL, mi RNA technology and transgenic approaches as well as NUE can be targeted to improve rice productivity. Yield being complex and multigenic trait linkages between carbon and nitrogen pathways are essential. An attempt on complex interactions between the two major physiological pathways linked by photosynthesis and photorespiration in global climate change for enhancing NUE in relation to rice yield was reviewed.

Keywords Nitrogen use efficiency · Rice · Biological approaches · miRNA · Hormones · Photorespiration

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Introduction

Nitrogen(N) is one of the essential macronutrients required to sustain plant life. As it is the key substrate in many important structural, genetic and metabolic compounds of plant cells. It is a major component of chlorophyll, the compound by which plants use sunlight energy to produce sugars from water and carbon dioxide by photosynthesis. It is also a major component of amino acids, the building blocks of proteins. N is a significant component of nucleic acids such as DNA, the genetic material that allows cells to grow and reproduce. As Nitrogen constitutes 70 % of the atmospheric gases, atmospheric nitrogen can also be used by plants after reduction by soil microorganisms. Soil contains both NH_4^+ and NO_3^- forms of N. They are available in the soil as mobile form 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, de nitrification, volatilization, and microbial consumption.The majority of crops except nitrogen-fixing legumes receive an application of nitrogen, the major requirements are for the production of seeds (Mengel et al. 2006) and forage (Kingston-Smith et al. 2006). There has been considerable interest in identifying the processes involved in regulating N uptake and metabolism within the plant (Andrews et al. 2004; Gallais and Hirel 2004).

Rice (*Oryza sativa* L.) leaf N content is about 75 % of total N present in plant and is physiologically important in dry matter production through photosynthesis (Dalling 1985). Higher yields are necessary to support rapidly increasing populations. Relation among growth, yield and N utilization in rice plant are becoming increasingly understood at physiological and molecular levels. 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 threefold by 2050 (Good et al. 2004). Conventional breeding efforts in the past few decades have significantly increased crop yield and also improved Nitrogen use efficiency (NUE).

Rice is the model crop in monocots; its genome has been sequenced. This review mainly focused on the numerous techniques involved for enhancing the NUE and also discussed on the mechanisms of N uptake, NH_4^+ assimilation during the plant life cycle. Finally, our knowledge on nitrogen metabolism in relation with carbon metabolism like photosynthesis and photorespiration is emphasized. References to work on other cereals, dicots and lower plants are made when similar information is not available for rice.

What is nitrogen use efficiency?

Nitrogen use efficiency has been defined as grain yield per unit of N available in the soil. (Good et al. 2004), NUE can be categorized as (a) uptake and (b) utilization efficiencies. Uptake efficiency drives biomass production and depends on amount of N uptake, storage, and assimilation into amino acids and other important nitrogenous compounds where as utilization efficiency involves N remobilization i.e. 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.

Improved NUE is an important goal in developing cropping system. Determination of NUE in cereal based ecosystems enabled broad assessment of management and environmental factors related to N use, grain yield and N accumulation, N in aboveground, N harvest index, and grain N accumulation are the key indicators of NUE (Huggins and Pan 2003). In general, total biomass production of *indica* cultivars is greater than that of *japonica* cultivars, whereas grain yield is less. For example, the mean value of one-spikelet weight on the main stem of Kasalath (*indica*) and Nipponbare (*japonica*) was 18.0 and 26.9 mg. The current average NUE in the field is approximately 33 % and a substantial proportion of the remaining 67 % is lost into the environment, especially in the intensively cropped area (Abrol et al. 2007).

Why NUE has to be increased ?

Nitrogen use efficiency is an important in modern agriculture, not only for crop growth and yield but also for reducing production cost. Moreover, one of the major negative environmental impacts of agricultural activities is associated with excessive nitrogen application. Improving NUE will ensure lower level of N fertilizer usage thus reduce environmental contamination. Organic farming is being increasingly proposed as another option to protect the environment. However, if all farmers adopt organic farming, the food produced will not be sufficient to feed the global population, as natural N_2 fixation and organic N recycling cannot keep pace with the N demand of high-yielding cultivars. Hence, it is predicted that the next few generation of humankind will continue to depend on reactive N generated in fertilizer factories (Smil 1997). Thus, there is an urgent need for the development of a comprehensive approach to optimize N utilization, managing the N fertilizer and improving the NUE of rice plant through different strategies. The challenges associated with N and the need for improved nutrient efficiency was reviewed (Pathak et al. 2008; Garnett et al. 2009; Townsend and Palm 2009). It is estimated that 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 utilization of N is achieved by two pathways. One of the pathways is through *nitrate* form and second pathway is through *ammonia*. The detailed mechanisms about these two pathways are discussed.

Mechanism of nitrogen uptake and transport system

Nitrate uptake

In rice (*O. sativa* L.), N is primarily available in the form of nitrate (NO_3^-) and ammonium (NH_4^+). The NO_3 is a most predominant form in agricultural soils (Crawford and Forde 2002) taken up by active transport through the roots, distributed through the xylem and assimilated by the sequential action of the enzymes nitrate reductase (NR) and nitrite reductase (NiR) followed by ammonium assimilation, amino acid biosynthesis, and protein synthesis. The nitrate produced by nitrification has been shown to be taken up with the diffusion of oxygen through the roots in

flooded soils (Kirk and Kronzucker 2005). In addition, rice grows better and utilizes nitrogen efficiently when nitrate and ammonium supplied together (Ta and Ohira 1981; Kronzucker et al. 1999). There are, however, complex interactions with many other aspects of nitrogen metabolism, including (i) the storage and remobilization of nitrate in different parts of the plant, (ii) de novo ammonium assimilation, (iii) the recycling of ammonium released during photorespiration, (iv) the distribution of nitrogen between the highly branched pathways of amino acid biosynthesis (Morot-Gaudry et al. 2001), and (v) the multifarious fates of amino acids, which can be exported, stored in the vacuole, used for protein synthesis, or diverted into secondary metabolic pathways leading to phenyl propanoids, alkaloids and tetra pyrrole. NR, which reduces NO_3^- to NO_2^- was considered the rate-limiting enzyme in the NO_3^- assimilation pathway, and was hence thought to be pivotal to the growth response of plants to nitrate fertilization.

Nitrate 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. Exposure of plant roots to NH_4^+ inhibits nitrate uptake, while NO_3^- in medium has little effects on NH_4^+ uptake (Samuelson et al. 1995; Kronzucker et al. 1999; Feng et al. 2003; Wang et al. 2003). NH_4^+ influences NO_3^- uptake probably through the feedback regulation of NO_3^- metabolism (Samuelson et al. 1995; Kronzucker et al. 1999; Zhuo et al. 1999; Orsel et al. 2002), or through the direct effect on the plasma membrane (Ullrich 1992; Ayling 1993).

Nitrate is transported by two systems namely NRT1, a low-affinity (millimolar nitrate) transporters (LATS) and NRT2, a high-affinity (micromolar nitrate) transporters (HATS). The HATS consists of two system, the nitrate inducible HATS (iHATS) and constitutive HATS (cHATS) (Wang and Crawford 1996; Crawford and Glass 1998; Forde 2000). It admits enough nitrate into the cell to induce the expression of transporter and assimilatory genes, and presumably plays a physiological role in the nitrate uptake only above a certain threshold. Both the cHATS and iHATS—become active when the concentration of nitrate in the soil/medium is low, i.e. below 1 mM. Both these HATS are up regulated in response to nitrate. cHATS provide a high affinity, low capacity pathway for nitrate entry in uninduced plants but their activity become three-fold on exposure to nitrate. The iHATS have been extensively studied and are known to be induced by nitrate or nitrite. In all the above-mentioned systems, each ion of nitrate is co-transported with two or more protons.

Low affinity transport system has been identified in *Arabidopsis thaliana* and rice wherein it may play a greater role in nitrate uptake. In some, but not in all cases, the HATS have been shown to be identical with that of nitrate transport, by uptake competition studies as well as voltage changes (Ullrich 1992). The nitrate uptake system of higher plants consists of a constitutive, LATS (possibly a carrier system or an anion channel), and an inducible, high affinity transport system regulated by cellular energy supply, and by intracellular nitrate consumption, and whose activity depends on the proton electrochemical gradient. The latter system is regarded as an H^+/anion co-transport carrier mechanism that produces transient plasma membrane depolarization upon addition of nitrate. The depolarization is counteracted by the plasma membrane H^+-ATPase (Ullrich 1992). The plasma membrane proton ATP-ase is induced by nitrate (Santi et al. 1995). Ammonium decreased the electrical polarized status and therefore inhibited nitrate uptake. Coexistence of ammonium and nitrate caused steady depolarization. Recent studies revealed that NRT2.1 high-affinity nitrate transporter plays major role in the regulation of root branching. (Little et al. 2005). The genes involved in nitrogen uptake may directly be able to influence NUE (Lea and Azevedo 2007). In rice, four *NRT2* family genes isolated from rice genome database of which three genes were nitrate inducible and one gene was expressed before nitrate induction.

The transcriptional regulation of nitrate responsive genes could involve *cis*-acting regulatory sequences or nitrate response elements (NRE) (Raghuram et al. 2006). However, the identification of putative *cis* elements that are responsive to C/N signalling interactions indicates the possible combinatorial role of different *cis*-regulatory elements. (Palenchar et al. 2004). Identification of such regulatory elements might provide an end-point for nitrate signalling and open up avenues for characterizing/manipulating the rest of the signalling pathways to enhance NUE. Another way to improve NUE is by manipulating *NR* and *NiR* genes. NR has been considered to be the rate-limiting step in nitrate assimilation. The similar strategy can apply to the rice crop for enhancing the NUE but the utility of transgenic over expression of NR/NiR for major improvements of NUE remains uncertain.

Ammonium uptake

Out of the two forms of N, in paddy field, ammonium (NH_4^+) form is more preferred rather than the nitrate (NO_3^-) form and it can be taken up directly through roots, even though uptake can occur in biphasic manner with

LATS and HATS (Glass et al. 2001). Ammonium generated from primary nitrate assimilation, re-assimilation of internal metabolites or other secondary sources, is incorporated into amino acids in a reaction catalysed by GS and then by GOGAT (Forde and Lea 2007). The NH_4^+ uptake in rice occurs in the root apex and in the region where secondary roots are being formed (Tatsumi 1982). NH_4^+ is taken up by plant roots through ammonium transporters (AMTs). The expression of AMTs is affected by N conditions, and strictly regulates the influx of ammonium in plants including rice (reviewed in Loqué and von Wirén 2004).

Glutamine synthetase (GS) serves for assimilation of NH_4^+ in rice roots. NH_4^+ is mainly stored and transported because of problem of toxicity and it is so first assimilated in root and then transported through the xylem to the shoots, mainly in the form of glutamine (Fukumorita and Chino 1982). Because Glutamine (Gln) serves as a major nitrogen source transported from root to shoot through the xylem (Kiyomiya et al. 2001). In rice roots, application of ammonium regulates two different types of AMT species, accompanied by a temporal increase in internal ammonium content; Os*AMT1;1* and Os*AMT1;2* were up-regulated, whereas Os*AMT1;3* was down regulated by ammonium (Sonoda et al. 2003b). For these reasons, a complex regulatory system is suggested to control ammonium transport and downstream assimilatory pathways in rice plants (Sonoda et al. 2003b).

It is of considerable physiological relevance whether neutral NH_4^+ or positively charged NH_4^+ is transported, since the gradient across the membrane is often opposite for both species. Toxicity symptoms frequently occur if crop plants are grown in ammonium in the absence of nitrate (Britto et al. 2001). Ammonium uptake systems have been well defined as concentrative, energy-dependent, and camer-mediated in algae (Smith and Walker 1978), fungi (Kleiner 1981), bacteria (Kleiner 1985), and cyanobacteria (Boussiba and Gibson 1991). The end-product, ammonium (NH_4^+), is incorporated into amino acids via the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle (Forde and Lea 2007).

GS is the central enzyme in ammonium assimilation in plants with a cytosolic (GS1) and a plastidic (GS2) isoform. Similarly, GOGAT has two isoforms, a ferredoxin-dependent plastidic isoform (Fd-GOGAT) and a NADHdependent cytosolic isoform (NADH–GOGAT). The plastidic isoforms of both the enzymes (GS2 and Fd-GOGAT) are involved in primary ammonia assimilation, while their cytosolic isoforms are involved in secondary assimilation. In rice leaves, two isoforms of GS, GS1 (cytosolic form) and GS2 (chloroplast form), have been identified by Suzuki et al. 1981. In rice concentrate GS1 in phloem companion cells to drive export of N from leaves. In the nodules, cytosolic GS1 is posttranslationaly regulated by phosphorylation in response to nitrogen fixation (Lima et al. 2006). Cytosolic GS also assimilates ammonium produced by endophytic diazotrophs colonizing the internal parts of some plants, such as sugarcane, without establishing symbiotic relationships (Nogueira et al. 2005) The GS1 gene family consists of several isoenzymes, and their physiological roles and regulations are rather complex compared with GS2 (Ishiyama et al. 2004).

In rice roots, reported only one form of cytosolic GS (GSr), but two isoforms of GS, GSra and GSrb, have been detected using native-PAGE and activity staining. The two isoforms of GS in rice roots may play different roles in the growth and development of rice plants. In roots, the cytosolic form (GS1) facilitates the assimilation of ammonium taken up from soils or provided from symbiotic nitrogen fixation (Hirel et al. 1987; Forde et al. 1989; Cock et al. 1990; Edwards et al. 1990; Miao et al. 1991; Sakakibara et al. 1996; Terce-Laforgue et al. 1999; Tobin and Yamaya 2001; Ishiyama et al. 2004).

As might be expected in rice, the regulation of the AMT genes was more complex. AMT1-1 exhibited constitutive and ammonium-promoted expression in shoots and roots and AMT1-2 was root specific and expression was induced by ammonium, while AMT1-3 was root specific and subject to nitrogen-derepressible expression (Sonoda et al. 2003a, b). In addition, AMT2-1 exhibited constitutive expression in both roots and shoots, while AMT3-1 was only weakly expressed in roots and shoots. In a different series of experiments in rice, Kumar et al. (2003) demonstrated an increase in expression of AMT1-1 and to a lesser extent AMT1-2 and AMT1-3, following transfer from 10 mM to 101 M ammonium. It was found that NH_4^+ uptake was decreased when the plants were supplied with glutamine or asparagine (Lea and Azevedo (2007). With the exception of rice, both a HATS and a LATS for ammonium uptake are present in plant roots that are constitutive and do not seem to be significantly induced by ammonium (Glass et al. 2002).

The NUE can be improved by enhancing the N uptake and N utilization of plant. These opportunities help to genetically improve the N uptake by plant. Some of the suggested option to improve the NUE through an improved root system was increased root to shoot ratio, high root vigor, N-induced root proliferation, root length density, root hairs, root exudates, microbial symbiosis and root N metabolism. The utility of these traits in isolation or in combination is largely determined by the target crop environment. Garnett et al. 2009, reported that very little is known about how the various N transporters contributed to the net N uptake by crops in field situation or how this changes over the life of the crop. Furthermore, the mechanisms regulating the distribution of NO_3^- and NH_4^+ from

root to shoot remain poorly understood. Research in these areas will make it easier to focus on targets for improving NUE.

Role of 14-3-3 Proteins

14-3-3 proteins are quite different in sequence and structural topology from other protein families in the database. The only significant homology with other proteins appears to be with tetratricopeptide repeat (TPR) helices as first observed in the structure of protein phosphatase 5 (Das et al. 1998). The TPR is a degenerate 34 amino acid sequence identified in a wide variety of proteins, present in tandem arrays of 3-16 motifs, which form scaffolds that can mediate protein–protein interactions and the assembly of multiprotein complexes. Structural studies on SMG7, which contains a TPR domain, have shown it to be a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway (Fukuhara et al. 2005). The plant 14-3-3 proteins are involved in many crucial processes interacting with numerous proteins involved in various biological processes. The 14-3-3 proteins play an important role in metabolic coordination between enzymes of C and N metabolism, modulating their activity by binding them in a phosphorylation dependent manner. NR, GS, sucrose-phosphate synthase (SPS), trehalose-phosphate synthase, glutamyl-tRNA synthetase, and an enzyme of folate metabolism have been found to bind to 14-3-3s in a phosphorylation-dependent manner (Das et al. 1998).

The control of NR expression is exerted by light and metabolites such as nitrate and sucrose, demonstrating cross-talk of primary carbohydrate and nitrogen metabolism. In addition, NR is subject to fine control via inactivation by phosphorylation. Phosphorylation occurs in the hinge 1 region of nitrate reductase, at Ser-534 in Arabidopsis and Ser-543 in spinach (Su et al. 1996). However, phosphorylation of the enzyme alone does not induce the inactivation. The process is completed after reversible binding of a nitrate reductase inhibitor protein (NIP) (Bachmann et al. 1995). Therefore it appears that NR is highly regulated, firstly by the steady-state level of the enzyme protein (Lillo 1994; Crawford 1995; Campbell 1996) and secondly by reversible protein phosphorylation (Mackintosh 1992). Not only do 14-3-3 proteins regulate enzymes of the nitrogen assimilation pathway and proteins involved in generating proton gradients which serve as the driving force for nutrient uptake, they also regulate the activity of enzymes involved in primary carbon metabolism. SPS is a good example of this. SPS catalyses the conversion of UDP-glucose and fructose-6-phosphate to sucrose-6-phosphate, the second last step in sucrose biosynthesis, which can be regulated by allosteric effectors and protein turnover (Huber and Huber 1996). The enzyme has several putative phosphorylation sites which regulate its activity by 14-3-3 dependent and independent mechanisms. Non-14-3-3 events include phosphorylation of SPS on Ser-424 and Ser-158 which are thought to be responsible for light/dark modulation and osmotic stress activation of the enzyme (McMichael et al. 1993; Toroser and Huber 1997) However, there is a site-specific regulatory interaction between 14-3-3 proteins and Ser-229 of spinach SPS which inhibits SPS activity (Toroser et al. 1998). The targeting and regulation of key enzymes of carbon and nitrogen metabolism by 14-3-3 proteins suggest a common 14-3-3 mediated mechanism in regulating and possibly co-ordinating the use and generation of metabolites in plants. It is worth noting that primary carbon and nitrogen metabolites can also be sensed, leading to physiological and developmental changes.

Experiments in transgenic potato plants indicate that repression of 14-3-3 proteins led to significant increases in NR and SPS activities (Fukuhara et al. 2005). More recently, the effect of repression of 14-3-3 genes on actual activity of NR in *Nicotiana benthamiana* leaves, was studied by silencing the Nb14-3-3a and Nb14-3-3b genes using virus induced gene silencing method, which implicated Nb14-3-3a and/or Nb14-3-3b proteins in the inactivation of NR activity under darkness in *Nicotiana benthamiana* (Xiao et al. 2002). The 14-3-3 proteins also interact with components of plant signalling pathways as observed in their interaction with RGS3, a negative regulator of the G-alpha subunits of heterotrimeric G proteins 40, suggesting a possible role in the regulation of G-protein signalling pathways, which in turn have been implicated in mediating light regulation of NR (Aitken 1999). 14-3-3 proteins regulate plant H^+-ATPase and ion channel activity. The Plasma membrane H^+-ATPase is known to play crucial roles in the plant cell by generating a proton gradient, thereby providing the driving force for nutrient uptake, phloem loading, water movements, stomatal closure, and opening. Its activity is regulated by many environmental stimuli and hormones.

What happens to the rice crop when nitrogen level is low?

Rice plants with nitrogen deficiency often have spindly stems and their growth is stunted. In addition, their leaves turn yellowish from lack of chlorophyll and flowering is delayed. Nitrogen deficiencies first appear on the lower leaves because it is a mobile element within the plant and is often transferred from older to younger tissues when uptake is limiting. As harvested plant parts such as seeds are high in nitrogen content and require sufficient supplies for optimal growth, nitrogen deficiency is particularly damaging to crop yields.The low nitrogen availability in rice leads to decline

in leaf N allocation, photosynthesis and water uptake, due to decreased demand by the plant. Similar patterns are observed in most other studies (Chapin 1980; Clarkson 1985). It has been observed that Nitrogen-limited plants have high carbohydrate status, and light-limited plants have high tissue-nitrogen concentrations (Evans 1989).

Nitrogen limitation of plant growth provides support for this hypothesis. Under conditions of high nitrogen availability, plants have a low potential to absorb nitrogen and a low allocation to roots. Under these circumstances, nitrogen demand by the plant has more effect on nitrogen uptake than does nitrogen availability in the soil (Clarkson 1985). By contrast, when growth is nitrogen-limited, nitrogen uptake is controlled by the rate of supply from the soil. (Maheswari 1986) were reported by application higher N fertilizers to increased the volatilization losses and free ammonium levels. During senescence, most of the nitrogen is mobilized from various parts to developing grains and also simultaneously lost from the canopy, these gaseous N losses depending on the species and environment.

How to increase the nitrogen use efficiency by using different approaches

Fertilizer nitrogen use efficiency (FNUE)

The doubling of agricultural food production worldwide over the past four decades has been associated with a seven-fold increase in the use of nitrogen (N) fertilizers. The use of fertilizers (N in particular) in agriculture, together with an improvement in cropping systems, mainly in developed countries, have provided a food supply sufficient for both animal and human consumption (Cassman and Pingali 1994). N is the most critical input that limits rice productivity (Sahu et al. 1997; Shrawat et al. 2008). The use of large amounts of N fertilizer for many crops helps to 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. Farmers are facing increased economic pressures with the rising fossil fuels costs required for production of N fertilizers. This challenge is particularly relevant to cereals for which large amounts of N fertilizers are required to attain maximum yield and for which NUE is estimated to be far less than 50 % (Raun and Johnson 1999).Fertilizer was supplied in the name of Urea for rice crop to meet the crop N demand, where the soil nitrogen is very low. Although 75 % of the variation in grain yield was explained by total N uptake, genotypic differences in NUE were observed (Inthapanya et al. 2000). The NUE has been increased up to 50 % only by the application of mineral N, rest of N lost through different mechanisms, including ammonia volatilization, denitrification, and leaching (Choudhury and Kennedy 2005).

The time of application of the fertilizer during crop growth plays a crucial role in determining the amount of N utilized. NUE of crops such as rice under irrigated condition can be affected by the form of N fertilizer used.The potential target to improve NUE is through minimizing the loss of applied nitrogen. Loss of applied N in the soil can be minimized through exploiting genetic viability in biological nitrification inhibition (BNI) by roots exudates of a crop (Subbarao et al. 2006). Utilization of biological N fixation (BNF) technology can decrease the use of urea-N, reducing the environmental problems to a considerable extent. Different BNF systems have different potentials to provide an N supplement, and it is necessary to design appropriate strategies in order to use BNF systems for efficient N supply to a rice crop. Research has been conducted around the world to evaluate the potential of different BNF systems to supply N to rice crops. The aquatic biota Cyanobacteria and Azolla can supplement the N requirements of plants, replacing 30–50 % of the required urea–N. BNF by some diazotrophic bacteria like Azotobacter, Clostridium, Azospirillum, Herbaspirillum and Burkholderia can substitute for urea–N, while Rhizobium can promote the growth physiology or improve the root morphology of the rice plant. Green manure crops can also fix substantial amounts of atmospheric N. Among the green manure crops, *Sesbania rostrata* has the highest atmospheric N_2-fixing potential, and it has the potential to completely substitute for urea–N in rice cultivation. The blue-green algae (BGA) and *Azolla* also plays a major role in supplying N to rice fields is well. They contribute significantly towards maintaining and improving the productivity of rice. BGA liberate extracellular organic compounds and photosynthetic O_2 during their growth, while *Azolla* prevent a rise in the pH, reduce water temperature, NH_3 volatilisation and suppress weeds; and both of them contribute the biomass. Finally basing on BNF technology yield improvement of rice is seems to be increased by about 20 % (Sprent and Sprent 1990). Since biological N_2 fixation is known to be inhibited by inorganic N, addition of BGA will reduce this effect in addition to the supply of nitrogen to the field.

According to FAOSTAT 2010, the world nitrogen fertilizer demand is expected to increase from a total of 103.9 million tonnes in 2010–111.6 million tonnes in 2014 at the annual growth of 1.8 %.

Genetic approaches

Using Genetic engineering approaches NUE can also be increased. The regulatory mechanisms and genes

responsible for N responses in plants have been investigated by using genetics. The first attempts to identify the limiting steps of plant NUE were largely facilitated following the development of genetic engineering techniques on both model and crop species (Good et al. 2004; Sinclair and Purcell 2005; Hirel and Lemaire 2005). Success in terms of increased NUE through genetic manipulation was done through over expression in roots of alanine aminotransferase (AlaAT), which is a downstream process in N assimilation (Good et al. 2007). In transgenic canola, yields were higher at low N associated with higher influx of N. In this material root alanine was increased and shoots glutamine decreased. Transgenic *Brassica napus* plants over expressing a barley AlaAT cDNA, driven by a Brassica root-specific promoter (btg26), showed improved NUE. Compared with wild type Brassica, transgenic plants showed increased biomass and yield in both the laboratory and field under low N conditions whereas no difference were observed under high N conditions. These changes resulted in a 40 % decrease in the amount of applied N fertilizer required under field condition to achieve yields equivalent to those of wild type plants (Good et al. 2007). A similar strategy was applied for improving NUE in rice (Shrawat et al. 2008). Nipponbore, a model rice plant, was genetically engineered by introducing barley AlaAT cDNA driven by rice tissue specific promoter(OsAntl).This modification increased the biomass and GY significantly in comparison with control plants when plants were well supplied with N content, indicating increased NUE. More recent discoveries were the master clock control gene *CCA,* which links organic N regulation and circadian rhythms (Gutiérrez et al. 2008), and micro RNA167, which Mediates the cell specific control of root development in response of N (Gifford et al. 2008). Most recently, a protein kinase AtCIPK8, was identified that is needed for nitrate response at high, but not at low, nitrate concentration (Hu et al. 2009), and a DNA binding protein, *AtNLP7*, which encodes the NIN like protein 7 (NLP7), nitrate regulation during nitrate assimilation (Castaings et al. 2009). *NLP 7*mutants have altered root growth longer primary roots and more lateral roots) typical of N starved plants and more resistant to water stress. However, this accumulated knowledge on the molecular aspects of N uptake is yet to be exploited for enhancing NUE in crop plants.

In Rice although several studies demonstrate that an increase in GS activity in transgenic plants has no effect on the phenotype, other researchers show a direct correlation between an enhanced GS activity in transgenic plants and an increase in biomass or yield, upon incorporating a novel *gs1* construct 6,8,15. The goal of molecular genetics researchers is to increase NUE through genetic approaches (Masclaux et al. (2001). The activities of specific enzymes involved in nitrogen metabolism within the plant have been targeted for transformation. For example, plants have been transformed to over express a glutamine synthetase gene, which is crucial in the assimilation of NH_3, but this either had no influence on nitrogen accumulation or decreased mass accumulation (Harrison et al. (2000). By using genetic engineering approaches it is possible to manipulate the corresponding genes and reintroduce them to produce transgenic plants with efficient nitrogen economy.

There are a few other examples of successful genetic modification of N metabolism using either structural or putative regulatory genes. When the bacterial enzyme glutamate dehydrogenase (GDH A) from *E. coli* was constitutively overexpressed in tobacco, biomass production of the transgenic plants was increased by about 10–15 %. In addition to the increase in biomass production GDHA had more overexpressed in leaves and their free amino acid content was higher, suggesting that both N and C metabolism were modified (Ameziane et al. 2000). The results of (Fuentes et al. 2001) concluded that, over expression of cytosolic glutamine synthetase (GS1) from alfalfa, which increased the photosynthesis and growth in tobacco and are able to utilize N more efficiently under N stress conditions. Similarly, Oliveira et al. (2002) also showed that in tobacco, the over expression of a gene encoding a pea GS1 lead to increased biomass production both under limiting and non-limiting N feeding conditions. In more recent studies the transgenic rice lines over expressing GS1 gene was improved harvest index and N utilization efficiency. However, these lines did not exhibit higher NUE under N-limiting conditions compared to non-limiting N conditions (Brauer et al. 2011). Jing et al. (2004) suggested that, in wheat, over expression of GS1 gene from bean led was increased the grain yield (grain weight in particular) about 20 % and therefore of NUE. Yanagisawa et al. (2004) studied that, a *Dof1* gene encoding a transcription factor from maize was overexpressed in Arabidopsis (*A. thaliana* L.), an increase in amino acid content and of N uptake was observed, especially when plants were grown at a low level of N supply and similarly gene was overexpressed in potato, transgenic plants accumulated more amino acids especially glutamine and glutamate. These two sets of experiments suggest that this gene could be used to improve the uptake and utilization of N in several species. In addition, the transgenic plants produced more biomass under low N supply and they did not exhibit symptoms of N deficiency in comparison to the untransformed control plants, which developed much earlier symptoms of senescence. Thus, overexpressing regulatory genes rather than structural genes, such as genes encoding GS, GOGAT or AlaAT appears to be an interesting alternative to improve plant NUE and overall plant growth and development in a more stable and balanced way across species. Same results are showed in maize that, the over expression of a native

gene encoding GS1 (*Gln1-3*) for Grain yield of the maize transgenic plants grown under greenhouse conditions was increased by about 30 %. However, grain N content and biomass production of the transgenic plants were not modified at maturity (Martin et al. 2006). A gene encoding NAD(H)-dependent GOGAT from alfalfa was constitutively expressed in tobacco, a significant increase in biomass production was observed (Chichkova et al. 2001). Over expression of the native NAD(H)-dependent GOGAT in rice led to an increase in grain weight (Yamaya et al. 2002; Tabuchi et al. 2007). A gene of unknown function OsENOD93-1, which is a N responsive gene identified following genome wide gene expression profiling in rice, led to an increase in grain yield, of 13–14 % and 19–23 % under limiting and non-limiting N nutrition conditions respectively (Bi et al. 2009).

Physiological approaches

Nitrogen use efficiency is affected by both N uptake efficiency and Physiological N use efficiency (PE). N uptake efficiency is nitrogen uptake relative to the supply and PE represents grain yield or plant biomass relative to nitrogen accumulation. PE is a key parameter for evaluating NUE in rice (De Datta 1986; Peng et al. 2002), which is related to the accumulation and redistribution of biomass and N in rice (*O. sativa* L.). It was observed that increasing the N rate has decreased the PE for biomass and grain yield.Grain yield and PE were about 20 and 18 % lower than those of high NUE cultivars. Differences in biomass, N accumulation and N redistribution were observed at the post-heading stage among rice cultivars with differing NUEs. PE is largely affected by two factors (i) the genetically determined mode of photosynthesis either the C_3 or C_4 photosynthetic pathway; and (ii) the grain N concentration-also under genetic control but affected by N supply as well. Both rice and wheat are C_3 plants while maize is a C_4 plant. The C_4 plants tend to have greater Physiological nitrogen use efficiency than C_3 plants because the C_4 pathway has a higher photosynthetic rate per unit leaf-N content (Sage et al. 1987), which results in greater biomass production per unit of plant-N accumulation. Many physiological processes affect N utilization efficiency for biomass production (NUEb) and NUEg (Ladha et al. 1998). NUE biomass is largely affected by critical concentrations (internal N requirement) for expansion and organ formation, N partitioning between leaves and stems, vertical N distribution in a canopy, efficiency of N use in converting CO_2 to carbohydrate through photosynthesis, rubisco activity and leaf senescence. Grain N concentration, sink capacity, unproductive tillers, HI and the ability to remobilize the absorbed N from straw to grain determine N harvest index (NHI) and NUEg. Leaf N plays a major role in biomass production through photosynthesis. Leaf N content is closely correlated with single-leaf photosynthetic rate (Peng et al. 1996). Increasing leaf N content and delaying N efflux from leaf (i.e., delaying leaf senescence), especially the flag leaf, could improve NUEb if the ratio of photosynthesis to respiration was not decreased. High plant N content delays leaf senescence and therefore increases photosynthetic duration (Makino et al. 1988). Rice generally has greater NUEb and NUEg than some other C_3 crops such as soybean and wheat, because of low Nitrogen straw % and Nitrogen grain %. The canopy is another approach to achieving high NUEb. In the leaf, N is concentrated in the chloroplasts, mainly as the enzyme protein rubisco. Rubisco accounts for more than 50 % of total soluble protein and over 25 % of total N of leaves. Therefore, leaf is a major storage organ for N. The efficiency in N remobilization from old to new leaf and from straw to grain will affect both NUEb and NUE. Rice has a lower efficiency than maize because it is a C_3 plant although its lower grain N concentration partially offsets this disadvantage. By using physiological approaches increasing the grain yield as well as nitrogen concentration in grain with efficient nitrogen economy is vital for increasing the NUE in rice.

Molecular approaches

The initial phase of molecular research for improved NUE in crop plants was based on the assumption that genes contributing to N uptake and N assimilation in plants are crucial. This has led to the accumulation of substantial knowledge on N uptake and assimilation mechanisms at the molecular level. Recent developments in molecular biology provide a new opportunity to improve NUE through crop improvement.The improvement of crop yield has been possible through the indirect manipulation of QTLs that control the heritable variability of the traits and physiological mechanism that determine biomass production and its partitioning. (Gallais 2005) Gallais and Hirel (2004), Hirel et al. (2007) reported a set of QTLs for NUE and for grain yield (GY) and its components at high and low N levels. QTLs mapped in clusters and those identified under low N were generally a subset of those identified under high N, except for grain protein content, for which a higher number of QTLs were detected in low N, a number of genes that encode enzymes involved in N and C metabolisms were close to QTLs for vegetative development and for GY and its components Gallais and Hirel (2004). These included genes for GS, Sucrose-P synthase, sucrose synthase and invertase. The most notable outcome of these studies were the collocation of as major GY QTL on chromosome 5 with the gene encoding cytosolic GS(gln4 locus) and the correlation between the expression levels of the gln4 alleles and the contributions of the respective QTL alleles at this locus. Other candidate genes encoding enzymes involved in N

metabolism that collocated with NUE QTLs were the two GS genes (gln1 and gln2) on chromosome 1 and the GS gene (gln3) on chromosome 4. Collectively, these results suggest that the increased productivity in maize genotypes under low N may be due to their ability to accumulate nitrate in the leaves during vegetative growth and to efficiently remobilize the stored nitrate during grain filling. NADH–glutamate synthase (NADH–GOGAT) is important in the utilization of N in grain filling, and its activity in developing grains is positively correlated with yield, Obara et al. (2001) used a backcross inbred-line population to detect putative quantitative-trait loci (QTLs) associated with the contents of GS1 and NADH–GOGAT. GS1 is a key enzyme in the mobilization of N from senescing leaves, and its activity in senescing leaves is positively related to yield. Seven chromosomal QTL regions for GS1 and six for NADH–GOGAT were detected. Some of these QTLs were related to N recycling from senescing organs to developing organs. In rice, QTL analysis with DNA markers, based on a well saturated genetic linkage map, has been employed to detect genomic regions associated with several traits exhibiting complex inheritance. In a more mechanistic approach, studying on QTLs related to GS and GOGAT activities, in a back-cross inbred line population, Obara et al. (2001) reported that few of the QTLs associated with the enzyme content were found to co-localize with those associated with nitrogen recycling process from senescing leaves to developing organs. Further they reported coincidence of one QTL for spikelet weight on chromosome 2 associated with the position of the structural gene for GS1. Yamaya et al. (2002) discussed that QTLs affecting the two enzymes are likely to be involved in nitrogen recycling and are involved in grain filling process, both of which are complexly regulated. These results were further confirmed by Obara et al. (2004). In a recent study, Vinod (2007) reported a low N sensitive locus on chromosome 3 which was found to harbour QTLs corresponding to shoot biomass and flag leaf length under low N conditions, which was lying in close proximity of a locus for GS activity. Laza et al. (2006) reported co-localization of QTLs affecting leaf N at mid-growth with that N content in the shoot at maturity on chromosome 2 and chromosome 9. This location on Chromosome 9 was earlier reported to be locus of a QTL affecting Rubisco content in the rice leaf (Ishimaru et al. 2001). Mickelson et al. (2003) reported that the loci on chromosomes 3 and 6 were particularly important in nitrogen storage and remobilization and were directly associated with grain filling.

Micro RNA technology

MicroRNAs (miRNAs) are endogenous approximate 22 nucleotide (nt) small non-coding regulatory RNAs that play important roles in plants by targeting mRNAs for cleavage or translational repression. There have also been reported studies of nitrate response reactions induced by re-supply of nitrate, after using ammonium as the N source or with deprived of N, which identified large numbers of genes in Arabidopsis, including genes that are directly involved in nitrate transport, nitrate reduction and nitrite reduction, ammonium assimilation, and generation of NADPH through the oxidative pentose phosphate pathway (Wang et al. 2000, 2003, 2004; Price et al. 2004; Palenchar et al. 2004; Scheible et al. 2004). However, knowledge is still lacking concerning gene expression and regulation of the plants in response to low N stress as frequently occurring in agricultural field conditions, while such knowledge is essential for formulating strategies for manipulating the genetic architecture of the plants to improve the NUE. It is also essential to identify the signal transduction pathways and the regulatory elements that function to regulate the genes involved in the N uptake and Assimilation pathways. Some miRNA studies revealed that how plant genes respond to low N Stress and approaches to manipulate genes for improving NUE through gene regulation. Analysis of expression profiles after low N stress were identified and described as follows: (1) the genes involved in photosynthesis and energy metabolism were down-regulated rapidly; (2) many of the genes involved in early responses to biotic and abiotic stresses were up-regulated while many other stress responsive genes were down-regulated; (3) regulatory genes including transcription factors and ones involved in signal transduction were both up- and down-regulated; and (4) the genes known to be involved in N uptake and assimilation showed little response to the low N stress.

The challenges for future studies are to characterize the functional roles of the low N stress responsive genes in N metabolisms, including the large number of genes presently with unknown functions.

What is the major role played by the nitrogen during grain filling

Grain filling is an important process that determines the ultimate yield of rice and a large amount of nitrogen is required for grain formation. In general, the amount of N absorbed by the plant during this period is much smaller than the amount of N accumulated in mature grains, and a large part of grain nitrogen is translocated from vegetative organs, especially from leaf blades, where 75 % of leaf N associated with chloroplasts, which are physiologically important in dry matter production through photosynthesis (Dalling 1985). Rice plants require N during the vegetative stage to promote growth and tillering, which determines the potential number of panicles as well as to carbohydrate

accumulation in culms and leaf sheaths during the pre heading stage and in grain during the grain-filling stage by being a component of photosynthesis (Mae 1997). Grain protein concentration is directly related to the N concentration in the grain (Mosse 1990). Leaf nitrogen dynamics also seriously affects grain filling. Leaf nitrogen is essential for maintaining photosynthetic capacity, while its remobilization to the panicle is also required for the formation of sound grains during grain-filling (Mae and Ohira 1981). Nitrogen recycling and grain-filling in rice are complex traits that depend on many factors such as rate of senescence to provide nitrogen source, photosynthesis and respiration to provide carbon skeleton and energy for amino acid biosynthesis and transport, re-utilization of precursors in sink organs for storage of protein and starch, and so on.

The activity of glutamine synthetase is positively correlated with the amount of protein nitrogen in single rice grain at significant levels (Yang et al. 2005). Studies indicated that the role of cytosolic GS in nitrogen remobilization for grain filling in rice and maize (Tabuchi et al. 2005; Martin et al. 2006). Another enzyme NADH–GOGAT is important in the utilization of N in grain filling, and its activity in developing grains is positively correlated with yield. Increasing the N concentration in rice plants requires NADH dependent GOGAT protein without which increase in grain yield was not observed (Hayakawa et al. 1993).

Role of hormones during grain filling

Plant hormones are considered as key regulators to seed development (Davies 1987; Brenner and Cheikh 1995). Plant hormones like cytokinin have been shown to mimic the N-dependent regulation of gene expression in photosynthesis, cell cycling and translational machinery (Sakakibara et al. 1998). Hormones are hinting at a possible role in communicating the availability of nitrogen from roots to leaves (Sugiyama and Sakakibara 2002). Additionally, N sensing and response also seem to be affected by the crosstalk between various plant hormones. Auxin synergistically affects cytokinin activity on cell division and organogenesis (Soni et al. 1995), while ABA antagonizes the cytokinin-mediated nitrogen signalling by negatively regulating cytokinin inducible response regulator genes. Unlike cytokinins, which are positively regulated by nitrate, ABA biosynthesis is down regulated by nitrogen sufficiency. Benzyladenine in combination with nitrate was shown to enhance NR-specific mRNA. Despite these findings, establishing the role of hormones in nitrogen signalling needs further characterization of the complete signalling pathway.

Relation of nitrogen metabolism to the different processes in the rice plant

Photorespiration versus NUE

The photorespiration plays major role in the assimilation of nitrate from soil. Nitrate assimilation could interact with photorespiration through limitations on nitrate reductase (NR) by NADH supplied to the cytosol via several shuttle mechanisms. The photo respiratory carbon and nitrogen cycles are not completely closed and photo respiratory metabolites can be provided as substrates for other processes (Keys et al. 1978). Glycine produced in the photo respiratory pathway can, for example, be used for the synthesis of glutathione (Noctor et al. 1997, 1998, 1999). The GS/GOGAT cycle plays a crucial role in reassimilating the large amount of ammonia released during photorespiration (Somerville and Ogren 1981; Kendall et al. 1983; Kozaki and Takeba 1996). Ammonia can also be assimilated by glutamate dehydrogenase (GDH). The amount of ammonia released during photorespiration is up to tenfold greater than is the amount of primary nitrogen taken up by the plant (Keys et al. 1978). Thus, efficient recapture of this photo respiratory ammonia is essential for survival of the plant. Manipulation of GS1 and NADH–GOGAT genes has been responsible for the improving NUE. GS1 plays major role in transport of N via phloem in senescing leaves. On the other hand, in case of developing leaf blades and spikelets, NADH–GOGAT was implicated in the utilization of glutamine transported from senescing organs. Transgenic over expression and under expression studies to modulate the expression of NADH–GOGAT in alfalfa and rice plants (Yamaya et al. 2002) was reported, through these genes of secondary ammonia assimilation appear to be more viable candidates for improving NUE. Manipulation of GS2 and Fd-GOGAT genes has been responsible for the improvement of NUE. Improvement via manipulation of plastidic GS2 and Fd-GOGAT genes has met with limited success and over expression of GS2 has also been reported in rice (Hoshida et al. 2000). GS2 were shown to have an improved capacity for photorespiration and an increased tolerance to high-intensity light. It was responsible for the improved re-assimilation of ammonia. Studies on barley mutants with reduced Fd-GOGAT revealed changes in various nitrogenous metabolites, decreased leaf protein, Rubisco activity and nitrate content. On the other hand, transgenic with reduced amount of GS2 had a diminished capacity for photorespiration and were photo inhibited more severely by high-intensity light compared to control plant. To improve the NUE, efforts should be made in manipulating the Rubisco activity by using transgenic strategy.

Photosynthesis

Nitrogen plays a crucial role in determining the photosynthetic capacity in both natural and agriculture environments. (Abrol et al. 1999). The availability of nitrogen is significant determinant of both photosynthetic capacity and crop yield. The leaf is the predominate site of nitrogen assimilation in many crop species, and stimulation of nitrogen assimilation by light reveals a close dependence on photosynthesis. Leaf N can affect the size and morphology of chloroplasts. In C_3 plants approximately 60–80 % of leaf nitrogen is invested in the chloroplast (Evans 1989). The photosynthetic nitrogen efficiency is defined as the rate of C assimilation per unit of leaf nitrogen. The amount of leaf nitrogen and Rubisco per unit leaf area is affected by the nitrogen supply. The greater nitrogen supply generally results in increased N and Rubisco per unit leaf area in developing leaves. Photosynthetic capacity per unit of leaf area is thought to be an important factor related to crop productivity Leaf N plays a major role in biomass production through photosynthesis and it is closely correlated with single-leaf photosynthetic rate.

Future perspectives

Various attempts have been made to increase NUE, which can be resulted from many more complex interactions, that NUE compress the N uptake efficiency and N utilization efficiency, The major challenge for improving the NUE include optimization of N supply and demand, maximization of crop N uptake and N assimilation, minimization of N losses & improved crop productivity. Improved NUE can be achieved by fertilizer management that enhances FNUE by crop and also by improving the genetic potential of plants to take up and utilize the applied N. A lot of efforts have been devoted to improve the N fertilizer management that has shown great potential to reduce the N use without affecting the yield substantially under given condition. An enhanced understanding of the mechanisms that determine NUE is required so that the current levels of nitrogen fertilization can be decreased. The primary process involves uptake and transport of nitrate, these transporters may contributed to N utilization efficiency. On the other hand genetic manipulations leading to efficient C–N metabolism, enhanced efficiency of enzymes associated with N metabolism and improved biomass partitioning to grain can contribute to improved NUE. Factors associated with C–N metabolism have a close relationship to crop yields and they are thus important current targets for improving NUE, particularly using QTLs. However, transgenic studies and QTL approaches seem to increasingly suggest that the enzymes of ammonia assimilation are better targets for manipulation to enhance the NUE. Forward and reverse genetic approaches, coupled to classical biochemistry and physiology, will remain essential tools for analysis of the roles of mitochondrial targets in improving NUE. Plant traits that are associated with high grain yield and high NUE should be identified so that breeders are able to use these traits easily as selection criteria in the breeding programme to develop N-efficient varieties without sacrificing rice yield potential.

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