Reactive Nitrogen Inflows and Nitrogen Use Efficiency in Agriculture: An Environment Perspective

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Khalid Rehman Hakeem, Ruby Chandna, Altaf Ahmad, and Muhammad Iqbal

Abstract

Increased use of nitrogenous (N) fertilizer has significantly altered the global N-cycle and produced nitrogenous gases of environmental consequence. While nitrous oxide (N_2O) emissions contribute to global greenhouse gas accumulation and the stratospheric ozone depletion, degradation of groundwater quality by N use in agriculture is fundamentally a nitrate leaching problem. Despite these evident negative environmental impacts, consumption of N fertilizer cannot be reduced in view of the food security for teeming population in the developing countries. Various strategies, from agronomic to genetic engineering, have been tried to tackle this problem. Split application of N, use of slow-release fertilizers, nitrification inhibitors, and the use of organic manures are some agronomic techniques adopted. One of the important goals to reduce N-fertilizer application can be effectively achieved by choosing N-efficient (i.e., which can grow under low N conditions), ensuring their optimum uptake of applied N by application of adequate amounts of fertilizer nutrients in a balanced manner and knowing the molecular mechanisms for their uptake as well as assimilatory pathways. Newer approaches like quantitative trait locus and proteomics could also help us in understanding these processes fully, hence could contribute greatly in enhancing nitrogen use efficiency and reduction of N pollution in the environment.

Keywords

Reactive nitrogen • NUE • QTL • Proteomics • N pollution

K.R. Hakeem (✉) • R. Chandna • A. Ahmad • M. Iqbal
Molecular Ecology Laboratory, Department of Botany,
Faculty of Science, Jamia Hamdard,
New Delhi 110062, India
e-mail: Kur.hakeem@gmail.com

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1 Introduction

Nitrogen (N) represents one of the most important nutrients found in terrestrial ecosystems. It is an important constituent of a number of complex organic molecules viz., proteins, nucleic acids, etc. Atmosphere is the main reservoir of nitrogen (N_2), which stores around one million times more N than contained in all the organisms. Oceans and organic matter in soil are the other major store houses of nitrogen. N is often considered as an important limiting nutrient for plant growth and development, despite its remarkable abundance in the atmosphere. This is the reason for the past half a century, supply of nitrogen through fertilizers has been an influential application for increasing the growth and yield of cultivated plants such as cereals. To meet the increasing demand for food, farmers apply more fertilizers in their bid to increase the agricultural productivity. Fertilizer nitrogen has provided food security particularly to developing nations including India, as the cereal production has kept pace with its ever-increasing population. Today, India occupies the third rank in the world in fertilizer N consumption and second in fertilizer N production (FAI 2008). The consumption of fertilizer nitrogen in India increased from a mere 55,000 metric tons in 1950–1951 to over 14.2 million tons in 2007–2008 and is still increasing (FAI 2008). With the current rate of N fertilization, the requirement of nitrogen will be 22–25 million tons/year in 2020 (FAI 2008). However, it is remarkable that utilization of applied fertilizer nitrogen in field by most cereal crops does not exceed 50% and around 70% of the total nitrogenous fertilizer is applied for rice and wheat cultivation (Abrol et al. 1999). Therefore, with the increase in agricultural food production worldwide in last 50 years, the N fertilization of crop plants has increased more than 20-fold (Shrawat and Good 2008). However, the use of this fertilizer is generally inefficient, as lesser amount of applied N (around 30–40%) is actually utilized by cereal crops, and the major part (60–70%) is lost from the plant–soil system which has caused severe impacts on the ecosystems of the non-agricultural neighboring bacteria, animals, and plants. As a result of leaching, the unused N fertilizer causes impacts like eutrophication of freshwater (London 2005) and marine ecosystems (Beman et al. 2005). In addition, gaseous augmentation of N oxides reacting and affecting with stratospheric ozone and the volatilization of toxic ammonia into the atmosphere (Stulen et al. 1998) has also been linked to unused N fertilizers. The toxic effects of nitrate are due to its endogenous conversion to nitrite and this ion has been implicated in the occurrence of methaemoglobinemia, gastric cancer, and many other diseases (Anjana et al. 2007).

Presently the human population is more than 6.5 million, which is expected to increase around 10 billion by 2025 (Hirel et al. 2007), therefore, the major challenge will be to reach a highly productive agriculture without degrading the quality of our environment. Efficient farming techniques and choosing plant varieties/genotypes that have better nitrogen use efficiency (NUE) could be the tools to tackle this problem. The development of such varieties/genotypes, through conventional plant breeding techniques or by using recombinant DNA technology, will be more proficient with a better understanding the physiological, genetic, and molecular bases of NUE among cereal crops. Therefore, there is an urgent need of a "second green revolution" that does not rely on exhaustive use of inorganic fertilizers rather would aim at improving crop yields in soils by developing varieties with better adaptation to low-fertility soils (Yan et al. 2006). In the present chapter, we have discussed the inflow and effects of reactive N in the environment and then summarized the strategies adopted to develop the crop varieties/genotypes with high NUE.

2 Reactive Nitrogen Inflow

Reactive nitrogen (Nr) is usually referred to all the nitrogen species that are biologically active, photochemically reactive, and radiatively important N compounds in the atmosphere and biosphere of the earth (Galloway 1995). Thus, Nr includes

reduced inorganic forms of N (NH_3, NH_4^+), oxidized inorganic forms (NO_x, HNO_2, N_2O, NO_3^-), and organic compounds (urea, amines, proteins, nucleic acids). There are numerous sources in environment that contribute to Nr and total nitrate content of natural waters, e.g., atmosphere, geological features, anthropogenic sources, atmospheric nitrogen fixation, and soil nitrogen. However, detailed hydro geological investigations conducted have indicated a heterogeneous pattern of nitrate distribution. Soils with low water-holding capacity (sandy soil) and high permeability, movement of pollutants like chloride and nitrate is much quicker than in clayey soil. This is probably the main cause for high nitrates in areas with sandy soil. Vegetables account for more than 70% of the nitrates ingested in the human diet. The remainder of nitrate in a typical diet comes from drinking water (21%), meat and meat products (6%) (Prasad 1999).

The form of added N plays a role in regulating N losses and influencing NUE. Among these forms, NO_3 is the most susceptible to leaching, NH_4 the least, and urea moderately susceptible. Ammonia and urea are more susceptible to volatilization loss of N than fertilizers containing NO_3. Urea is the most widely used N fertilizer in India. The studies showed the importance of selecting ammonium-based N fertilizer early in the season to reduce N leaching due the mobility of urea and nitrate source in irrigated rice and wheat systems (Prasad and Prasad 1996). Nitrate containing fertilizers when applied to rice proved less efficient because nitrate is prone to be lost via denitrification and leaching under submerged soil conditions in normal and alkali soils (Prasad 1998). In saline soils, however, it is beneficial to use NO_3 containing N fertilizers as it compensates the adverse effects of Cl^- and $SO4^{2-}$ on absorption of NO_3 by plants (Choudhary et al. 2003).

Nitrogen losses from soil–plant system. Once inorganic N has appeared in the soil, it can be absorbed by the roots of higher plants or still metabolized by other microorganism during nitrification. This process is carried out by a specialized series of actions in which a few species of microorganisms oxidize NH_4^+ to NO_2 or NO_2^- to NO_3^-. Ammonium ion reacts with excess hydroxyls in soil solutions, which leads to N losses to the atmosphere by NH_3 volatilization (Wood et al. 2000). This represents an important source of N loss in agricultural soils under favorable conditions. Due to extensive use of N fertilizers and nitrogenous wastes, the amount of N available to plants significantly exceeds the N returned to the atmosphere by gaseous losses of N through volatilization and denitrification (Martre et al. 2003). Minimizing drying of surface soil and providing additional source of urease enzyme can minimize NH_3 volatilization. A portion of this excess N is leached out in the soil profile as NO_3^- or carried in runoff waters. These are conductive conditions for N losses in agricultural soils, thus reducing the NUE (Delgado et al. 2001). With transport of N in water ways and neighboring ground-water systems, the N concentration could exceed the levels acceptable for human consumption. Nitrate in soil profile may be leached into groundwater when percolating water moves below the rooting depths of crop and provides leaching potential. Paramasivam et al. (2002) have reported a potential leaching of NO_3^- in arid regions and sandy soils. Losses of N by leaching are affected by local differences in rainfall, water-holding capacity of soil, soil-drainage properties, and rates of mineralization of soil organic N (Delgado et al. 1999). Processes such as adsorption, fixation, immobilization, and microbial assimilation of added NH_4-N in soils are of great importance as they affect NUE and have the corresponding environmental repercussions (Kissel et al. 2004).

In many field situations, more than 60% of applied N is lost due in part to the lack of synchrony of plant N demand with N supply. The remainder of the N is left in the soil, or is lost to other parts of the environment through leaching, runoff, erosion, NH_3 volatilization, and denitrification. The cereal NUEs are 42% in developed and 29% in developing countries (Raun and Johnson 1999). Many ^{15}N studies have reported N fertilizer losses in cereal production from 20 to 50% with higher values in rice than in wheat (Ladha 2005). Prasad (1998) reported that apparent recovery of N applied to wheat varies from 40 to 91%. It has been estimated that rice and wheat N recovery efficiency ranging from 30 to 40% are occurring

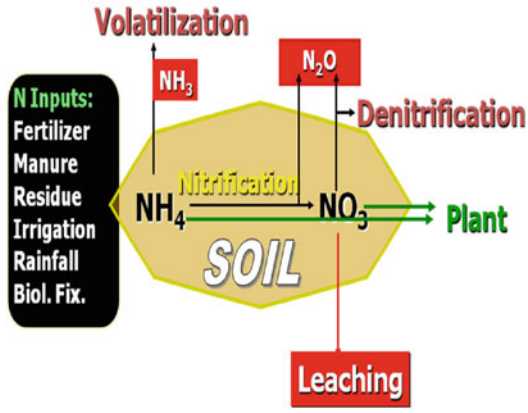

Fig. 10.1 Diagrammatic representation of N inflow and N loss in ecosystem

in irrigated conditions. An N recovery efficiency exceeding 40% is expected to occur in response to improved N management practices. In a rice–wheat cropping system of Punjab, recovery of ^{15}N by the first wheat crop was 30–41%, the soil at wheat harvest retained 19–26%, and the succeeding rice recovered 5.2% of the 120 kg N ha^{-1} applied (Singh and Singh 2001). Total losses of applied N (not recovered from soil–plant system) were about 42% in rice and 33% in wheat grown on a typical sandy loam soil in north-west India.

The main causes of for low N recovery are usually attributed to (1) ammonia volatilization, (2) denitrification, (3) leaching, and (4) runoff and erosion (Fig.10.1). Loss of N via NH_3 volatilization can be substantial from surface-applied urea in both rice and wheat, which can exceed 40%, and generally greater with increasing soil pH, temperature, electrical conductivity, and surface residue (Singh et al. 2003; Choudhary et al. 2003). Water management in rice and wheat fields influences the extent of N losses due to nitrification–denitrification and NH_3 volatilization. Available research results from ideal rice soils suggest that NH_3 volatilization rather than denitrification is an more important gaseous loss mechanism for fertilizer N applied to continuously flooded, puddled rice soils of the tropics. The picture is quite opposite in highly permeable porous soils under rice. There exist two mechanisms in such soils due to which losses due to denitrification assume more importance than NH_3 volatilization losses. Firstly, in porous soils under rice it is difficult to maintain continuous flooding. Rather there occur very frequent alternate aerobic–anaerobic cycles, which lead to very fast formation of nitrate under aerobic conditions and their subsequent denitrification under anaerobic conditions that develop due to application of irrigation (Singh and Singh 2001). Secondly, due to high permeability of coarse textured porous soils, urea as such is rapidly transported to subsoil where even after it is hydrolyzed to NH_4, it is not prone to losses via NH_3 volatilization (Sangwan et al. 2004a). Sangwan et al. (2004a, b) have shown that NH_3 volatilization losses from urea increases with the increase in soil salinity, sodicity, and the rate of N applied. The losses of fertilizer N as NH_3 in rice decreased with increasing floodwater depth and depth of placement (Singh et al. 1995a), and with the application of organic manures (Sihag and Singh 1997). Alkalinity, pH, and NH_3 concentration in flood water control the extent of NH_3 loss from flooded soils (Singh et al. 2003). Sarkar et al. (1991) reported a loss of 15–20% of applied N when urea was broadcast in a wheat field. Prasad (1999) reported a marked reduction in the loss of applied N when the urea was deep placed as compared with surface broadcast on a moist soil. They have reported 13.5% N losses as ammonia after 1 week of urea application under submerged conditions. The high pH or alkalinity resulted in high losses of ammonia by volatilization, which can be nearly 60% of applied N at field capacity. Submergence decreases pH as well as losses to 35% of applied N. The reclamation of sodic soils using gypsum has been found to decrease N losses through ammonia volatilization (Choudhary et al. 2003). The timing of fertilization and irrigation could further influence the losses of urea applied to porous soils. If applied on the wet soil surface following irrigation, as much as 42% of the applied ^{15}N was lost, most likely due to volatilization (Sangwan et al. 2004a). Singh et al. (1995b) showed that application of urea before irrigation increased the NUE by 20% as compared to its surface application after irrigation or broadcast application and surface mixing of urea at field capacity in a clay loam soil.

In nonideal porous soils under rice, there exist every possibility that applied urea N is preferentially lost via denitrification rather than NH_3 volatilization. Direct measurement of denitrification losses made by Aulakh et al. (2001) showed that denitrification is a significant N loss process under wetland rice amounting to 33% of the applied N. In excessive N fertilizer application (i.e., at rates in excess of that needed for maximum yield in cereal crops), NO_3 leaching can be significant, particularly from the coarse-textured soils. Residual N is then available in soil profile for potential leaching. High levels of NO_3-N in the region's groundwater have been reported by Singh et al. (1995b). There is not much information available on leaching losses of N. In a pot culture study, the leaching loss was 11.5% of the applied urea N and was reduced to 8.7% when urea was coated with neem cake (Prasad and Prasad 1996). In a field study at Pantnagar on a silty clay loam soil, 12% of the applied N was lost by leaching and these losses were reduced to 8% when urea was blended with neem cake (Singh et al. 1995b).

3 Nitrogen Removal by Crops

From the human nutrition point of view, rice and wheat are the most important cereals and their production in north-west India in rice–wheat cropping system, which covers about 10 million hectares, is the backbone of the India's food security (Prasad 2005). Rice–wheat cropping system produces 5–14 ton/ha/year grain and this depends heavily on nitrogen fertilization which ranges from 100 to 150 kg N/ha/crop or even more, especially in rice. From the animal nutrition point of view, maize, sorghum, and pearl millet stovers which contain 27–51% of nitrogen harvested by the crop in stover are more important both for milch as well as draught cattle. On the contrary, rice and wheat straw is low in nitrogen content and is a poor protein source. Nevertheless, they meet majority calorie requirements of the cattle. Also most sorghum and pearl millet is grown in rainfed areas where nitrogen application rates are low and even response to N application is low. Nitrogen removal per metric ton as well as its percentage in grain in pulses depends very much upon the plant stature and its vegetative growth. For example, Prasad (2004) reported a removal of 50.6 kg/ton in chickpea and 92.1 kg/ton in pigeon pea; these are the two major pulse crops in India. Most of this nitrogen is obtained by N fixation by *Rhizobia* as very little fertilizer N is applied to pulses. Again depending upon the plant stature and vegetative growth, 63.3% of total N removed by chickpea was contained in its grain, while the values for pigeon pea, a tall and heavily fertilized plant, was 31.6%. The protein-rich pulse foliage is widely used for enriching rice or wheat straw fed to cattle. Before the mechanization of Indian agriculture which is even now limited mostly to north-western India, draught animals were the major source of farm power and the Indian agriculture provided a characteristic "humans–animals–crops" ecosystem where man survived on the grains and the animals on the straw/stover. Taking an average N contribution by grain legumes at 30 kg N/ha, about 0.66 million metric tons of N is annually added to soil on 22 million hectares occupied by them. Another 0.34 million metric tons N may be added by leguminous trees and plants in forests and grasslands, and by leguminous oilseed crops such as groundnut. Thus the N contribution of legumes in Indian soils can be roughly estimated at least at 1 million metric tons, it is likely to be much more. In addition, some N is added by rains and use of N-fixing biofertilizer such as *Azotobacter*, *Azospirillum*, *Acetobacter*, Blue-green algae, and *Azolla*.

4 Concept of NUE

NUE at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity (Raghuram et al. 2006). As a concept, NUE includes N uptake, utilization, or acquisition efficiency, expressed as a ratio of the total plant N, grain N, biomass yield, grain yield (output) and total N, soil N, or N-fertilizer applied (input) (Pathak et al. 2008). NUE is quantified based on apparent nitrogen recovery using physiological and agronomic parameters. Agronomic

efficiency is an integrative index of total economic outputs relative to the available soil N (native and applied). Apparent nitrogen recovery is related to the efficiency of N uptake; physiological NUE deals with N utilization to produce grain or total plant dry matter. NUE in the context of photosynthesis is called as photosynthetic nitrogen use efficiency (PNUE), which is determined by the rate of carbon assimilation per unit leaf nitrogen (Kumar et al. 2002). The most suitable way to estimate NUE depends on the crop, its harvest product, and the processes involved in it.

5 Strategies for Minimizing N Pollution in Agriculture

Various strategies were adopted to minimize the N loss from the agricultural fields. Split application of N, use of slow-release fertilizers, nitrification inhibitors (NIs), and the use of organic manures are some agronomic techniques used. Bulk of the fertilizer nitrogen in India is broadcast on surface and both surface runoff (on sloppy lands) and ammonia volatilization lead to N losses. This can be easily overcome by deep placement of N a few centimeters below soil surface. For example, Sarkar (2005) showed that in wheat surface broadcast application of urea as band or top dressing caused 15–20% loss of N due to agriculture volatilization. Surface broadcast application followed by its mixing with top soil reduced the volatilization loss to 10%, while side band placement of urea reduced it further to only 5%. Thus the farmers need to be told about the advantage of incorporation in surface soil or if possible its placement using a *ferti-drill* or a pore in upland crops. Split application is a well-established technique for increasing NUE. In wheat and maize, studies with ^{15}N showed that application of 40 kg N/ha as basal followed by 60 kg N/ha at crown root initiation (CRI) gave significantly higher yield than all basal application and other split application combinations (Sachdev et al. 2000; Narang et al. 2000). Havangi and Hegde (1983) showed in pearl millet also two or three split applications were found to be better than a single application. In rice, two split applications are recommended for short and medium duration varieties, while three split applications are recommended for long duration varieties (Prasad 1999). Another way is NIs, these are a group of chemicals that are toxic to *Nitrosomonas* sp. and *Nitrosomonas* sp. involved in the conversion of NH_4 to NO_2^- as well as to *Nitrobacter* sp. involved in the conversion of NO_2 to NO_3 and therefore, inhibits nitrification, which reduces losses due to leaching and denitrification. The most widely tested NIs are 2-chloro-6-trichloromethyl pyridine (N-serve), 2 amino-4-chloro-6 methyl pyrimidine (AM), dicyandiamide (DCD), and sulfathiazole (ST) (Prasad and Power 1995). Research on the use of NIs for reducing N losses and increasing NUE from the soil was initiated in India by Prasad (1999) at the Indian Agricultural Research Institute (IARI), New Delhi, with a field experiment on rice. Treatment of ammonium sulfate with N-serve significantly increased rice yield and nitrogen uptake by the rice crop. Prasad (2005) showed from a laboratory experiment that N losses due to denitrification could be considerably reduced by treating ammonium sulfate with NIs N-serve and AM. Prasad and Prasad (1996) showed through field experiments that treatment of urea with NIs, N-serve, and AM significantly increased rice yield and N uptake. Das et al. (2004) showed the effect of N-serve and AM on nitrification under field capacity moisture (upland) and water-logged (low-land paddy) conditions at New Delhi. Both the NIs were effective in retarding nitrification. The nitrification rate (nitrates expressed as percentage of total mineral N) after 40 days of incubation was 78% with N-serve at 2 ppm and 76% with AM at 10 ppm (mg/kg) as against 100% with untreated urea. Slow-release N fertilizers (SRFs) were developed with an aim to slowdown the dissolution of applied N so that most of it is taken up by crop plants rather than be subjected to N-loss mechanisms. There are two kinds of SRFs, namely, coated fertilizers and inherently slow dissolution rate materials. The examples of coated SRFs are sulfur-coated urea (SCU) (developed by TVA, USA), lac-coated urea (developed by Indian Lac Research Institute), polymer-coated urea, and to some extent neem cake-coated urea. The other

kind of slow-release fertilizers are generally urea–aldehyde condensates, e.g., urea-form (urea and formaldehyde products developed in USA), isobutylidene diurea (IBDU, urea and isobutyraldehyde product developed in Japan and USA), and CD-urea (urea and crotonaldehyde product developed in Germany) (Prasad 2005). After 20 days of incubation under field capacity conditions, the mineral N (NH_4^+ NO_3^-) in soil was 67, 43, 31, and 27 ppm (mg/kg soil) with urea, oxamide, IBDU, and SCU, respectively. As would be expected under submerged conditions, NO_3-N was not detected and the NH_4^+-N content in soil after 20 days of incubation was 67, 61, 46, and 15 ppm with urea, oxamide, IBDU, and SCU, respectively. Thus, of the three SRFs oxamide released, the N the fastest and SCU the slowest.

6 Physiological and Molecular Aspects for Improving NUE

NUE at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity. In terms of agriculture, it is the optimal utilization of nitrogenous manures or fertilizers for plant growth, yield, and protein content, as atmospheric nitrogen gas is not utilized by higher plants, except symbiotic legumes. The inherent efficiency of the plant to utilize available N for higher productivity needs to be tackled biologically (Abrol et al. 1999; Abdin et al. 2005). This includes uptake, assimilation, and redistribution of nitrogen within the cell and balance storage and current use at the cellular and whole plant level. Moreover, since N demand and its actual availability tend to vary in time, space, and environmental conditions, the regulation of plant nitrogen metabolism must be responsive to nutritional, metabolic, and environmental cues.

6.1 Regulation of Nitrate Uptake

Plants have evolved an active, regulated, and multiphasic transport system making their NO_3^- uptake scheme efficient enough to transport sufficient NO_3^- to satisfy total nitrogen demand of the plant in face of varying external NO_3^- concentrations. Plants can also take up other forms of nitrogen, such as amino acids and ammonium ions. Root NH_4^+ uptake is carried out by both high-affinity and low-affinity NH_4^+ transporters that are encoded by a multigene family (Glass et al. 2002). However, nitrate is the most abundant form of nitrogen available to the plant roots in aerated soils. Nitrate influx is an active process driven by the H^+ gradient and can work against an electrochemical potential gradient (Vidmar et al. 2000). The uptake involves high- and low-affinity transport systems, also known as HATS and LATS, respectively (Forde 2000). One of the high-affinity systems is strongly induced in presence of NO_3^- and is known as inducible high-affinity transport system (or iHATS), while the second high-affinity system (the cHATS) and LATS are constitutively expressed (Aslam et al. 1993; Glass and Siddiqi 1995; Forde 2002). The Km values of iHATS, cHATS, and LATS for nitrate are in the ranges of 13–79 μM, 6–20 μM, and >1 mM, respectively.

The iHATS is a multicomponent system encoded partly by genes of the NRT2 family or nitrate–nitrite porter family of transporters. Recently, two dual affinity transporters have been identified in *Arabidopsis*, AtKUP1, and AtNRT1.1, of which the latter is induced as HATS by phosphorylation at threonine residue 101. This family of transporters is recognized as being exceptional in both the variety of different substrates which its members can mobilize (oligopeptides, amino acids, NO_3^-, chlorate) and in the ability of individual transporters to handle substrates of very different sizes and charges. Nitrate acts as a regulator for its own uptake, a specific property which is not seen in other ion transport systems such as phosphate, sulfate, etc. On exposure of the cells to external NO_3^-, the uptake capacity increases after a lag period of 0.5–1.5 h and reaches a new steady state after 4–6 h. Use of RNA and protein synthesis inhibitors provided early evidence that induction of the iHATS involves gene expression and the synthesis of new transporter protein (Aslam et al. 1993). The evidence that the inducer of iHATS is indeed

nitrate ion and not its downstream metabolite came from NR-deficient mutants of *Arabidopsis* and *N. plumbaginifolia* (Krapp et al. 1998; Lejay et al. 1999). Studies in the last decade have shown that enhancing the uptake of N by overexpressing transporters may not necessarily improve NUE. For example, transgenic overexpression of a CHL1 cDNA (representing the constitutive HATS) driven by the cauliflower mosaic virus 35S promoter in a chl1 mutant, recovered the phenotype for the constitutive phase but not for the induced phase (Liu et al. 2003). Similarly, the NO_3^- contents in transgenic tobacco plants overexpressing the NpNRT2.1 gene (encoding HATS), were remarkably similar to their wild-type levels, despite an increase in the NO_3^- influx. These findings indicate that genetic manipulation of nitrate uptake may not necessarily lead to associated improvement in nitrate retention, utilization, or NUE, though it remains to be seen whether different plants respond differently to the overexpression of different transporters (Pathak et al. 2008). Light as an important abiotic factor is known to enhance NO_3^- uptake in a number of plant species and diurnal changes in nitrate uptake have been observed (Anjana et al. 2007). These changes seem to be linked to the imbalance between nitrate uptake and reduction due to the light regime and as well as to the rate of photosynthesis in shoots. Reduced nitrate uptake during darkness could be reversed by exogenous supply of sugars (Raghuram and Sopory 1995). Recent evidence on the upregulation of AtNRT1.1 gene expression by auxin (Li et al. 2007) suggests that nitrate transporters may also be regulated by hormones.

6.2 Physiology of Nitrate Reduction in Crops

A portion of the nitrate taken up is utilized/stored in the root cells, while the rest is transported to other parts of the plant. Due to the abundant availability of photosynthetic reductants, leaf mesophyll cells are the main sites of nitrate reduction. This is initiated by the NAD/NADP-dependent NR enzyme, which converts nitrate to nitrite by catalytic reaction in the cytosol. Nitrite is transported into the chloroplast, where it is further reduced into ammonium ion by a ferredoxin-dependent NiR. Being the first, irreversible, and often rate-determining step of the N-assimilatory pathway, nitrate reduction has been a favorite step for physiological and biochemical approaches to optimize fertilizer N use.

6.3 Developing Plants with Transport Gene Systems Using Genetic Engineering Tools

Plants receive N from the soil in the form of nitrate or ammonia, however, some may utilize amino acid as an important sources of N. Specific transporters located in the root cell membrane are responsible for uptake of N from the soil. Subsequent to its uptake, NO_3^- is assimilated via a series of enzymatic steps. Nitrate reductase being the first enzyme in nitrate assimilatory pathway and thus an important gene for manipulation. NR activity in leaf blades, express either as seasonal average or converted into seasonal input of reduced N, has been related to total reduced N, grain N, and grain yield of cereals. The pattern of nitrate assimilation from different plant parts, viz. the main shoot of wheat, developing ear of wheat plants grown at different soil N levels, and in the leaf blades at different stages of growth has revealed a direct positive correlation between increasing NR activity and increasing rates of nitrogenous fertilization. Most plant tissues have the capacity to assimilate nitrate, though their NR activity varies widely. Several endogenous as well as exogenous factors have been found to influence the expression of NR genes at both translational as well as transcriptional levels.

Andrews et al. (2004) reported that overexpression of either the NR or the NiR gene often affects N uptake by increasing mRNA levels in the plants. However, this does not seem to increase the growth or yield of plants, irrespective of N source. It is believed to be due, in part, to the complex regulation of both NR and the

pathway as a whole. Transcriptional regulation of NR has only minor influence on the levels of free amino acids, ammonium, and nitrate, whereas posttranslational regulation of NR strongly affects these compounds (Lea et al. 2006). The light/dark conditions affect NR activity; heterotrophic nitrate assimilation in darkness is closely linked to the oxidative pentose phosphate pathway and the supply of glucose-6-phosphate. Under photoautotrophic conditions, glucose-6-phosphate dehydrogenase is inhibited by reduction with thioredoxin in light, thus replacing the heterotrophic dark nitrate assimilatory pathway with regulatory reactions functioning in light. These studies as well as bioenergetic calculations have indicated that both yield and N harvest or protein can be increased to some extent with adequate nitrogen supply by altered management practices, thus improving the fertilizer NUE. Genotypic differences in the NR levels also provide insight in the relation of varietal differences in N assimilation. The genotypic differences in NR expression have been reported in corn, wheat, sorghum, and barley. In sorghum, a positive relationship between decline in the height of the plant and enhancement of NR activity was observed, though no such relationship was evident in tall and dwarf cultivars of wheat, *T. aestivum*. Wheat genotypes revealed over twofold variability in NR activity, which supports genetic findings that the enzyme level is highly heritable, its differences are reflected in N harvest and that hybrids could be bred with predictable NR levels by selecting parents appropriately. In the high NR genotypes, higher levels of NR activity were found under low N levels, often with significantly higher N concentration in the grains. They also have sustained activity at later stages of growth, such as flag leaf emergence and anthesis. The reasons for these genetic differences are not fully understood, except that the regulation operated at the level of gene expression and that low levels of NADH might limit NR activity in low NR genotypes. Similarly, overexpressing NiR genes in *Arabidopsis* and tobacco resulted in increased NiR transcript levels but decreased enzyme activity levels, which were attributed to posttranslational modifications.

6.4 Glutamine Synthetase and Glutamate Synthase (GOGAT) Gene Systems

Glutamine synthetase (GS) catalyzes the critical incorporation of inorganic ammonium into glutamine. In higher plants, it is represented by two groups of protein – the cystolic and plastidic forms (Miffin and Habash 2002). Cystosolic GS (GS1) is known to be encoded by a complex multigene family, whereas plastidic GS (GS2) is encoded by a single gene. Glutamate synthase (Glutamine (amide): 2-oxoglutarate aminotransferase, GOGAT) catalyses the reductive transfer of the amide group of glutamine (produced by GS) to 2-oxoglutarate (α-keto glutarate) to form two glutamate molecules (Lea and Ireland 1999). GS/GOGAT pathway is of crucial importance since the glutamine and glutamate produced are donors of amino groups for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Lea and Ireland 1999; Hirel and Lea 2001). A direct correlation was reported between an enhanced GS activity in transgenic plants in some cases, which is depicted by an increase in biomass or yield by transforming novel GS1 construct. Similarly, Kozaki and Takeba (1996) constructed transgenic tobacco plants enriched or reduced in plastidic glutamine synthetase (GS2, a key enzyme in photorespiration). Ectopic expression of GS1 has been shown to alter plant growth (Fuentes et al. 2001; Oliveira et al. 2002) and the overexpression of GS1 in transgenic plants could cause the enhancement of photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (Fuentes et al. 2001). The overexpression of soybean cytosolic GS1 in the shoots of *Lotus corniculatus* was reported to accelerate plant development, leading to early senescence and premature flowering, particularly when plants were grown under conditions of high ammonium (Vincentz et al. 1993). Man et al. (2005) provided additional empirical evidence for enhanced nitrogen-assimilation efficiency in GS1 transgenic lines. However, differences in the degree of ectopic GS1 expression have been reported (Fuentes et al. 2001) and attributed to

positional effects, effectiveness of chimeric constructs, or differences in growth conditions. This may be due to lack of correlation between the enhanced expression of GS1 and concomitant growth (Vincentz et al. 1993; Ortega et al. 2001). A significant increase in leaf area, plant area, plant height, and dry weight has been recorded in poplar trees transformed with conifer gs1a gene. Striking differences were observed at low nitrate concentration. Furthermore, higher rates at ^{15}N incorporation into the transgenic plants demonstrate that the transformed plants have increased NUE (Man et al. 2005). Transgenic overexpression and antisense technology have been employed recently to modulate the expression of NADH-GOGAT in alfalfa and rice plants (Yamaya et al. 2002). The studies on transgenic rice plants expressing antisense RNA for either GS1 or NADH-GOGAT point towards the possible involvement of GS1 in the export of N via phloem in senescing leaves. On the other hand, in case of developing leaf blades and spikelets, NADH-GOGAT was implicated in the utilization of glutamine transported from senescing organs (Yamaya 2003). While these genes appear to be good candidates for improving NUE in the short run, the degree of improvement may vary with the crop and cropping conditions. Therefore, the utility of transgenic overexpression of N-assimilatory genes for major improvements of NUE remains uncertain, though the possibility that different crops respond differently cannot be ruled out yet.

6.5 Other Gene Systems Regulating N Metabolism and Their Manipulation

Enzymes like asparagine synthetase (AS), that catalyzes the formation of asparagine (Asn) and glutamate from glutamine (Gln) and aspartate. In higher plants, AS is encoded by a small gene family (Lam et al. 1998). Together with GS, AS is believed to play a crucial role in primary N metabolism. The observation made by Carvalho et al. (2003) that the levels of AS transcripts and polypeptides in the transgenic nodules of *Medicago truncatula* increase when GS is reduced suggests that AS can compensate for the reduced GS ammonium assimilatory activity. However, it was also demonstrated that GS activity is essential for maintaining the higher level of AS. Thus, GS is required to synthesize enough Gln to support Asp biosynthesis via NADH-GOGAT and AspAT (Carvalho et al. 2003). A reduction in GS activity in transgenic *Lotus japonicas* is also correlated with an increase in Asn content (Harrison et al. 2007), supporting the hypothesis that when GS becomes limiting, AS may be important in controlling the flux of reduced N into plants. With the aim of increasing Asn production in plants and to study the role of AS, several researches have attempted to clone AS genes and to examine the corresponding gene expression in plants. Lam et al. (2009) showed overexpression of the ASN1 gene in *Arabidopsis* and demonstrated that the transgenic plants have enhanced soluble seed protein content, enhanced total protein content, and better growth on N-limiting medium. *Arabidopsis* plants overexpressing the ASN2 gene accumulate less endogenous ammonium than wild-type plants when grown on medium containing 50-mM ammonium. This study indicates that signaling processes may provide an attractive route for metabolic engineering. In comparison to GS/GOGAT enzymes, the physiological role of glutamate dehydrogenase (GDH) has been less clear (Dubois et al. 2003). In an attempt to investigate the role of GDH by expressing a bacterial gdhA gene from *E. coli* in tobacco, Ameziane et al. (2000) found that biomass production is consistently increased in gdhA transgenics, regardless of whether they are grown under controlled conditions or in the field.

7 Signaling and Regulation of Nitrogen Metabolism

It is a well-known concept in signal transduction that whenever multiple genes are subject to transcriptional regulation by a common signal, it is mediated through a regulatory sequence that exists in all the genes that respond to the signal.

These signature sequences, commonly known as response elements, are identified by mutations that abolish their function, and their conserved nature as revealed by homology comparisons. Early experiments in transgenic Nicotiana plants using GUS gene fused to NR and NiR promoter sequences clearly demonstrated for the first time that nitrate induction of gene expression requires some sequence(s) associated with the NR and NiR promoters (Raghuram et al. 2006). Subsequent studies in transgenic tobacco incorporating the 5′ flanking regions of the nitrate reductase genes NR1 and NR2 (designated NP1 and NP2), in case of *Arabidopsis thaliana*, demonstrated that 238 and 330 bp of NP1and NP2, respectively, are sufficient for nitrate-dependent transcription (Lin and Demain 2006). These nitrate-responsive elements (NREs) are composed of several copies of a core A[G/C]TCA sequence motif preceded by an ~7-bp AT-rich sequence present in the 5′ flanking regions of nitrate reductase (NR1 and NR2) genes. This particular sequence motif was also found to be very well conserved in the 5′ flanking regions of NR and NiR genes from eight other plants. Sarkar (2003) compared the flanking sequences of all available plant nitrate-responsive genes and found that the NRE core sequence (A[C/G]TCA) was present in multiple copies on both strands in all the known nitrate-responsive genes in many dicots, monocots, and cyanobacteria. Though most of the NREs examined contained both the core sequence and a proceeding AT-rich sequence, there were some cases which had GC-rich regions or did not reveal any AT/GC bias. A more detailed bioinformatic analysis of the entire *Arabidopsis* genome in our lab revealed that the proposed NREs are randomly distributed, with no difference between nitrate-responsive genes and the presumably nonresponsive genes and intergenic regions in the rest of the genome (Raghuram et al. 2006). These findings raise doubts on the validity of the proposed NRE as comprising of (A[C/G]TCA) elements preceded by AT-rich sequence. Further work in this area will need a combination of bioinformatic and experimental approaches to redefine the NREs that mediate the expression of all nitrate-responsive genes in all plants. The discovery of NREs is important, as it provides an end point for nitrate signal transduction.

8 QTL Approach to NUE

NUE in plants is a complex quantitative trait that depends on a number of internal and external factors in addition to soil nitrogen availability, such as photosynthetic carbon fixation to provide precursors required for amino acid biosynthesis or respiration to provide energy. Although this trait is controlled by a large number of loci acting individually or together, depending on nutritional, environmental, and plant developmental conditions, it is possible to find enough phenotypic and genotypic variability to partially understand the genetic basis of NUE and thus identify some of the key components of yield for marker-assisted breeding. Thus the development of molecular markers has facilitated the evaluation of the inheritance of NUE using specific quantitative trait loci (QTLs) that could be identified. In maize, Hirel et al. (2001) and Masclaux et al. (2001) analyzed recombinant inbred lines for physiological traits such as nitrate content, NR and GS activities. When the variation in these traits and yield components were compared, it was found that there was a positive correlation between nitrate content, GS activity, and yield. When the loci that govern quantitative traits were determined on the map of the maize genome, the positions of QTLs for yield components and the locations of the genes for cytosolic GS (GS1) coincided. In maize, studies on different genotypes or populations of recombinant inbred lines based on NUE components, chromosomal regions, and putative candidate genes have hinted at some factors that might control yield and its components directly or indirectly, when the amount of N fertilizers provided to the plant is varied (Hirel et al. 2007).

Similar results were obtained in rice by Obara et al. (2001), confirming the earlier indications that the GS1 enzymatic activity in the leaf cytosol is one of the major steps controlling organic matter reallocation from source to sink organs

during senescence and for grain-filling in cereals. Previous studies have already demonstrated that when GS1 is over expressed in *Lotus*, nitrogen remobilization was prematurely induced leading to early senescence of the plant (Vincentz et al. 1993). In rice (Yamaya et al. 2002) and wheat (Habash et al. 2001), preliminary investigations with enhanced or decreased GS1 activity indicated that grain yield and grain nitrogen content were modified. In other species such as tobacco (Migge et al. 2000) or poplar (Gallardo et al. 1999), overexpression of GS2 or GS1 significantly increased plant biomass production at early stages of plant development. With these experiments, two out of seven QTLs for GS1 protein content were detected in different regions from other physiological and biological traits. In maize, QTLs for the activities of acid-soluble invertase and sucrose-phosphate-synthase were detected in the regions where each structural gene was mapped (Ishimaru et al. 2001).

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

9 Proteomics Approach to NUE

The ability of crop plants to cope up with the variety of environmental stresses depends upon a number of changes in their proteins, which may be up- and downregulated as a result of altered gene expression. Under a stressful condition, the modifications in the expression levels of these proteins could provide us valuable information about the nature of stress factor as well as the physiological and molecular state of a biological system. Hence, provides us some clues to understand the nature of defensive mechanism and adaptability, besides stress monitoring in these biological systems.

Proteomic-based technologies have been recently applied for the systematic analysis of the induced gene products in a number of plant species subjugated to a wide range of abiotic and biotic challenges. Proteome analysis is becoming a powerful tool in the functional characterization of plants. Due to the availability of vast nucleotide sequence information and based on the progress achieved in sensitive and rapid protein identification by mass spectrometry, proteome approaches open up new perspectives to analyze the complex functions of model plants and crop species at different levels. Improvements in proteomic technology regarding protein separation and detection, as well as mass spectrometry-based protein identification, have an increasing impact on the study of plant responses to salinity stress (Parker et al. 2006; Qureshi et al. 2007; Caruso et al. 2008). Proteomics has provided valuable information in various fields of plant biology. Construction of several plant protein databases is in progress for *Arabidopsis*, rice, maize, and some trees, where different genetic, cellular, and physiological information is available, such as expression in various organs or tissues, response to treatments, cellular localization, and genetic bases (Thiellement et al. 1999). Recent advances in MS techniques will facilitate protein identification so that in the future this will not be a limiting factor in the interpretation of variations detected on 2D gels. By providing information on affected and unaffected proteins, large-scale protein identification will simplify determining the consequences of mutations, plant transformation, or natural polymorphism for plant metabolism, as well as interpreting the effects of protein changes on development, or in response to biotic and abiotic stress. Studies in *Saccharomyces cerevisiae*, for which hundreds of proteins have been identified, show the power of the proteomic approach in the study of the regulation of metabolic pathways. Schiltz et al. (2005) studied that during seed filling, the accumulation of proteins in the seeds relies on the nitrogen supply from the mother plant, and a proteomic approach was used to study the mobilization of proteins from the leaves to the filling seeds in pea. Two contrasting N-responsive wheat varieties have differential expressions of root as well as leaf

proteins when grown under controlled conditions at different N levels (Bahrman et al. 2004, 2005). These proteins were grouped into two categories, one involved in carbon metabolism and the other associated with other pathways and functions like thiol-specific antioxidant proteins, etc. This study revealed that levels of gene expressions are modified with the varying levels of nitrate supply, even if only a few polypeptides appear, disappear, or change. Sarry et al. (2006) have demonstrated the protein level changes associated with nitrogen and sulfur metabolism, and their interaction. With the help of high throughput proteomic tools, they were able to detect various enzymes including ATP sulfurylase, sulfite reductase, cysteine synthase, S adenosylmethionine synthase, glutamine synthase, aspartate aminotransferase, GDH, etc., involved directly or indirectly in S and N metabolism. Recently a study for the detection of low nitrogen-responsive proteins in cultivated rice species was done by Kim et al. (2009). Studies at constructing 2-D gel reference map for use in comparative proteomics among cultivars for N-responsive proteins might provide an insight for precise identification of potential molecular protein markers to assist the breeders for screening N-efficient genotypes and help in understanding how crop adapts to low N availability. Correlations between the level of expression and NUE might bring information on the possible role of the genes involved in nitrogen metabolism.

10 Conclusion and Future Perspectives

Present review provides an overview of plant nutriomics, which is still at a conceptual stage. Although considerable efforts are in progress with the aim at enhancing plant nutrient efficiency through molecular and genetic approaches. We have focused here largely on nitrogen with which we have been working on along molecular biology lines. Crop response to N and NUE is very low in developing countries including India. Use of NIs and slow-release nitrogen fertilizers and efficient crop and fertilizer management can significantly increase NUE. It is clearly evident that optimizing the plants, NUE goes beyond the primary process of uptake and reduction of nitrate, involving quality of events, including metabolite partitioning, secondary remobilization, C–N interactions, as well as signaling pathways and regulatory controls outside the metabolic cascades. Despite the various attempts to manipulate each of the above steps in some plant or the other, we are far from finding a universal switch that controls NUE in all plants. However, transgenic studies, QTL, and proteomics approaches seem to increasingly suggest that the enzymes of secondary ammonia remobilization are better targets for manipulation, followed by regulatory processes that control N–C flux, rather than the individual genes/enzymes of primary nitrate assimilation. There is an urgent need of large-scale, co-ordinated research on plant nutriomics, involving sincere efforts from both national and international researchers to develop the nutrient-efficient, high-yielding, and stress-tolerant genotypes/varieties that will contribute to both environmental safety as well as food security worldwide.

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