Nitrogen Regulation and Signalling in Plants

6

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Abstract

Nitrogen (N) is an essentially critical element which is involved in signalling and can affect plant growth and development. The plants have evolved different strategies involving short- and long-ranged signalling pathways to cope with the changes in N regulations in the soil. These pathways work at cell and plant level for coordination of N metabolism, growth, and development in accordance with the external and the internal N status. Currently, identification and characterization of local and systemic signalling has been emphasized, but information about integrating coordination and organization of N response of the plants is still lacking. Tracing out the full N pathway could help us to devise and produce high N-efficient genotypes and increase Nitrogen use efficiency (NUE). In this chapter, we discuss the physiological as well as molecular means to understand the mechanisms involved in local and systemic nitrogen responses and how these responses are coordinated.

Keywords

Nitrogen • Signal molecule • N-metabolic pathway • N regulation • NUE

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6.1 Introduction

Nitrogen (N) is a macronutrient required for the normal plant growth and development. Nitrogen inputs actually define the yield and productivity of crops (Hakeem et al. 2011, 2012a). It has been estimated that the use of nitrogenous fertilizers has increased sevenfold which has doubled food production during the last century. Globally, demand for nitrogenous fertilizer has increased tremendously during the last century, and it will continue to rise at the rate of 1 % per annum (FAO 2007). Nitrogen use efficiency (NUE), the grain yield per unit of nitrogen available from the soil, including the residual N present in the soil and the fertilizers (Moll et al. 1982), under field conditions is about 30–40 %, and the remaining N is lost into the environment particularly in the intensive agriculture. The remaining 60–70 % of unused nitrogen (N) creates severe environmental as well as health problems. The unused nitrate leaches to ground water and causes several health effects, such as methemoglobinemia and gastric cancer, among others (Hakeem et al. 2011). Emission of unused N, from agricultural fields, in the form of NO_x contributes to global warming and other harmful effects to the environment (Hakeem et al. 2012b). The Delhi Declaration of the International Nitrogen Initiative (http://initrogen.org/index.php/publications/delhi-declaration) showed the concern about rising N losses from agriculture and insisted for devising a comprehensive strategy for efficient N management in agricultural and industrial sectors. Nitrogen use efficiency can be enhanced by optimizing interaction between fertilizer soil and water.

It is evident that plants use different strategies in response to the variations in N supply (e.g., localized NO_3^- in the soil, uniform high NO_3^-, and N starvation). The sensing and signalling for deprivation of nutrients are not well characterized particularly for N deprivation. Signal transduction cascade involved in phosphorus-deprivation response has been identified which is controlled by sumoylation. Two microRNAs control the gene expression under P and S deficiency. Understanding of short-term and longer-term responses is necessary for progression of signalling events under limited internal and external supply of nutritions.

In this chapter we tried to gather the latest information about the nitrogen metabolism and its regulation, also to trace out its signalling pathway.

6.2 Nitrogen: An Important Plant Nutrient

Mineral elements play an important role in growth and development of plants. There are 17 essential elements, of which six are required in large quantities. During the past half century, supply of mineral nutrients, especially N, P, and K, through fertilizers has been considered as an important input in agriculture sector for crop production. Nitrogen occupies a unique position among these mineral elements since it is an important component of different organic compounds and structures in plant cells. Nitrogen has a role in energy transfer by being part of compounds, like ATP (adenosine triphosphate) which is responsible for conservation and use of energy during metabolism by the cells. It is a component of nucleic acids (DNA, RNA), proteins, vitamins, and hormones. Nitrogen supply determines the nature and diversity of plants, the population of grazing animals and their predators, plant productivity, and the cycling of carbon and soil minerals in many terrestrial and aquatic systems (CFAITC 2008). In agriculture, application of nitrogen influences yield and quality of crops. Thus, it has greatly influence food security, economic development, and environmental quality. Nitrogen deficiencies lead to chlorosis and reduced photosynthesis which ultimately result in lower yields. The deficiency of N causes a significant reduction in the levels of phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase, and Rubisco and a concomitant decrease in the level of their respective mRNAs in developing maize leaves (Khamis et al. 1990). Proteomics studies also revealed that proteins like glutamine synthetase, phytoene synthase,

enoyl-CoA hydratase, 2-cys peroxiredoxin BAS 1, and enolase-2 protein were involved in conferring NUE to the N-efficient rice cultivars/genotypes (Hakeem et al. 2012a).

6.3 Nitrogen in Plants

Nitrogen exists in the form of molecular N_2, volatile ammonia, oxides of N, NO_3^-, NH_4^+, and organic N (urea and amino acids). However, NO_3^- and ammonium are the preferred source of N by most plant species (Leleu and Vuylsteker 2004; Hakeem et al. 2011). Under aerobic soil conditions, although ammonium is also present but nitrate is the dominant N form. It is the most abundant which is under cultivation of annual crops. Generally, field crops prefer a mixture of NH_4^+ and NO_3^-, but proportionally uptake of ammonium is more than NO_3^- compared to their proportion in the soil solution. It was reported after the analysis of 35 soil samples from agricultural fields that NO_3^- in soil solution was 6.0 mM compared to 0.77 mM of NH_4^+ (Wolt 1994). Uptake and assimilation of N are described in the following sections.

6.4 Nitrogen Uptake

The first step in N acquisition and its use by plants is the nitrate (NO_3^-) transport from soil through plasmalemma of epidermal and cortical cells of the root (Miller and Cramer 2004). According to Forde (2007), NO_3^- can directly affect the gene expression associated with NO_3^- uptake, transport, and assimilation. It thus acts as both a nutrient and a signal. Although most of the higher plants can reduce NO_3^- in roots and shoots, the reduction of NO_3^- is more efficient in leaves than in roots due to easy availability of reductants, energy, and carbon skeletons produced by photosynthesis (Chen et al. 2004). NO_3^- uptake is an energy-dependent process triggered by the gradient of proton or the protonmotive force maintained by H^+-ATPase. Two or more protons are cotransported along with every NO_3^- ion (Santi et al. 1995). In many higher plants, there exists a biphasic relationship between NO_3^- uptake rate and external NO_3^- concentration. It is suggested that there are two different types of transporter systems in higher plants, namely, high-affinity transporter system (HATS) and low-affinity transporter system (LATS). HATS are further categorized into *inducible high-affinity transporter system (iHATS)* and *constitutive high-affinity transporter system (cHATS)* (Okamoto et al. 2006).

6.5 Nitrogen Assimilation

Once the NO_3^- has been taken up by the plant cell, the next step in the N-assimilation pathway is the reduction of NO_3^-. The NO_3^- entering the plant cell is assimilated in a series of steps involving the action of a number of different enzymes (Crawford et al. 2000). This reduction is catalyzed by NO_3^- reductase (NR) and nitrite reductase (NiR). NR reduces the NO_3^- into nitrite, which is then reduced to ammonium by NiR.

$$NO_3^- + NAD(P)H + H^+ \xrightarrow{NR} NO_2^- + NAD(P)^+ + H_2O$$

$$NO_2^- + 6\,Fd_{red} + 8H_3O^+ \xrightarrow{NiR} (6e^-)\,NH_4^+ + 6Fd_{ox} + 10H_2O$$

In plants, NH_4^+ formed after reduction of NO_3^- in the plant or absorbed directly from soils is rapidly converted into nontoxic organic compounds. In plants, NH_4^+ assimilation to synthesize amino acids is mediated by two enzymes: glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyzes the first step of NH_4^+ assimilation into glutamate for synthesis of glutamine (Suzuki and Knaff 2005). The reaction is as follows:

$$\text{Glutamate} + ATP + NH_4^+ \xrightarrow{GS} \text{Glutamine} + ADP + Pi$$

GOGAT catalyzes the synthesis of two molecules of glutamate. The reaction is as follows:

$$\text{Glutamine} + \text{2-oxoglutarate} \xrightarrow{GOGAT} 2\text{Glutamate}$$

GS/GOGAT pathway is important as glutamine and glutamate synthesized produced serve as donors of amino groups for the amino acid biosynthesis, nucleotides, chlorophylls, polyamines, and alkaloids (Hirel and Lea 2001).

Alternatively, glutamate formation involves reductive amination of 2-oxoglutarate by NH_4^+, catalyzed by mitochondrial glutamate dehydrogenase (GDH). The reaction is as follows:

$$\text{2-oxoglutarate} + NH_4^+ + \text{NAD(P)H} \xrightarrow{\text{GDH}} \text{Glutamate} + H_2O + \text{NAD(P)}^+$$

6.6 Molecular Physiology of Nitrate Uptake

6.6.1 Biochemical Characterization

Inducible high-affinity transporter system (iHATS) is substrate inducible and is responsible for uptake at low concentrations of NO_3^- (below ~1 mM) that are characterized by low K_m values (5–200 μM). Constitutive high-affinity transporter system (cHATS) is responsible for uptake at low concentrations of NO_3^- and provides a low-capacity pathway in uninduced plants but operates simultaneously with *iHATS* in the induced state. Their activity becomes threefold on exposure to NO_3^- (Crawford and Glass 1998). It is characterized by low values of both K_m (6–20 μM) and V_{max}. Low-affinity transporters form a constitutive transport system which is responsible for uptake at high external NO_3^- concentrations (>1 mM). Despite of showing linear kinetics, it is a transport system which is an active H^+-dependent system (Kronzucker et al. 1995). LATS are characterized by high K_m values (>0.5 mM). It allows enough NO_3^- into the cell which is sufficient to cause the expression of transporter and genes and probably plays a physiological role in the NO_3^- uptake only above a certain threshold. Molecular studies of NO_3^- transporters in plant suggest that NO_3^- transporters belong to two different families, NNP and PTR (Forde 2000).

It is suggested that high- and low-affinity transporters are functional at early growth stages but the high-affinity transporters are also functional at later stages when the soil N concentration is low. Studies on oilseed rape showed that HATS accounted for about 89 % of the total NO_3^- uptake (18 and 79 % for cHATS and iHATS, respectively) when no fertilizer was applied (Malagoli et al. 2004). They also found that LATS accounted for a minor proportion of the total NO_3^- uptake. It is suggested that NO_3^--inducible part of HATS functions mainly as a sensor for NO_3^- availability to the roots (Miller et al. 2007a).

6.6.2 Genomic Organization

Nrt1 and *Nrt2* gene families characterize two classes of membrane proteins which might involve in low- and high-affinity NO_3^- transport, respectively (Okamoto et al. 2003). Unkles et al. (1991) isolated the first eukaryotic NO_3^- transporter gene from the fungus, *Aspergillus nidulans*. Later on, a NO_3^- transporter gene (*AtNrt1:1)* was identified in *Arabidopsis thaliana* (Tsay et al. 1993). Later, this gene was used as a probe to isolate two more genes (*LeNrt1:1)* and (*LeNrt1:2)* in tomato (Lauter et al. 1996). Here, *Nrt1:2* is shown to be NO_3^- inducible and its expression restricted to roots except in stem or leaves, but *Nrt1:1* is not limited to roots and is constitutively expressed. Nitrate transporter genes have been cloned from wide range of plants. *BnNrt1:2* is identified in *Brassica napus* also (Crawford and Glass 1998). *AtNrt1:4* is expressed in a specific pattern petiole of leaf and plays an important role during accumulation of NO_3^- in these tissues (Chiu et al. 2004). *AtNrt1:3* expression was NO_3^- induced in the leaf, but in roots it contributed significantly to LATS (Okamoto et al. 2003). According to Li et al. (2007), *AtNrt2:1* was the main contributor to iHATS and cHATS.

Ammonium (NH_4^+) transport shows a normal homeostatic tendency, but the range of the concentration at which absorption occurs is very limited due to the potential toxicity at elevated NH_4^+ concentrations. Like NO_3^-, ammonium is also transported by transporter protein located in the plasma membrane. AMT-type transporters handle NH_4^+ influx and mediate the uniport of this ion. First NH_4^+ transporter *AtAMT1;1* was isolated from *Arabidopsis thaliana* (Ninnemann et al. 1994) and then another five homologous sequences, *AtAMT1;1* to *AtAMT1;5* were discovered.

6.6.3 Regulation of Transporters

Signals derived from NO_3^- for regulating NO_3^- uptake trigger changes in gene expression which cause N-metabolism reprogramming for facilitating the absorption and incorporation of NO_3^- and amino acid synthesis. Available NO_3^- and reduced N regulates the assimilatory pathway of NO_3^-. It was reported that increasing concentration of NO_3^- increased the NO_3^- uptake in strawberry (Taghavi and Babalar 2007). Many LATS- and HATS-related genes are inducible by NO_3^-, and one HATS-related gene *NpNrt2:1* is also repressible for reduced N (Quesada et al. 1997). The cHATS gives a high affinity having less capacity pathway for nitrate entry into uninduced barley and white spruce plants. Nevertheless, activity of cHATS is controlled by exposure to NO_3^- by three times (Trueman et al. 1996). In barley, the fully induced iHATS flux was about 30 times higher than that resulted from the cHATS (Quesada et al. 1997). It has been observed that increasing transcription increases NO_3^- absorption rate (Imsande and Touraine 1994). It is reported that in citrus seedlings, LATS is controlled by feedback mechanism induced by the N contents of plant. The absorption of NO_3^- is reported to decrease due to the application of amino acids (Glu, Asp, Asn, Gln) to the external solution (Cerezo et al. 2000). The importance of biosynthesis of proteins for NO_3^- uptake is evident because of using of chemical inhibitors in physiological studies (Aguera et al. 1990). Cerezo et al. (2001) proposed mechanism of degradation for transporter protein in Arabidopsis. The presence of conserved protein kinase carbon recognition motifs in the nitrogen and carbon domains of *HvNRT2:1* (Forde 2000) indicated that phosphorylation activities are involved in regulation of *AtNrt2:1* activity in response to different factors of environment. Remans et al. (2006) reported that *AtNrt2:1* played an important role as a major NO_3^- absorption under N-deficient conditions and influenced initiation and development of lateral root system with external NO_3^- availability.

6.6.4 Regulation of Nitrogen Metabolism

Competition for N resources by crops causes nutrient deficiency in the soil during the interval between fertilizations. Nitrogen nutrition index (NNI) is used for assessment of sub- versus supraoptimal N supply in agriculture (Richard-Molard et al. 2008). Study of these processes helps environmentally safe production of the crops which is the demand of sustainable agriculture. New bioinformatics tools (sun gear software) can be used nowadays for detailed study of NO_3^- response in plants. These analyses have identified many genes and pathways (Gutierrez et al. 2007). One might think that NO_3^- is used in a linear pathway involving absorption and transport, followed by its assimilation, and biosynthesis of amino acids and protein. But, complex interactions including storage and remobilization of NO_3^-, assimilation of de novo NH_4^+, and distribution of N in highly branched pathway of amino acid synthesis are affected by temporal changes in expression of gene, enzymatic activities, metabolite levels, and fluxes. Leaves contained high contents of NIA transcription at the end of night when grown in high NO_3^- and favorable light conditions (Kaiser et al. 1999) which led to around three times increase during initial hours of exposure to light (Scheible et al. 1997). The NO_3^- assimilation rate surpasses net flux through GOGAT pathway by 25 % and results in assimilation of reduced N in products such as NH_4^+ and glutamine, photorespiratory metabolites, glycine, and serine. Usually, assimilation of NO_3^- and NH_4^+ is controlled through transcriptions due to the balance between NO_3^- influx and its incorporation and posttranscriptionally due to the signals from nitrogen metabolism. Increase of NH_4^+ and glutamine on feeding glutamate indicates the important role of glutamate in the sensitive feedback mechanism responsible for regulation of NH_4^+ assimilation. Concentration of amino acids in plants regulates the NO_3^- uptake by plants by indicating N status by signalling (Miller et al. 2007b). It is reported that acids, including cysteine and asparagine, play an important role in feedback regulation on NO_3^-

assimilation. Glutamate, cysteine, and asparagine have important positions in metabolism of amino acid for feedback inhibitors of NO_3^- reduction.

6.7 Molecular Physiology of N-Assimilatory Enzymes

6.7.1 Nitrate Reductase

6.7.1.1 Biochemical Characterization and Localization

Nitrate reductase (NR) is the most important enzyme which catalyzes the first step of NO_3^- assimilation in plants (Leleu and Vuylsteker 2004). It reduces NO_3^- to nitrite with pyrimidine nucleotide in higher plants. This is a soluble enzyme in plants that is present in the cytosols of epidermal cells of roots and cortical cells and mesophyll cells of shoot. It exists as a homodimer metalloprotein of 110-kD subunits. NO_3^- reduction to nitrite is catalyzed by NR by transferring two electrons from NAD(P)H to NO_3^- via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor in a 1:1:1 stoichiometry per subunit. Each redox center is attached to an independent functional domain of the enzyme having activity unaffected by other domains. NR is a substrate-inducible enzyme which is the most important limiting step in N assimilation. For this reason, NR activity is considered as a selection criterion for grain yield and N-assimilation potential. Nitrate reductase in plants exists in three major forms which are characterized by electron-donor source, either NADH and/or NADPH (Miller and Cramer 2004).

6.7.1.2 Genomic Organization and Regulation

NO_3^- reductase is regulated in both shoots and roots. Most plants have two or more genes for NR. Clones of both genes have been isolated and mapped (Sivasankar and Oaks 1995). Molecular and genetic analyses have indicated that in plants there are two or more structural genes for NR, the only known exception being *Nicotiana plumbaginifolia*, that has a single gene for NR, that encodes an NADH-dependent NR (Caboche and Rouze 1990). In barley, the NADH-specific NR is encoded by *nar*I gene, while the NADPH-bispecific NR is encoded by *nar*7 gene. Clones of both genes have been isolated and mapped (Miyazaki et al. 1991) and their induction properties have been compared (Sueyoshi et al. 1995). Although, the two proteins are distinct, the genes respond similarly to NO_3^-.

NR activity can be controlled either by modifying the activity of already present enzyme or by maintaining the amount of enzyme by producing new enzymes and degradation of the old one. Many factors regulate this enzyme. NO_3^- triggers transcription of inducible genes (NIA) encoding NR. De novo synthesis of new NR, stimulated by NO_3^-, is one of the mechanisms for controlling enzyme level when combined with NR-protein degradation (Stitt 1999). NO_3^--induced increase in the NR activity and NR protein is due to the enhanced steady state level of NR-mRNA (Miyazaki et al. 1991; Sueyoshi et al. 1995), and this induction is shown to be repressed by downstream N-assimilation products like glutamine and asparagine (Vincentz et al. 1993; Sivasankar et al. 1997). Sucrose addition (Sivasankar and Oaks 1995) and light also enhance NR protein and mRNA induction. It appears that NR expression is regulated by light via phytochrome after it is triggered by NO_3^-. The role of light in induction is probably more related to the activity of the enzyme rather than to the activation of the NR gene. In another study, NR-protein kinase was used to identify the apparent key serine residue in spinach NR (Bachmann et al. 1996).

Many environmental factors initiate the modulation and posttranslational regulation of NR. The total amount of NO_3^- reductase depends simultaneously on its synthesis and degradation. Activity level can be controlled by mechanisms involving phosphorylation of the NR protein and binding of Mg^{2+} or other divalent cation and an inhibitor protein (Stitt 1999). Light has a very important effect though it is not a direct signal for activation of NR, but photosynthetic process is needed for activation of NR. It has been reported that the availability of oxygen and light is the important external factor for rapid and reversible

modulation of NR activity, and feeding of sugar in the leaves in darkness activated the NR. Sugar and/or phosphates of sugars are the internal signals regulating the protein kinase(s) and phosphatase. The roots usually do not change their reduction rate as rapidly as shoots. However, during sudden anoxia, the enzyme is rapidly modulated, being activated within minutes (Kaiser and Huber 2001).

6.7.2 Nitrite Reductase (EC 1.7.7.1)

6.7.2.1 Biochemical Characterization and Localization

The second enzyme in the sequence, nitrite reductase (NiR, ferredoxin nitrite oxidoreductase), catalyzes the six-electron transfer reaction from reduced ferredoxin to NO_3^-, leading to the synthesis of NH_4^+. It is localized in chloroplasts in leaf and in plastids of root cells (Sechley et al. 1992). It is a monomeric protein of about 63 kDa containing sirohaem and a 4Fe4S center as prosthetic groups (Faure et al. 2001). Reduced ferredoxin acts as the electron donor in both leaves and roots. This enzyme gains reducing power from NADPH, produced by the oxidative pentose phosphate pathway present in the plastids of the roots (Bowsher et al. 1989). It is reported that ferredoxin and the NADPH-dependent ferredoxin:NADP-oxidoreductase increase in isolated pea root plastids due to NO_3^- application.

6.7.2.2 Genomic Organization and Regulation

The gene for the NiR apoprotein has been cloned in at least six different plant species. There is one NiR apoprotein gene per haploid genome in barley and spinach, two in maize, and four in *N. tabacum* (Kronenberger et al. 1993). The four NiR apoprotein genes in tobacco are reported to be involved in encoding of two different isoforms in shoots and in roots, as evidenced by the gene expression (Kronenberger et al. 1993). The promoter region of NiR gene has been fused to β-glucuronidase (GUS), and transgene has then been successfully introduced into tobacco (Rastogi et al. 1993).

This gene appears to be very responsive to NO_3^- additions and to the additions of sucrose, glutamine, or asparagine. The experiments of Rastogi et al. (1993) provide evidence that induction of this gene by NO_3^- is a transcriptional event. However, the addition of asparagine or glutamine results in a repression of induction, whereas sucrose enhances the induction (Sivasankar and Oaks 1995). Light is also an important environmental cue in the NiR induction (Wray 1993).

6.7.3 Glutamine Synthetase (EC 6.3.1.2)

6.7.3.1 Biochemical Characterization and Localization

Glutamine synthetase (GS) mediates the ATP-dependent transformation of inorganic N (NH_4^+) to an organic form like glutamine. This enzyme along with GOGAT represents the major pathway for incorporation of ammonia (toxic to plant function) into amino acids (Hirel and Lea 2001; Fei et al. 2006). There are two types of GS: type-I GS, which is dodecameric with subunits of about 52 kDa, and type-II GS that is octameric and composed of about 40 kDa subunits (Nogueira et al. 2005). Type I is found mainly in bacteria and type II is best identified in higher plants. In plants, this is present as a chloroplastic (GS2) and a cytosolic (GS1) enzyme (Scarpeci et al. 2007). Nitrogen, which is moved through a small number of transport compounds, is added to and removed from proteins in different organs during different stages of plant development. A major portion of N is released as ammonia and reassimilated via GS (Miflin and Habash 2002).

6.7.3.2 Genomic Organization and Regulation

Molecular analysis of GS genes reveals a multigene family whose individual members encode several distinct cytosolic GS (GS1) polypeptides and a single chloroplastic GS (GS2) polypeptide. Li et al. (1993) have identified five distinct cDNA clones of GS in maize. Six distinct genes encoding for GS in maize (Li et al. 1993) and five in sugarcane (Nogueira et al. 2005) have been identified. It has been demonstrated that GS occurs in an organ-specific manner; roots and

nodules generally contain proportionally more cytosolic GS, while leaves contain more chloroplastic GS (Becker et al. 1992). Genetic study of GS has helped in explicating the role of each isoform. Chloroplastic GS is considered to be involved in the reassimilation of photorespiratory NH_4^+.

The genes GS2 and GS1 are controlled by the external N application, but the extent of control is a plant species dependent, N source, and plant organ/tissue (Cren and Hirel 1999). Nitrogen assimilation whether in the form of NH_4^+ and/or NO_3^- has a regulatory effect on gene expression in rice, maize, tobacco, tomato, sunflower, and mustard (Zozaya-Hinchliffe et al. 2005). Nitrogen and carbon metabolites could also control the GS expression in the leaf of Arabidopsis (Oliveira and Coruzzi 1999) and tobacco (Masclaux-Daubresse et al. 2005). According to Zozaya-Hinchliffe et al. (2005), light and metabolic factors associated with light also regulate the expression of this enzyme. Detailed studies on *Pinus sylvestris* by Elmlinger et al. (1994) have shown that regulation of GS2 expression through light occurs crudely at transcriptional level, but fine regulation occurs at the posttranscriptional level. Similar observations were made in seedlings of tomato (Migge et al. 1998). Some mechanisms controlling the stability and activity of GS have been identified. Finnermann and Schjoerring (2000) proposed a model that can control GS1 reversibly by phosphorylation and dephosphorylation which incorporated the roles of ATP, Mg^{2+}, and 14-3-3 binding. This model is based on the major role of ATP/AMP ratio in the light where it is suggested that levels of ATP/AMP are high in dark, and therefore GS1 is phosphorylated and binds 14-3-3 proteins to protect its deterioration. Conversely, in the light, GS1 is unphosphorylated and becomes susceptible to damage. Riedel et al. (2001) have also demonstrated that GS2 is phosphorylated in tobacco. Many workers have shown that important factors affecting GS activity are light, C status, and N nutrition. Experiments with white, red, far-red, or blue light by Becker et al. (1992) and Migge et al. (1998) have shown that the phytochrome and the blue light photoreceptors are responsible for positive response to light. Carbon compounds, important in the stimulation of GS1 and GS2 production, include sucrose and 2-oxoglutarate (Oliveira et al. 2002). Studies on dark-adapted *Arabidopsis* seedlings have shown that sucrose enhances the expression of GS2, thus representing the light effect. Woodall et al. (1996) conducted experiments on pea and barley plants and concluded that temperature is a crucial factor that controls the GS expression. Within 2 days of keeping the plants in 15 °C instead of 25 °C, they observed 50 % reduction in GS2 activity, while GS1 activity remained unaffected. There are indications that substrate availability (Ortega et al. 1999) or phosphorylation (Moorhead et al. 1999) may be an important factor controlling the enzyme turnover and activity respectively.

6.7.4 Glutamate Synthase (E.C. 1.4.1.13)

6.7.4.1 Biochemical Characterization and Localization

Glutamate synthase (Glutamine (amide):2-oxoglutarate aminotransferase, GOGAT) mediates the transfer of the amide group of glutamine (produced by GS) to 2-oxoglutarate (α-keto glutarate) to form two glutamate molecules through reduction (Ireland and Lea 1999). The discovery of NAD(P)H-dependent GOGAT in bacteria (Tempest et al. 1970), ferredoxin (Fd)-dependent GOGAT in pea chloroplast (Lea and Miflin 1974), and NAD(P)H-dependent GOGAT in carrot cell cultures (Dougall 1974) established a route, GS-GOGAT cycle, for the incorporation of NH_3 into organic compounds. The synthesized glutamate may be utilized either to fulfill the glutamate pool for subsequent GS catalysis or to donate its amino group to form other N-containing compounds. One important fate of glutamate and glutamine is the synthesis of aspartate and asparagine. These amino acids are important N-transport compounds in many plants (Temple et al. 1998). In higher plants, GOGAT are present as two different isoforms, NADH-GOGAT (EC 1.4.1.14) and Fd-GOGAT (EC 1.2.7.1), and these differ in molecular mass, subunit composition,

enzyme kinetics, and metabolic functions (Gregerson et al. 1993; Sakakibara et al. 1991). Fd-GOGAT, an iron-sulfur flavoprotein, generally functions as a monomer with subunit molecular mass of 130–180 kD. The presence of Fd-GOGAT isoform in maize roots is different from the enzyme present in leaves, and this suggested that these are encoded by separate genes. The root isoform has been involved in NH_4^+ assimilation which is derived from soil NO_3^- (Redinbaugh and Campbell 1993). NADH-GOGAT is also an iron-sulfur flavoprotein and is found primarily in nongreen tissues. In higher plants, it occurs as a monomer with a native subunit mass of 225–230 kDa and has a pH-optimum range from 7.5 to 8.5 (Lea et al. 1990).

6.7.4.2 Genomic Organization and Regulation

GOGAT is found in all types of organism, and its amino acid sequence is remarkably well conserved (Temple et al. 1998). The expression pattern of the genes encoding cytosolic GS and NADH-GOGAT appears to be synchronized in nonlegumes where the function of proteins coupled with processes such as primary NH_4^+ assimilation derived from soil nitrate and reassimilation of ammonium released due to catabolism of amino acid. cDNA clones for Fd-GOGAT have been isolated from a number of species including barley (Avila et al. 1993), maize (Sakakibara et al. 1991), and *A. thaliana* (Coschigano et al. 1998). Full-length cDNA and genomic clones of NADH-GOGAT have been isolated from alfalfa (Trepp et al. 1999) and rice (Goto et al. 1998).

Light and a variety of metabolites exert major regulatory controls over metabolic pathways. Evidence by Suzuki and Rothstein (1997) indicates that light exerts a positive regulatory effect on the expression of Fd-GOGAT (GLU1). GLU2 expression is also induced by light, but the induction of this gene by sucrose in dark indicates that light-induced expression may in part be caused by increased concentration of C metabolites. The Fd-GOGAT activity increases with the start of photosynthesis and photorespiration during the expansion and development of a new leaf (Emes and Tobin 1993). In barley, enzymatic activity, mRNA, and protein increased on the leaf emergence and expansion and decreased aging of the leaf (Pajuelo et al. 1997). Nitrate also acts as a signal resulting in wide range changes in the expression of key genes in N-metabolism pathway, including Fd-GOGAT (Scheible et al. 1997). Gene expression study in developing alfalfa nodules suggests that NADH-GOGAT is uniquely regulated, as compared with other genes of N metabolism (Vance et al. 1994). The expression of NADH-GOGAT occurred in effective nodules and in ineffective nodules, and roots was only 12–20 % of the maximum. These results show that active N fixation and NH_4^+ itself or a downstream product of its metabolism is required for maximum NADH-GOGAT gene expression. Fd-GOGAT proteins from *Arabidopsis* and maize contain a presequence with many of characteristics of plastid transit peptides. Similarly, both rice and alfalfa (Gregerson et al. 1993) NADH-GOGATs contain presequences that are thought to be responsible for targeting of plastids. Interestingly, presequences are found in all characterized eukaryotic GOGAT proteins. As shown by the experiments of Yamaya (2003), NH_4^+ ions and glutamine could serve as signals for the transcription increase.

6.7.5 Glutamate Dehydrogenase (EC 1.4.1.2)

6.7.5.1 Biochemical Characterization and Localization

Glutamate dehydrogenase (GDH) can release N from amino acids to generate keto acid and ammonia which can be recycled separately for use in respiration and amide formation, respectively. It is thought to be an alternative pathway for the glutamate formation involving reductive amination of 2-oxoglutarate by NH_4^+. Its role in plant cells remains controversial (Miflin and Habash 2002). It is yet to be clearly established that the enzyme has an important role in NH_3 assimilation or in recycling of carbon (Dubois et al. 2003). Studies have shown that it is involved

in the deamination of glutamate for energy supply and return carbon from amino acids into metabolism of carbon during shortage of carbon or energy (Miflin and Habash 2002). However, Dubois et al. (2003) have still speculated the physiological role of GDH in plants. GDH is capable of synthesizing or deaminating glutamate, but the direction of activity depends on specific environmental cues (Pahlich 1996). One isoform of enzyme, localized in mitochondria in roots and leaves, uses NADH as the electron donor (Sechley et al. 1992). Another isoform, having specific requirement for NADPH, is present in chloroplasts of photosynthetic tissues. The primary role of GDH could be replenishment of TCA cycle intermediates via its oxidation to 2-oxoglutarate. Glutamate is deaminated to 2-oxoglutarate in isolated mitochondria; however, in the presence of aminooxyacetate, glutamate no longer contribute to mitochondrial respiration (Sechley et al. 1992). This observation indicates that GDH does not oxidize glutamate.

6.8 Improving NUE Through Manipulation of Signalling Targets

The failure to improve NUE in transgenic plants by overexpression of enzymes of NO_3^- and NH_3 incorporation has supported the theory that metabolic flux via such pathways may be regulated by regulatory switches outside these pathways. The NO_3^- sensing and signalling is not well understood. Posttranslational control of some nitrate-responsive enzymes is carried out by 14-3-3 proteins despite the fact that these control light effect and other signals, rather than NO_3^-. Some elements which could be related with NO_3^- signalling are Ca^{2+} and protein kinases/phosphatases. These elements are involved in mediation of the NO_3^- signal which is responsible for expressing NR, NiR, and GS2 mRNAs (Sakakibara et al. 1992; Sueyoshi et al. 1999). Hartwell et al. (1999) observed that Ca^{2+}-free PEPCase protein kinase is a member of the Ca^{2+} calmodulin-regulated group of protein kinases. Krapp et al. (2002) explained the specific roles of these groups in mediation of NO_3^- and other interacting signals. Study of mutants associated with signal-transfer cascade can lead to better understanding of the nitrate-signalling cascade from NO_3^- to the NR gene (Ogawa et al. 2000). It reveals some intermediary and potential sites for the management of NUE. Light is an another signal which is responsible for the regulation of the expression of many nitrate-responsive genes (Raghuram and Sopory 1995; Chandok et al. 1997; Lillo and Appenroth 2001). In green plants, light controls signalling and NUE via the photosynthetic process and sugars (Lillo and Appenroth 2001). At the posttranslational level, light modulates the enzyme phosphorylation status, in connection with 14-3-3 proteins.

Transcription of NO_3^--responsive genes by NO_3^- as a signal needs cis-acting regulatory sequences or NO_3^--responsive elements (Raghuram et al. 2006). Zhang and Forde (1998) reported the presence of ANR1, a putative signalling and NUE transcriptional factor which is similar to the MADS-box family in *Arabidopsis thaliana*. ANR1 is NO_3^- inducible and root specific which is responsible for lateral-root proliferation due to NO_3^- in transgenic plants (Forde 2007). However, this type of transcription is not related with known NO_3^- responses even in the root. The manipulation of N by Dof1 overexpression shows that understanding of the signalling mechanisms may lead to new goals and techniques for metabolic-engineering activities in future (Lochab et al. 2007). Yanagisawa et al. (2004) developed such transgenic *Arabidopsis* lines that can overexpress Dof1. Dof1 is a maize protein of Dof family of plant-specific transcriptional factors which is responsible for activation of different genes responsible for C-metabolism related to metabolism of organic acid. The transformants indicated approximately 30 % more mRNA and enzymatic activities for PEP carboxylase and pyruvate kinase, without decreasing GS, NR, and GOGAT RNAs. If Dof1 is not induced by NO_3^-, it will indicate numerous transcriptional factors which may mediate in the coordination of gene expression responsible for nitrogen and carbon metabolism.

6.9 Understanding Nitrogen Regulation Through Proteomics

The complete genome sequences and technologies have improved monitoring of the transcriptional reprogramming of cells due to environmental factors. However, further studies show that transcriptional factors are not perfect indicators of levels of protein in vivo fluxes (Griffin et al. 2002; Washburn et al. 2003; Daran-Lapujade et al. 2004), and thus it brings poor comprehension of complete biological systems. The use of sensitive and quick techniques to identify and technical improvement of protein by two-dimensional gel electrophoresis (2-DE) have made these techniques sophisticated for analysis. Proteomics is used for relatively fast, sensitive, and reproducible investigation of changes in the protein profile caused by introduction, mutations, or gene silencing due to various stress factors. This technique is important for generating information on physiological, biochemical, genetic, and architectural aspects of cells. Proteomics is an important technique for characterizing lines, individuals, and estimation of inter-/intrapopulation genetic variability, establishing genetic differences for use in phylogenetic studies, and characterizing the mutants with localized encoding of genes that indicated proteins (Thiellement et al. 1999). This is necessary for decoding the role and function of genes in plant genome sequencing projects. Use of proteomics can tremendously increase agricultural productivity (Dhand 2000; Xu et al. 2006). It is the most important method used for identification of proteins repressed, induced, or modified through posttranscription during the development as complex as senescence. Protein synthesis and accumulation in the seeds is dependent upon N supply from the mother plant during seed development. In legumes, sources of N could be (1) exogenous N absorbed from the soil N and/or N fixed by the symbiotic relationship with microbes and (2) from N mobilized from vegetative parts. Nitrogen fixed by microbes is not sufficient to fulfill the high N demand for seed development (Sinclair and de Wit 1976; Salon et al. 2001). Nitrogen mobilization for seed development closely related the shedding of vegetative parts which is due to the decrease in protein and chlorophyll and subsequently leaf yellowing.

Amount of available N and its source affected the complex and poorly understood metabolic rearrangement (ter Schure et al. 2000; Wek et al. 2004). Both limitations and availability of N had overrepresented the proteins in the category "metabolism" (42 proteins), which resulted in rearrangement of metabolic processes in yeast for adaptation under changed nutrient availability (Kolkman et al. 2006). Cánovas et al. (2004) characterized symbiotic proteins and defined the modifications in their metabolisms while interacting with each other. Proteome maps from control *Melilotus alba* roots, wild-type nodules, and cultured *Sinorhizobium meliloti* bacteroids were developed and compared. Sequencing of N present in amino acid and MALDI-TOF-MS peptide mass fingerprint analysis along with database searching were used for putative identification of about 100 nodule, bacterial, and bacteroid proteins. Deregulated proteins between the N-deficient media of C/N ratio and control have been identified to better understand the metabolism and regulation of N in the cultured *Monascus* cells under limited N supply (Lin et al. 2005). This study demonstrated that proteomics helps in the investigation of biological processes through systematic analysis of many expressed proteins that is a good approach to associate sequencing with functional genomics.

6.10 Conclusion

Nitrogen uptake, assimilation as well as utilization, is highly controlled by signalling pathways.

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