Cellular Biology of Nitrogen Metabolism and Signaling

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Abstract This chapter summarizes major aspects of N-nutrition in plants. N distribution within a plant varies widely according to the organ, the development stage, and mostly to the environmental conditions. Within the cell, the different N forms are stored in different compartments and the pool sizes are controlled in contrasting manner. Plants can take up nitrate, ammonium, urea, and other organic N forms. Various transporters for these compounds have been characterized, and the localization and properties of these proteins give rise to a complex pattern of N fluxes within the plant. The further assimilation of nitrate is well described, but the *in planta* role of all proteins, as for example GS1 and GDH, is far from being evident. Some are involved in N remobilization which is an important N source for example during seed filling.

Regulation of N assimilation occurs at the transcriptional and post-transcriptional levels, and regulation of the different steps is highly coordinated. However, only very few molecular players are known. As a special case in N-signaling, NO, a side product of N assimilation, is considered in some detail.

1 Introduction

Nitrogen is the mineral nutrient required in highest amounts by plants and is most frequently limiting growth and yield. Inorganic or organic N forms participate to plant nutrition in a variable extent depending on plant parameters as well as soil

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characteristics. In temperate climatic conditions, inorganic N forms are predominant, and fertilizers are often supplied as nitrate, ammonium, or urea (http://www.fertilizer.org/ifa/). However, the soil solution may contain different organic N forms such as soluble proteins or amino acids derived from proteolytic processes. A variety of plant species are able to use organic N forms in artic, boreal, temperate, Mediterranean shrub-land, or alpine natural ecosystems (reviewed in Näsholm et al. 2009). In legume plants, atmospheric N_2 is fixed in the nodule, a specialized organ resulting from the interaction between bacteria and roots (Gordon et al. 2001). In the same manner, nutrient use efficiency is increased by symbioses between fungi and plants, the mycorrhizal system being involved in nutrient uptake and the plant partner providing reduced carbon to the fungus (Martin et al. 2001).

Although such symbioses are important in natural ecosystems, this chapter describes only direct N uptake by root cells. We give an overview of (1) N distribution within the plant and more precisely within a plant cell, (2) the molecular elements involved in different fluxes or in assimilatory steps, and (3) the regulatory mechanisms that control these processes. N metabolites, such as nitrate, ammonium, and glutamate act as signal molecules as well. However, this is out of the scope of this chapter and has been reviewed recently (Walch-Liu et al. 2005); instead, we extend this chapter by (4) a detailed description of the synthesis and mode of action of NO.

2 Distribution of N Forms in Plant Cells

2.1 N in Different Tissues

The N forms and N quantities within a plant vary widely according to the organ, the development stage, and the environmental conditions. The root is obviously the predominant organ where large exchanges of a variety of N forms occur between root cells and the soil solution. The differential expression and localization of channel- or transporter proteins (see below) led to a complex picture of the root cellular organization, with specialized uptake functions for lateral root caps or epidermis/cortex, and horizontal transport toward the vasculature for endodermis/pericycle and stele lines (see below 1.2.2 ammonium transport). Inorganic N forms can then enter the xylem to be transported to the shoots. N assimilation and remobilization take place in roots and shoots, and organic N forms are then distributed to sinks organs (Brouquisse et al. 2001).

2.2 N Cellular Distribution

Within the cell, N forms are stored in different compartments (Fig. 1).

Cytoplasmic ammonium pools originate not only from ammonium uptake across the plasma membranes but also from amino acid catabolism occurring during

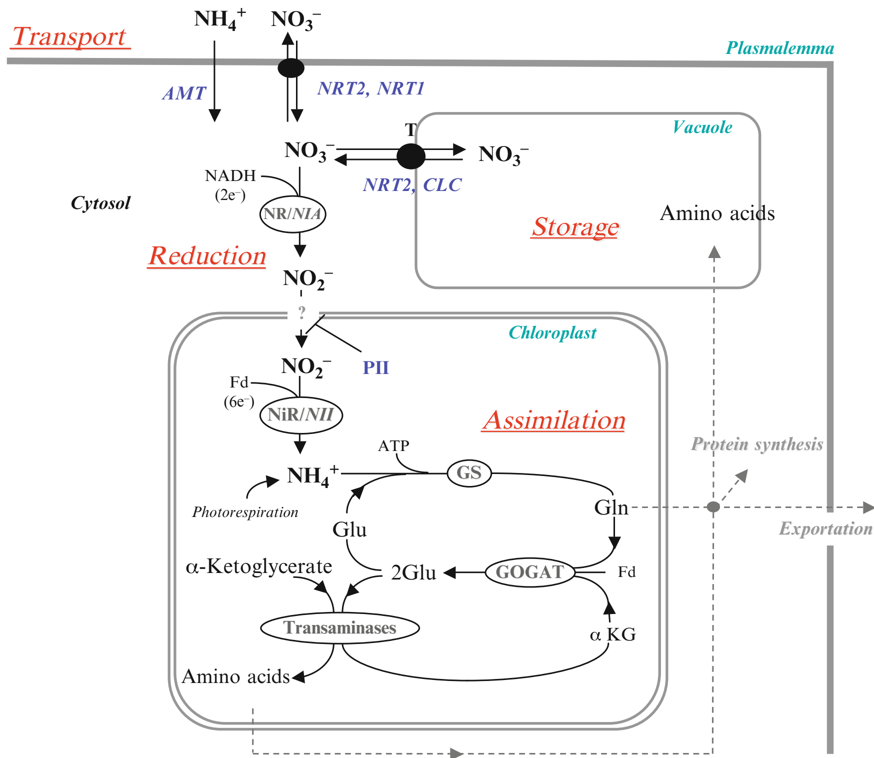

Fig. 1 *N Storage in different compartments*. Nitrate and ammonium enter the cell and can be either stored in the vacuole, transported to other tissues, or assimilated in the cytosol and the chloroplast. Small italic letters: nitrogen assimilation steps. Capital letters: enzymes. Capital italic letters: genes. NR: Nitrate reductase. NiR: nitrite reductase/ GS: Glutamine synthethase. GOGAT: glutamate synthase, AMT: ammonium transporter. NRT: nitrate transporter; CLC: chloride channel

photorespiration in illuminated leaves (Leegood et al. 1996) or in senescent tissues (Matsson and Schjoerring 2003). Ammonium concentrations have been measured using analysis of ^{1}H-coupled ^{14}N-NMR signals (review in Mesnard and Ratcliffe 2005) or with ammonium-selective microelectrodes (Wells and Miller 2000). In both cases, the cytoplasmic ammonium concentrations were no more than a few millimolar (8–15 mM), but this concentration could be increased in maize roots when ammonium assimilation was blocked (Lee and Ratcliffe 1991). In vacuoles, ammonium concentrations vary between 1 and 45 mM in nonstressed plants (Miller et al. 2001), indicating a possible role of this compartment for the storage of ammonium.

The global nitrate concentrations in leaves or roots are highly dependent on external N supply, and nitrate, among all N-compounds, disappears most quickly in response to N starvation (Richard-Molard et al. 2008). The pool of nitrate associated with purified chloroplasts remains remarkably constant under various

conditions (Schröppel-Maier and Kaiser 1988). In contrast, the vacuolar nitrate pools show a positive correlation with the external nitrate supply (Miller and Smith 2007; van der Leij et al. 1998). The pool size varies also with the cell type and is higher in epidermal than in mesophyll cells in barley leaves (Karley et al. 2000) and higher in cortical compared to epidermal cells in barley roots. In roots, remobilization of vacuolar nitrate occurs more slowly from cortical cells than from epidermal cells (van der Leij et al. 1998). This tissue heterogeneity revealed by single-cell techniques implies that knowledge obtained for vacuoles from one type of tissue cannot be necessarily transferred to vacuoles from other tissues, as also shown for gene expression (Gifford et al 2008). A striking characteristic of the cytosolic NO_3^- pool is its low size (in the order of 3–4 mM). In contrast to vacuoles, cytosolic nitrate is maintained at a remarkably stable value that is independent of changes in the external nitrate concentration (Miller and Smith 2007; van der Leij et al. 1998).

The global amino acid contents in leaves depend on external N supplies and can vary from 150 to 45 nmol/mgDM when *Arabidopsis* plants are fed with 10 or 3 mM nitrate, respectively (Loudet et al. 2003). Subcellular volumes and amino acid concentrations have been analyzed using non-aqueous fractionation in spinach (Winter et al. 1994), barley (Winter et al. 1993), or potato (Leidreiter et al. 1995). In all cases, the concentration of amino acids is much lower in the vacuoles than in the cytosol (1.7/40 mM for glutamate in barley, for example). These concentrations are quite similar between cytosol and stroma.

3 N Fluxes Within a Plant Cell

3.1 Nitrate and Nitrite Fluxes

Two nitrate transport systems have been shown to co-exist in plants and act co-ordinately to take up nitrate from the soil solution and distribute nitrate within the whole plant (Fig. 2) (review in Daniel-Vedele et al. 1998; Tsay et al. 2007).

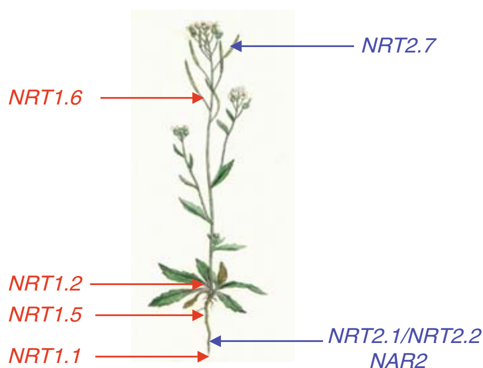

Fig. 2 *Schematic presentation of the known localization of NRT1 and NRT2 genes in Arabidopsis*. Two nitrate transport systems have been shown to co-exist in plants and act co-ordinately to take up nitrate from the soil solution and distribute nitrate within the whole plant

It is generally assumed that the NRT1 gene family mediates the root low-affinity transport system (LATS), with the exception of the At*NRT1.1*, which is a dual affinity transporter (Wang et al. 1998; Liu et al. 1999). In *Arabidopsis*, 53 genes belong to the NRT1 family. Among them 51 genes are expressed and exhibit different tissue expression patterns in the whole plant (Tsay et al. 2007), suggesting a specialized and unique function for at least some of them. The most extensively studied gene is the first one isolated, *AtNRT1.1* (formerly *Chl1*; Tsay et al. 1993). The gene is expressed in epidermis of the root tips and in the cortex and endodermis in the more mature part of the root (Huang et al. 1996) but also accumulates in nascent organs (Guo et al. 2001). *AtNRT1.1* is also considered as a nitrate sensor that could regulate other processes like regulation of other components of nitrate uptake (Krouk et al. 2006), stomatal opening (Guo et al. 2001), relieving of seed dormancy (Alboresi et al. 2005), or stimulation of root proliferation by nitrate (Remans et al. 2006a). Beside this gene, the *AtNRT1.2* gene is constitutively expressed only in the root epidermis and belongs to the constitutive low-affinity system (Huang et al. 1999). *AtNRT1.5* is located on the plasma membrane of root pericycle cells close to the xylem. The protein is a low-affinity, pH-dependent bidirectional nitrate transporter and is involved in long distance transport of nitrate from the root to the shoot (Lin et al. 2008). The *AtNRT1.4* gene is only expressed in leaf petioles, and the nitrate content is twice lower in the petiole of the mutant compared to that of the wild type (Chiu et al. 2004). Recently, *AtNRT1.6* was shown to be involved in embryo development. The gene is expressed in the vascular tissue of the silique. Expression in oocytes and mutant phenotypes suggest that the protein could deliver nitrate from maternal tissue to the developing embryo (Almagro et al. 2008). A striking particularity of the NRT1 family is that certain members belonging to the group II (reviewed in Tsay et al. 2007) are able to transport not only nitrate but also di or tripeptides in heterologous systems, while OPT proteins transport tetra/pentapeptides.

The high-affinity transport system (HATS), acting when the external nitrate concentration is low, relies on the activity of the so called NRT2 family (reviewed in Williams and Miller 2001). *AtNRT2.1* is a major component of the iHATS in *Arabidopsis*, as shown by the fact that a mutant disrupted for the *AtNRT2.1* gene has lost up to 75% of the inducible high-affinity NO_3^- uptake activity and showed a lower leaf nitrate content (Cerezo et al. 2001; Filleur et al. 2001). As a consequence, growth of these mutants is severely impaired at low NO_3^- concentration (Orsel et al. 2004; Orsel et al. 2006). Li and coworkers showed that the *AtNRT2.2* makes only a small contribution to iHATS under normal growth conditions (Li et al. 2007).

Nitrate can also be exported from the cytosolic pool by an efflux mechanism. Segonsac and co-workers have identified an *Arabidopsis* excretion transporter, localized at the plasma membrane of cortical root cells and encoded by the *NAXT1* gene belonging to the *NRT1* family (Segonzac et al. 2007).

Regarding vacuolar nitrate pools, classical experiments using indirect assay of H^+ transport provided evidence for the presence of a NO_3^-/H^+ antiporter in the tonoplast (Schumaker and Sze 1987). Recently, De Angeli et al. (2006)

demonstrated that the AtCLCa protein, localized in the vacuolar membrane, behaves as a NO_3^-/H^+ exchanger, allowing the accumulation of nitrate within the vacuole. Residues important for nitrate/proton coupling have been identified in plant and mammalian CLC transporters (Eun-Yeong et al. 2009; Zifarelli and Pusch 2009). Insertion mutants within the *AtCLCa* gene exhibit normal development but show a reduced capacity to store nitrate but not other anions (Geelen et al. 2000). This phenotype was also recently found when the expression of the vacuole-located nitrate transporter AtNRT2.7 was affected. This *AtNRT2* gene is expressed in aerial organs and also highly induced in dry seeds. In two allelic *atnrt2.7* mutants, less nitrate is accumulated in the seed. In contrast, seeds from plants overexpressing the *AtNRT2.7* coding region accumulate more nitrate, and as a consequence they are less dormant than the corresponding wild type seeds (Chopin et al. 2007).

Finally, little is known on potential channels or transporters that could be involved in fluxes towards the chloroplast (reviewed in Weber et al. 2005). Fusion proteins with the GFP marker revealed the chloroplastic subcellular localization of the AtCLCe protein. The *atclce* mutants display a phenotype linked both to photosynthesis (Marmagne et al. 2007) and nitrate content (Monachello et al. 2009). The flux of nitrite, the product of nitrate reduction in the cytosol, into the chloroplast could also play a role in the flux of nitrate towards the chloroplast and thus in the homeostasis of cytosolic nitrate. A nitrite transporter belonging to the NRT1 family has been recently identified in cucumber and *Arabidopsis* (Sugiura et al. 2007).

3.2 Ammonium Fluxes

Since the cloning of the first gene involved in ammonium transport (Ninnemann et al. 1994), five other genes belonging to the same family were found in *Arabidopsis* (Gazzarrini et al. 1999; Sohlenkamp et al. 2000), ten in rice (Sonoda et al. 2003), a species adapted to ammonium nutrition, and 14 in poplar (Couturier et al. 2007). Focusing on the results obtained in *Arabidopsis*, kinetics properties of the AMT proteins expressed in oocytes showed Km values ranging from 34 mM for *AMT1;1* (Wood et al. 2006) to 140 mM for *AMT1;2* (Neuhäuser et al. 2007). Among the six genes, *AMT1;1*, *AMT1;2*, *AMT1;3,* and *AMT2;1* are highly expressed in roots (Loqué and von Wirén 2004) and encode proteins that are located in the plasma membranes (Loqué et al. 2006; Yuan et al. 2007). In order to analyze the function of each of this genes separately *in planta*, physiological and ammonium influx studies were carried out on single, double, triple, and quadruple mutants (Yuan et al. 2007). Additive contribution of AMT1;1 and AMT1;3 was shown, while a second saturable transport is thought to be coded by the *AMT1;5* gene. A complex picture is now emerging from these studies (Fig. 3). There is a spatial organization of AMT1 proteins, the transporters possessing the highest ammonium affinities being located in outer root cells or root hairs where they can uptake ammonium from the soil solution (*AMT1;1*, *AMT1;3*, *AMT1;5*). The lower

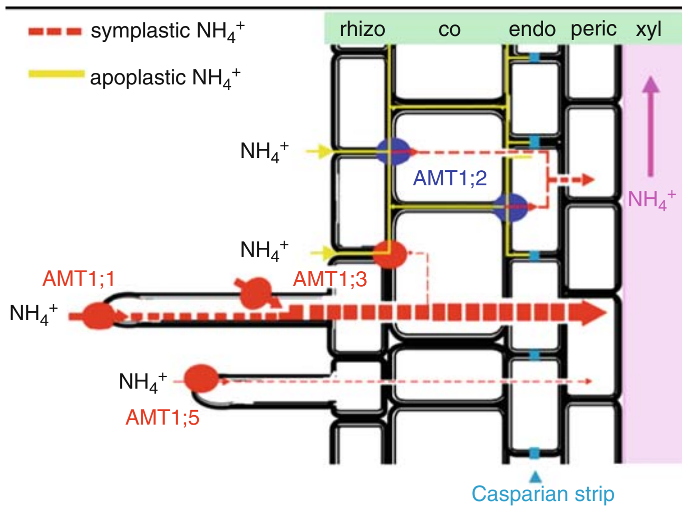

Fig. 3 *Model summarizing the functions of AMT1-type transporters in high-affinity ammonium uptake in Arabidopsis roots* (from Yuan et al. 2007). This schematic representation shows the contribution to ammonium uptake and spatial expression in root tissues of AMT1;1, AMT1;3, AMT1;5 (all in *red*), and AMT1;2 (*blue*) under nitrogen deficiency. AMT-dependent ammonium influx is proportionally represented by the size of their arrows. rhizo, rhizodermis; co, cortex; endo, endodermis; peric, pericycle; xyl, xylem

affinity of *AMT1;2* and its location in the endodermis along the root hair zone suggest a function in the retrieval of ammonium that is released from the cortex, or that enters the root via the apoplastic route.

The electrochemical gradient between vacuole and cytosol would drive NH_3 import to and NH_4^+ export out of the vacuole. Indeed, tonoplast intrinsic proteins of the TIP family were shown to play a role in NH_3 transport into the vacuole (Loqué et al. 2005). Vacuolar loading with NH_4^+ should require an electrogenic ammonium transporter, which has not yet been identified.

3.3 Urea Transport

Although urea is the major nitrogen form supplied as fertilizer in agricultural plant production, its uptake by plant roots or leaves before its hydrolysis has been a matter of debate for a long time. However, studies in crop plants (Merigout et al. 2008a) and *Arabidopsis* (review in Kojima et al. 2006) showed the uptake of urea. The identification of the high-affinity urea transporter *AtDUR3* by Liu et al. (2003a) and of the *AtTIP* urea permeases (Liu et al. 2003b) led to new insights regarding the molecular basis of urea uptake in plants. Growth of mutant lines carrying T-DNA insertions in *AtDUR3* is impaired when urea is the sole nitrogen source. (Kojima

et al. 2007). Physiological and transcriptomic analyses were performed in *Arabidopsis* plant to assess the interactions between urea and ammonium or nitrate uptake and assimilation (Merigout et al. 2008b).

3.4 Organic N Transport

So far, plant putative amino acid transporters have been identified as members of at least five gene families, comprising for example in *Arabidopsis* at least 67 genes (reviewed in Ortiz-Lopez et al. 2000; Rentsch et al. 2007). We will focus here on amino acid transporters shown to be clearly involved in uptake or distribution of amino acids within the plant.

Forward and reverse approaches were used to identify transporters involved in root amino acid uptake (Hirner et al. 2006; Svennerstam et al. 2007). Both studies led to the conclusion that LHT1 (Lysine/histidine transporter) is crucial for root uptake of acidic and neutral amino acids. The AAP1 protein was also shown to transport uncharged amino acids, but only when they are supplied at high concentrations in the external medium (Lee et al. 2007b). Uptake of cationic amino acids like L-Lys or L-Arg is mediated by AAP5 within the concentration range relevant for field conditions (Svennerstam et al. 2008). Näsholm et al. (2009) suggests a hypothetic mode of root amino acid uptake in nonmycorrhizal plants. Although expression of many seed amino acid transporters precedes storage protein synthesis during seed maturation, only a few organic N transporters, among them AtOPT3, have been shown to be essential for seed loading or development (Stacey et al. 2002).

Intracellular transport is expected to be important particularly in the case of amino acid transport. Indeed, plastids are key compartments for amino acid biosynthesis, some of them being exclusively synthesized there (phenylalanine, tyrosine, tryptophan, and lysine) whereas others (glutamine, aspartate, and serine) are produced in multiple compartments. Strikingly, only one protein, Dit2.1, is so far clearly localized at the inner envelope membrane and functions as a glutamate/malate exchanger, essential for the photorespiratory pathway (Renné et al. 2003). Similarly, only transporters for basic amino acids have been localized in the mitochondrial membrane (Catoni et al. 2003; Hoyos et al. 2003). Some transporters have been localized at the tonoplast and their function remains to be demonstrated. The concentration of amino acids in the vacuole is lower than in the cytosol, but so far a vacuolar export system has been shown only in *Chara* vacuoles (Martinoia et al. 2000).

4 N Assimilation Pathways

As described before, the main nitrogen sources taken up by higher plants are nitrate or ammonium as inorganic N sources, and eventually amino acids under particular conditions. Here, we will briefly describe the main steps of nitrate or ammonium

assimilation in growing cells and summarize recent results obtained for source organs when N is remobilized.

4.1 N Assimilation

A global overview of N assimilation in plants is given in Fig. 1. Nitrogen assimilation requires the reduction of nitrate to ammonium, followed by ammonium assimilation into amino acids.

Nitrate reduction into nitrite is catalysed in the cytosol by the enzyme nitrate reductase (NR). This enzyme is a homodimer, each monomer being associated with three prosthetic groups: flavin adenine dinucleotide, a haem, and a molybdenum cofactor (MoCo). Characterization of mutants resistant to chlorate, which can be reduced into toxic chlorite by NR, identified two classes of genes, the *NIA* genes encoding the NR apoenzyme and the *CNX* genes encoding the MoCo cofactor (Pelsy and Caboche 1992; Crawford and Arst 1993). Since 1993, a lot of work has been done to characterize the NR in different species (reviewed in Meyer and Stitt 2001). Although the NR enzyme is thought to be localized in the cytosol (Solomonson and Barber 1990), an association with the plasma membrane (PM-NR) has been found in some species like in corn roots (Chen and Wang 1995) or barley roots (Ward et al. 1989). The structural characteristics and the potential role of this PM-NR have been intensively studied in *Chlorella* by Tischner and collaborators (reviewed in Tischner 2000). Nitrite is then translocated to the cytosol where it is reduced to ammonium by the second enzyme of the pathway, nitrite reductase (NiR). The *NII* genes encoding the NiR enzyme have been cloned from various species, the number of genes varying from one to two copies (Meyer and Stöhr 2002).

Ammonium, originating from nitrate reduction, photorespiration, or amino acid catabolism, is assimilated in the chloroplast by the so-called GS/GOGAT cycle (Lea and Miflin 2004). The glutamine synthetase fixes ammonium on a glutamate molecule to form glutamine. This glutamine reacts subsequently with 2-oxoglutarate to form two molecules of glutamate, this step being catalysed by the glutamine 2-oxoglutarate amino transferase (or glutamate synthase GOGAT). Two classes of genes code for GS: the *GS2* gene, present as a single nuclear gene in all species studied so far, codes for a chloroplastic GS, involved in the assimilation of ammonium stemming from nitrate reduction or photorespiration. Conversely, the *GS1* nuclear gene family codes for cytosolic GS isoforms, present in different organs such as roots or stems and thought to be involved in ammonium recycling during particular developmental steps such as grain filling or leaf senescence (reviewed in Hirel and Lea 2001; Corruzzi 2003). Two different forms of glutamate synthase are present in plants: the Fd-GOGAT and NADH-GOGAT use ferredoxin and NADH as electron donors, respectively. Fd-GOGAT is predominantly localized in leaf chloroplasts, while NADH-GOGAT is primarily located in plastids of non-photosynthetic tissues, such as roots or etiolated leaf tissues. The structural, mechanistic, and

regulatory properties of GOGAT enzymes and their role in amino-acid metabolism have been recently reviewed by Suzuki and Knaff (2005).

4.2 N Remobilization

Although nitrogen uptake still operates at the reproductive stage (Gallais et al. 2007), it is generally assumed that seeds receive a large part of nitrogen from remobilization of different N forms present in source organs (Feller and Keist 1986). During senescence, a re-distribution of amino acids, free or produced by proteolysis of proteins (Patrick and Offler 2001) leads to an increase of asparagine in pea (Rochat and Boutin 1991) and an increase in glutamine in other species, in the phloem sap (Herrera-Rodriguez et al. 2006; Masclaux-Daubresse et al. 2006). Some amino acid transporters of the AAP family are putatively involved in phloem loading (see above). During these particular developmental stages, specific enzymes related to N metabolism are activated (reviewed in Masclaux-Daubresse et al. 2008). Induction of cytosolic GS1 as well as induction of glutamate dehydrogenase appears in a large variety of plants. The latter, catalysing glutamate deamination as well as glutamate synthesis, carried out the de-amination reaction in source leaves (Masclaux-Daubresse et al. 2006). This N remobilization during senescence is also triggered in response to environmental factors such as drought, nutrient limitation, or pathogen attack (Pageau et al. 2006).

5 Regulation of N Uptake and Metabolism

N uptake by the roots and N assimilation are integrated to match the nutrient demand of the whole organism. Regulatory mechanisms that modulate the expression and/or the activity of transport systems and enzymes, according to the nutritional status of the plant and to external stimuli or stresses, ensure both rapid adjustments of metabolism and long term adaptations (Fig. 4).

5.1 Regulation at the mRNA Level

Patterns for changes in mRNA abundance of many components of N uptake and N assimilation have been observed, which allow coordinated regulation of N metabolism. Two main metabolic cues operate in the control of N uptake and assimilation.

The first mechanism includes the induction by substrates and repression by endogenous N assimilates, mediating a negative feedback regulation by the N status of the whole plant (Gazzarrini et al. 1999; Cerezo et al. 2001). This results in up regulation when N is low and down regulation when N is high. Accordingly,

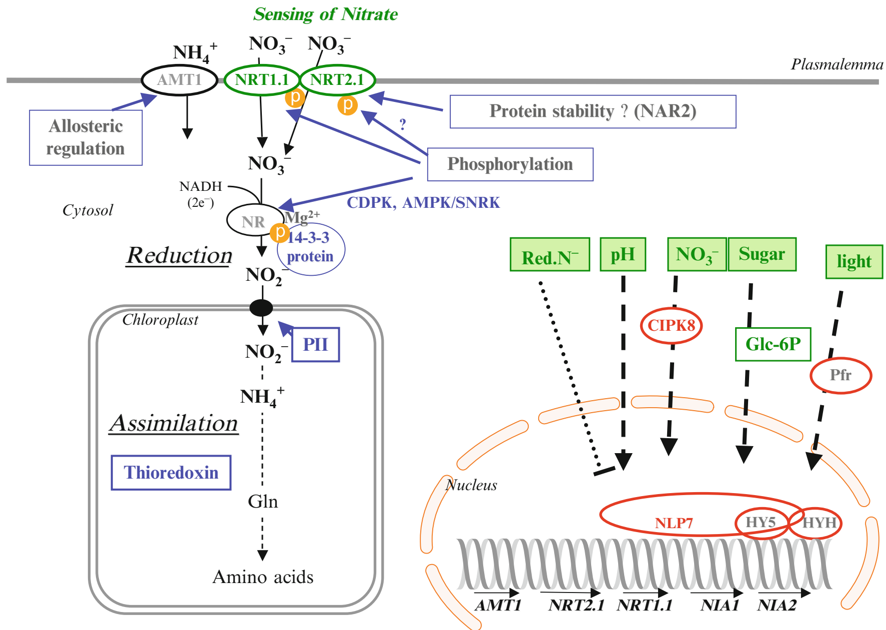

Fig. 4 *Regulatory mechanisms that modulate the expression and/or the activity of transport systems and enzymes*. Components acting on protein level are given in blue, transcriptional regulators in red, and signals and sensors in green (for abbreviation see 1.4)

several *NRT2* and *AMT1* transporters as well as *NIA* and *NII* were found to be repressed at the mRNA level by N metabolites such as amino acids (Tsay et al. 2007). Further studies using the glutamate synthase inhibitor, AZA, or exposure to NH_4^+ or various amino acids suggested that glutamine plays an important role in the down regulation of *NRT2.1* (Nazoa et al. 2003, Zhuo et al. 1999).

In response to N deprivation, *AMT1.1*, *AMT1.3,* and *AMT1.5* (Gazzarrini et al. 1999; Gansel et al. 2001; Loqué et al. 2006), as well as *AtNRT2.1*, *AtNRT2.2,* and *AtDUR3,* are induced (Lejay et al 1999; Scheible et al. 2004). Interestingly, two genes of the NRT2 family are slowly but steadily induced by starvation (Orsel and Krapp, unpublished data). Resupply of nitrate re-induces *NRT2.1,* and *NRT2.2* as well as *NIA* and *NII* expression after long term starvation (Scheible et al. 2004), whereas expression of *NRT2.4* and *NRT2.5* is repressed by the resupply of any N source (Okamoto et al. 2003).

Transcriptional regulation of genes involved in LATS for NH_4^+ and NO_3^- is less documented. *NRT1.1* shares many regulatory features with *NRT2.1*. *NRT1.1* is rapidly induced by nitrate and by starvation but less subjected to regulation by N metabolites (Tsay et al. 1993), while *AtNRT1.2* is constitutively expressed (Huang et al. 1999); *AtNRT1.5* is much more slowly induced by nitrate, and is in addition regulated by potassium. *AtNRT1.1* and *AtNRT1.5* are both regulated by pH (Tsay et al. 1993; Lin et al. 2008).

Global transcriptome studies (Wang et al. 2003; Scheible et al. 2004) confirmed transcriptional regulation of N uptake and assimilation by nitrate and showed a broad action spectrum of nitrate as a regulator of gene expression, coordinating for example C and N metabolism. Using NR mutants (Wang et al. 2004) it was shown that nitrate itself acts as signal. Another study (Wang et al. 2007) investigating gene regulation by nitrite showed an overlap between nitrate and nitrite regulated genes. Nevertheless, specific regulation by nitrite was shown for several genes of N uptake (e.g. *NRT2.5, AMT1.3*). Nitrite was already discussed by Loqué et al (2006) as signaling molecule for the regulation of *NRT1.1* and *NIA1*.

The second major regulation of N uptake and N assimilation corresponds to the stimulation by photosynthesis (Lejay et al. 2003), which ensures that N uptake is harmonized with the C status. A major common feature is the diurnal fluctuation of N uptake and N reduction. This control has often been attributed to the regulatory action of sugars produced by photosynthesis and transported downward to the roots, as shown by the positive effect of CO_2 concentration on NO_3^- uptake (Gastal and Saugier 1989; Delhon et al. 1996) Diurnal fluctuations in uptake and assimilation, or stimulation by sugars, are generally correlated with the expression of genes encoding transporters and enzymes. This has been shown for NH_4^+ transporters (Gazzarrini et al. 1999; von Wirén et al. 2000; Lejay et al. 2003), NO_3^- transporters (Lejay et al. 1999; Ono et al. 2000; Matt et al. 2001), and NR and NiR (Vincentz et al. 1993). In *Arabidopsis*, genes tested by Lejay et al. (2003, *AtNRT2.1* and *AtNRT1.1)*, showed 5–10 times higher expression during the light period compared with the dark period. Nitrate uptake, measured using $^{15}NO_3^-$ also increased after the onset of light. The increase was approx. two-fold during the photoperiod. The decrease in *AtNRT2.1* and *AtNRT1.1* mRNA levels and nitrate uptake during the dark period was prevented by supply of 1% sucrose to the roots, which is a further indication for the role of sugars during diurnal regulation. This regulation seems to be independent of the known sugar regulation pathways, such as hexokinase signaling (Lejay et al. 2003). Recently Lejay et al. (2008) showed that up-regulation of nitrate transporters (*AtNRT2.1* and *AtNRT1.1*) was related to the concentration of glucose 6-phosphate. Contrary to that of the transporters, the diurnal regulation of *NIA* transcripts is not only governed by sugars but also by light regulation via phytochrome (Rajasekhar et al. 1988). In addition, *NIA* expression is controlled by signals from photosynthetic electron flow, which adds to the picture of intracellular cross-talk between chloroplasts and the nucleus (Sherameti et al. 2002).

Despite the very important regulation of transcript abundance by external and internal factors, information about the molecular players such as transcription factors, miRNA, etc. is still rather rare. Lately two bZIP (basic leucine zipper) transcription factors have been discovered as being involved in the light regulation of N metabolism (Jonassen et al. 2008): HY5 and its homolog HYH are essential for phytochrome dependent light-activated expression of NR. ChIPchip analyses showed a binding site for HY5 in the *NIA2* promoter (Lee et al. 2007a). Interestingly also the *NRT1.1* promoter has three binding sites for HY5, but HY5 has a negative effect on transcription in this case (Lillo 2009). However, not all light regulation of N metabolism is governed by the HY5/HYH system (Lillo 2009).

Camargo et al. (2007) identified CrNIT2 as a main regulator of *NIA* expression in *Chlamydomonas,* and Castaings et al. (2009) showed that *Arabidopsis* mutants in a homologous gene (NLP7) are defective in the nitrate induction of *N*IA genes, *NRT2.1* and *NRT2.2*. Both proteins belong to a class of putative transcription factors homologous to a protein first identified in *Medicago* and essential for nodulation (NIN = nodulation inception). The CrNIT2 protein has been shown to bind to multiple sites of the *NIA* promoter, but no target genes are yet known for the AtNLP7 protein. Interestingly, mutants in the *CIPK8* gene which encode a protein kinase (Hu et al. 2009), are also unable to fully induce expression of several genes by nitrate, such as the *NIA* genes, *NRT2.1, NRT1.1,* and several others. It is tempting to speculate that CIPK8 might be involved in the same regulation pathway than NLP7. *NLP7* belongs to a gene family with nine different members, but the functions of the other NLP proteins are still unknown.

5.2 Regulation at the Protein Level

N metabolism has to respond fast to external stimuli. This can be achieved by rapid post-translational protein modification.

The best studied case of post-translational regulation in N metabolism is the regulation of higher plant NR. NR is inactivated via a two step process that involves phosphorylation of ser^{543} in spinach and the subsequent magnesium-dependent binding of an inhibitory 14-3-3 protein to NR (Bachmann et al. 1996; Moorhead et al. 1996). This activation/inactivation process is linked to the production of C assimilates that thus control NR activity (De Cires et al. 1993; Kaiser and Huber 2001). Both CDPK (calcium-dependent protein kinases) and AMPK/SNRK (SNF1-related kinase)-related protein kinases are able to phosphorylate NR at least in vitro (McMichael et al. 1995; Douglas et al. 1997; Sugden et al. 1999; Ikeda et al. 2000). The inactive phosphorylated form is re-activated by dephosphorylation probably by PP2A (MacKintosh 1992).

Protein phosphorylation may act as a trigger for protein degradation, as well as for binding of the inhibitory 14-3-3 proteins. When a modified form of NR with a truncated N-terminus that was not susceptible to post-translational dark inactivation was overexpressed, the resulting protein did not decline in the second part of the photoperiod (Nussaume et al. 1995). There is also a correlation between the phosphorylation state or the activation state of NR and the rate at which NR protein decreases (Geiger et al. 1998; Kaiser and Huber 1997; Scheible et al. 1997; Weiner and Kaiser 1999).

Post-translational regulation of nitrate transporters has recently been described. The nitrate transporter NRT1.1 is regulated by phosphorylation. When phosphorylated, AtNRT1.1 functions as a high affinity transporter, whereas it is active in the low affinity range when dephosphorylated (Liu and Tsay 2003). Recent data show that NRT1.1 acts not only as a transporter, but is also involved in N signaling (Remans et al. 2006; Walch-Liu and Forde 2008). Interestingly in one case, only the phosphorylated form is an active signaling component (Walch-Liu and Forde

2008). Nitrate transporters from the NRT2 family are also subjected to post-transcriptional regulation. First indications of putative phosphorylation of NRT2 proteins came from their amino acid sequences (Forde 2000). In addition, several of the NRT2 proteins have been identified in global phosphoprotein studies (Benschop et al. 2007). Such a post-transcriptional regulation may explain why high affinity NO_3^- influx is down-regulated by NH_4^+ in transgenic plants expressing *NpNRT2.1* cDNA under the control of a constitutive, root specific promoter (Fraisier et al. 2000). Recently, Wirth et al (2007) showed that despite strict transcriptional regulation of *AtNRT2.1* , NRT2.1 protein levels are rather constant in response to light, sucrose, or nitrogen treatments that strongly affect both *NRT2.1* mRNA level and HATS activity. Again post-translational regulation processes are required to explain these observations. One such mechanism could correspond to the cleavage of NRT2.1 C terminus, which results in the presence of both intact and truncated proteins in the plasma membrane (Wirth et al. 2007). Several forms of the protein seem to co-exist in cell membranes (the monomer and at least one higher molecular weight complex). However, the monomer is the most abundant form of NRT2.1, and seems to be the one involved in $NO3^-$ transport (Wirth et al. 2007). Interestingly, AtNRT2.1 is only present and active at the plasma membrane in the presence of AtNAR2.1 (Orsel et al. 2006; Wirth et al. 2007). The mechanism by which NAR2.1 affects NRT2.1 is so far unknown, but might open a new level of regulation by protein stability or protein transport.

A different form of post-translational regulation has been revealed for ammonium transporters allowing rapid shut-off in order to avoid toxic accumulation of ammonium. Loqué et al (2007) showed that the soluble carboxy terminus of the oligomeric AtAMT1 serves as an allosteric regulator essential for function. It is suggested that this C terminus interacts physically with cytosolic loops in the neighboring subunit with phosphorylation as a regulating mechanism.

Less is known about nitrite transport and its regulation. In *E. coli*, the PII protein regulates nitrite transport. This regulation seems to be conserved in plants. The chloroplastic PII protein might be involved in the regulation of nitrite uptake by chloroplast as mutants affected in the gene exhibit a nitrite sensitive phenotype (Ferrario-Méry et al. 2005). This hypothesis was re-enforced by the increased nitrite uptake by chloroplasts isolated from PII mutants (Ferrario-Méry et al. 2008).

Several chloroplastic enzymes of nitrogen assimilation such as NIR, GS2, and Fd-GOGAT are redox regulated through the thioredoxin system (Lemaire et al. 2007; Lichter and Häberlein 1998). In addition NR is also regulated by NO, a by-product of its own activity. NO production and the broad mode of action are described in the following paragraph.

6 N- Signaling: Nitric Oxide – A Special Case

Nitrate and other low molecular weight intermediates of nitrogen metabolism are not only substrates, but also act as signals regulating the interaction between metabolic pathways of growth and differentiation, or plant interactions with the

environment. Among these nitrogen signals, nitric oxide has gained specific attention during the last decade. Therefore, the role of this N-compound will be considered in more detail in context with N-metabolism.

6.1 Sources for NO in Plants

NO (+2) may be formed either by reduction of higher N-oxidation states, preferentially nitrite, or by oxidation of more reduced N-forms (for review see del Rio et al. 2004). Figure 5 summarizes pathways for NO production.

Reductive NO formation: Nitrate reduction appears always linked to the production of trace amounts of NO, originating from a one-electron reduction of nitrite. The reduction can be mediated by NR, or, at least in non-green plant tissues, by mitochondrial electron transport (Planchet et al. 2005; Gupta et al. 2005). In both cases, nitrite competes with the "normal" substrates (e.g. nitrate in the case of NR or oxygen in the case of mitochondrial ET), and therefore rather high nitrite concentrations are required for appreciable rates of NO production. Cytosolic nitrite concentrations are usually low (10–20 μM). Nevertheless, nitrate-fertilized plants emit NO into NO- free air at rates that can be detected and quantified by sensitive analytical methods such as gas-phase chemiluminescence. For example, with illuminated tobacco leaves, NO emission was 0.3 nmoles/g FW h (Rockel et al. 2002). Rates were lower in the dark, because NR activity is down regulated. As NO is rather reactive, real NO production rates inside leaf cells could be much higher, but this is not known with certainty. NR is activated by light or by anoxia in the dark, whereas nitrite reduction becomes very low under anoxia in the dark, presumably because NADPH production via oxidative pentose phosphate cycle ceases. In consequence, nitrite accumulates in anoxic cells and tissues to millimolar concentrations, and therefore anoxic NO emission can become 1,000-fold higher than in air (Rockel et al. 2002; Planchet et al. 2005). In NiR-deficient tobacco mutant leaves, which always accumulate nitrite even in air (*light*), NO emission was as high in air (*light*) as in nitrogen (*dark*). In NR-free *nia1nia2* double mutants, NO emission in air and in nitrogen was absent (Planchet et al. 2005). The oxygen-dependent NOS reaction appeared not to contribute to this normal "bulk"-NO emission from leaves.

Plants possess yet another PM-bound NR plus a nitrite::NO reductase, which together can also produce NO (Stöhr and Stremlau 2006). No genes for these two enzymes have been identified so far, and their physiological role is still under investigation.

NO generation from nitrite may also occur non-enzymatically in acidic compartments at pH-values below 5. Such compartments might be either the mesophyll apoplast or vacuoles. While apoplastic NO formation has been localized by DAF-2 fluorescence (Bethke et al. 2004), no vacuolar NO production has been reported so far, which is actually astonishing.

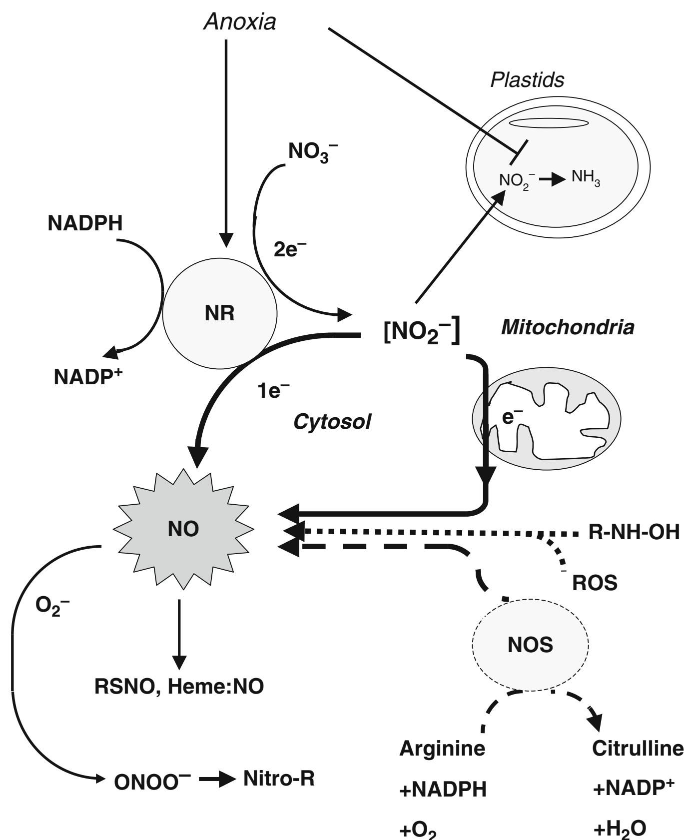

Fig. 5 *Pathways of nitric oxide (NO) synthesis, and basic reactions of NO with different targets.* NO can be synthesized by nitrite reduction, mediated either by NR itself of by mitochondrial electron transport (the latter only in roots). Nitrite to NO reduction requires high nitrite concentrations, which can become especially high under anoxia, when NR is highly active (dephosphorylated), and nitrite reduction is impaired. Also shown are the two oxidative pathways for NO synthesis; one is the (probably non-enzymatic) oxidation of hydroxylamines by reactive oxygen, the other one is the oxidation of L-arginine by a NOS-like activity. NO may either react directly with heme groups of enzymes forming Fe:NO adducts, or it may react with thiol groups to form nitrosothiols. At least in theory, NO may also react with superoxide radicals to form the highly reactive peroxynitrite, which may nitrosate aromatic amino acids

Oxidative NO formation: In animals the major NO source is L-arginine, which is oxidized to NO and L-citrulline in a complex process catalyzed by the family of NOS-enzymes (nitric oxide synthases), using NADPH and O_2 as further substrates. No gene homolog to the animal NOS family has been detected so far in *Arabidopsis*. Nevertheless, there are numerous indirect hints on NOS-like activities in plants, on the basis of effects of NOS-inhibitors, and also of enzyme activity measurements using NO measurement by EPR, of nitrite + nitrate

production, or of conversion of labeled L-arginine into L-citrulline (for review see del Rio et al. 2004). Recently, an enzyme converting L-arginine to citrulline and NO has been purified from *Arabidopsis* shoots. The activity depends on the typical NOS-cofactors BH4 and Calmodulin (R. Tischner, pers. communication). Sequence information on that preparation may give a first insight into the nature of plant NOS.

Another substrate for oxidative NO formation are hydroxylamines, which can be oxidized by plant cells to NO, probably using superoxide and/or H_2O_2 as oxidants (Rümer et al. 2009). Although it is not yet clear whether the reaction is physiologically relevant, there is little doubt that plants are able to produce NO not only via nitrite reduction, but also via oxidation of amine-N.

Concentrations of NO and its "bioavailability" in cells will also depend on NO consumption (Vanin et al. 2004). NO oxidation involving reactive oxygen species (ROS), or O_2-dependent oxidation catalyzed by hemoglobins (Dordas et al. 2003) should be among the most important reactions consuming NO. In addition, reversible binding of NO to thiols may be an important aspect regulating cellular NO levels (see below).

6.2 Mechanisms Through Which NO Affects Targets

In the complex cellular environment, NO may undergo various oxidation and/or dismutation reactions, yielding compounds like NO_2, N_2O_3, the nitrosonium cation (NO^+), or the nitroxyl anion (NO^-). Some of these products may rapidly and reversibly nitrosylate protein- or non-protein thiols, or form nitrosyl-iron complexes with metal ions, e.g. in heme-proteins. Peroxynitrite ($ONOO^-$) may be formed from the reaction of NO with superoxide anions. However, it is not clear to what extend the reaction occurs under natural conditions in vivo. Peroxynitrite may serve as a substrate for oxidation or nitration of aromatic amino acids. Nitration appears less easily reversible than nitrosylation. Because 3-tyrosine nitration occurs on the same position (3) that is also the site for phosphorylation, it can be assumed that tyrosine nitration has important consequence for regulation mediated via tyrosine protein kinases/phosphatases.

Cysteine-*S*-nitrosylation (also called nitrosation) appears as the most widespread way in which proteins are post-translationally modulated by NO (Fig. 5). More than 100 redox-sensitive proteins were identified in *Arabidopsis* as putative candidates for cysteine *S*-nitrosylation (Lindermayr et al. 2005). In animals, NO was shown to regulate by *S*-nitrosylation signaling-related proteins including soluble guanylate cyclase, the GTP-binding protein p21ras, Ca^{2+} permeable channels, and protein kinases (for review see Courtois et al. 2008, and literature cited). Already a decade before, Stamler et al. (1997) had suggested a general "nitrosylation motiv" consisting of three or four basic or acidic amino acids surrounding the regulatory cysteine, which would permit an acid-base-autocatalyzed *S*-nitrosylation and denitrosylation. In general, the actual

nitrosylating agent appears to be the nitrosonium cation NO^+, and hence *S*-nitrosylation would require an electron acceptor.

Glutathion in its reduced form is major cellular antioxidant. It reacts readily with NO to form the acid-stable *S*-nitrosoglutathione (GSNO), which may act as a NO donor to other cellular thiols. Such transnitrosation would include transfer of NO^+ to another reduced thiol (Dutton et al. 2005), or RSNO may be homolytically cleaved to release free NO and disulfide (Singh et al. 1996). GSNO can be metabolized by *S*-nitrosoglutathione reductase (GSNOR), yielding, e.g. GSSG, hydroxylamine, and NH_3 (Jensen et al. 1998). Hydroxylamine can be oxidized back to NO, probably involving ROS (Rümer et al. 2009). The relevance of GSNOR and GSNO levels for stress tolerance was recently demonstrated. Transgenic plants *Arabidopsis* with decreased GSNOR levels showed enhanced resistance against *Peronospora parasitica* correlated with higher intracellular GSNO levels (Rustérucci et al. 2007). The *Arabidopsis* HOT5 encoding a mutated GSNOR was unable to acquire thermotolerance and also had other important developmental defects (Lee et al. 2008).

NO also induces complex changes in the expression of many genes involved, e.g. in defense and cell death, transport, basic metabolism, and ROS production or degradation. Here again, *S*-nitrosylation of proteins acting as transcription factors might be the way for transcriptional control by NO. Seven families of transcription factor binding sites, among them WRKY-, GBOX-, and OCSE-elements, have been identified, which are preferentially located in the promoter regions of NO regulated genes, and co-expression of many genes can be explained by the cooperation of a set of such transcription factors (Palmieri et al. 2008).

As NO may be too short lived to diffuse via longer distances within tissues or even within single cells, it has been suggested that NO production (preferentially by NOS) and NO reception may be organized within supra molecular structures in which NO signaling occurs within highly localized environments and with minimal diffusion of free NO (Kone et al. 2003). Although this is an attractive idea, today there is no experimental evidence in context with NO that such supra molecular structures would exist and function in plants.

NO-regulated reactions in plants. The list of physiological processes in plants that are (probably) regulated by NO includes the induction of the hypersensitive response in resistance to incompatible pathogens, ABA-induced stomatal closure, seed germination and breakage of seed dormancy, iron homeostasis, flowering induction, and response to abiotic stresses such as drought, UV-B, salinity, chilling, or high temperatures (for recent reviews see Hong et al. 2008; Courtois et al. 2008; Neill et al. 2008). In spite of these many putative NO-regulated processes, today only few plant enzymes have been proven experimentally to be regulated by *S*-nitrosylation, hemoglobin 1, GAP-dehydrogenase, *S*-adenosyl synthethase, metacaspase, and potassium channels in guard cells being among them (summarized by Palmieri et al. 2008).

As mentioned, “regulatory” NO is either stemming from a NOS-like reaction or from nitrite to NO reduction. Involvement of nitrate metabolism in production of regulatory NO has been evidenced in a few cases only. For example, ABA-induced

stomatal closure in *Arabidopsis* is impaired in the *nia* double mutant. Tungstate, which prevents synthesis of functional NR, also inhibited stomatal closure, whereas nitrite addition induced stomatal closure (Bright et al. 2006; Neill et al. 2008, and literature cited). Similarily, Chitosan-induced stomatal closure in *Pisum sativum*, which may prohibit easy entry of pathogens into the leaf, was impaired by tungstate treatment, which would again suggest some role for nitrite-dependent NO (together with NOS-derived NO) (Srivastava et al. 2009). ABA-induced stomatal closure was also reduced in a nia1::DS deletion mutant, indicating that only *NIA1*, but not *NIA2* was required for effective ABA signal transduction (Bright et al. 2006). This is surprising, as *NIA1* is thought to contribute only about 10% to total nitrite production (Wilkinson and Crawford 1991), and because a specific response to *NIA1* would require a mechanism by which cells can distinguish between nitrite and NO derived from one or the other protein.

Another connection between nitrate reduction, NO production, and a physiological response seems to exist for the induction of the HR in *Arabidopsis* by incompatible strains of *Pseudomonas syringae*. Here, the HR was impaired in the *nia1nia2* mutant compared to WT, and was restored by addition of nitrite (Modolo et al. 2006). However, the *nia* mutants had significantly lower arginine contents compared to WT, which might limit their NOS activity. Thus, it appeared possible that this was an indirect response to the low arginine and not directly related to the lack of nitrite.

Recently it was suggested that NO produced from nitrite would enhance NR activity in roots of *Brassica chinensis* L, thereby forming a positive feedback loop. The conclusion was based on the observation that treatment of roots with NO gas, NO donors, or NO scavengers modified extractable NR activity in the roots. In addition, treatment of purified NR or of NR in root extracts of tomato with NO in vitro also increased NR activity (Du et al. 2008; Jin et al. 2009), suggesting a direct interaction of NO with NR. It is not known yet in detail how NO modifies NR, i.e. whether NO interacts with heme-iron of the cytochrome domain or whether it forms a nitrosothiol. One consequence appears to be an increase in V_{max}, of all partial reactions of NR.

7 Conclusion

Plants use a multitude of N forms, and their uptake, transport in the plant, and assimilation are taken care of by numerous transporters and enzymes. Their quantity, localization, and the regulation of their activity enable plants to adapt quickly and finely their N acquisition and utilization strategies to developmental and environmental changes. The availability of full genome sequences, in addition to new tools and resources for functional genomics, allows the use of systems biology in the last decade to give an entire view of this important metabolic pathway in plants. Still some effort is needed to reach a virtual plant. The *in planta* function of many of the proteins is still to discover and the actors implicated in the regulation

on mRNA and protein levels are just about to emerge. The N metabolite NO is implicated in many regulatory processes, but its synthesis pathways and their control, as well as the exact mode of interaction of NO with multiple targets, still need to be elucidated.

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