

# Nitrate: Nutrient and Signal for Plant Growth

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## INTRODUCTION

The mineral nutrient needed in greatest abundance by plants is nitrogen. Plants, however, must compete for nitrogen in the soil with abiotic and biotic processes such as erosion, leaching, and microbial consumption. Soil nitrogen is also lost when crops are harvested and plant material is removed from the soil. To be competitive, plants have evolved several mechanisms to acquire nitrogen at low concentrations and to use a variety of forms of nitrogen. Plants can assimilate inorganic forms, such as nitrate and ammonia, and organic forms, such as urea. Some plants, including legumes, can fix dinitrogen gas in association with symbiotic bacteria (see Mylona et al., 1995, this issue). This review focuses on the assimilation of nitrate, a transient yet critical form for plants.

Two key questions arise when considering nitrate assimilation. First, how do plants acquire optimal quantities of nitrate when the soil nitrate concentration can vary from 10  $\mu\text{M}$  to 100 mM? Second, how is nitrate assimilation integrated into the overall metabolic program of plants so that optimal amounts of energy and carbon are provided? What is known about nitrate assimilation at the cellular level is summarized in Figure 1. Once taken up, nitrate is either stored in the vacuole or reduced to nitrite by nitrate reductase (NR). Nitrite enters the chloroplast (or plastid in the root) and is then reduced to ammonia by nitrite reductase (NiR). Ammonia can then be fixed into carbon by the action of glutamine synthetase (see Lam et al., 1995, this issue). Reducing energy is provided in the form of NAD(P)H for NR and reduced ferredoxin for NiR. Glutamate provides the immediate carbon skeleton. Because nitrate assimilation produces several hydroxide ions, organic acids are also required for pH homeostasis. Coordinating these steps is a regulatory network that is responsive to both internal and external signals, some of which are diagrammed in Figure 1. Ultimately, it is the understanding of this regulatory network that will answer the aforementioned key questions.

Genetic analysis of nitrate assimilation has been possible because mutants blocked in the pathway can be rescued by providing ammonia as a source of nitrogen. Such mutants are obtained either by screening directly with an *in vitro* assay for plants defective in nitrate reduction or by selecting plants that are resistant to chlorate, the chlorine analog of nitrate. When chlorate is taken up and reduced by NR, toxic chlorite is produced. In higher plants, chlorate-resistant mutants are impaired in nitrate/chlorate reduction because they have either a defective

nitrate reductase structural gene (*NIA*) or a defective molybdenum cofactor gene (*CNX*). The one exception is the *chl1* mutant of *Arabidopsis*, which is defective in nitrate/chlorate uptake. Only in fungi and algae have regulatory mutants been described.

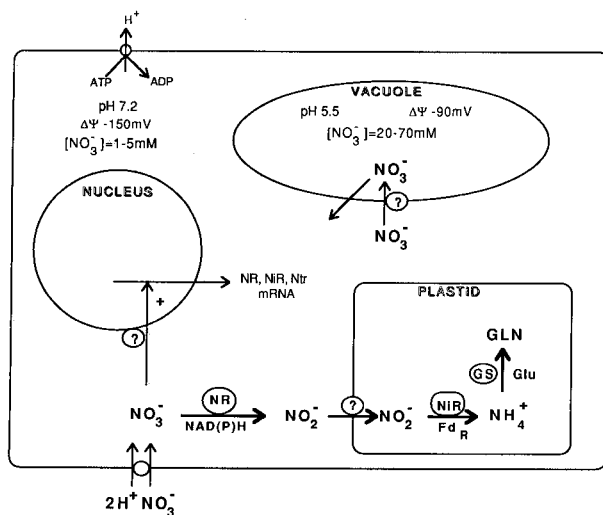
There are many excellent recent reviews of the nitrate assimilation field (Pelsy and Caboche, 1992; Warner and Kleinhofs, 1992; Cove, 1993; Crawford and Arst, 1993; Marzluf, 1993; Crawford, 1994; Hoff et al., 1994; Huppe and Turpin, 1994). This review provides an update on recent molecular advances that have uncovered genes and mechanisms responsible for nitrate uptake, reduction, and regulation.

## NITRATE UPTAKE

The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen in the face of external nitrate concentrations that can vary by five orders of magnitude. To function efficiently in the face of such environmental variation, plants have evolved a transport system that is active, regulated, and multiphasic. The energy that drives nitrate uptake comes from the proton gradient maintained across the plasma membrane by the  $\text{H}^+$ -ATPase (Figure 1). The individual components of the nitrate uptake system are just now being discovered, and they are revealing interesting parallels with uptake systems from algae, fungi, and mammals.

The initial uptake of nitrate occurs across the plasma membrane of epidermal and cortical cells of the root (Larsson and Ingemarsson, 1989). Subsequent transport can occur across the tonoplast membrane (Granstedt and Huffaker, 1982; Blumwald and Poole, 1985; Miller and Smith, 1992) and the plasma membrane of cells in the vascular system and leaf (Jackson et al., 1986). This discussion is restricted to the initial uptake process, that is, transport of nitrate across the plasma membrane of root cells.

The transport of anionic nitrate into a root cell must occur up an electrical gradient with potentials that are typically between  $-100$  and  $-250$  mV (negatively charged inside the cell; Figure 1). Given an external nitrate concentration of 2 mM and an electrical potential of  $-110$  mV, one would expect that the



**Figure 1.** Schematic Diagram of the Nitrate Assimilation Pathway.

The  $H^+$ -ATPase in the plasma membrane pumps protons out of the cell, producing pH and electrical ( $\Delta\Psi$ ) gradients. The nitrate transporters (Ntr) cotransport two or more protons per nitrate into the cell. Nitrate can be transported across the tonoplast and stored in the vacuole. Nitrate in the cytosol is reduced to nitrite, which enters the plastid and is reduced to ammonia. Ammonia is fixed into glutamate (Glu) to produce glutamine (GLN) by the action of glutamine synthetase (GS). Nitrate also acts as a signal to increase the expression of nitrate reductase (NR), nitrite reductase (NiR), and Ntr genes.

cytoplasmic concentration of nitrate would be 0.028 mM, based on the Nernst equation. In barley root cells, however, cytoplasmic nitrate concentrations have been recorded between 1 and 5 mM, that is, 35 to 175 times the predicted level (Zhen et al., 1991; King et al., 1992). This indicates that energy is required to drive the transport of nitrate into the cell.

Current evidence strongly indicates that nitrate uptake in plants is driven by electrogenic proton cotransport. During nitrate uptake, the plasma membrane undergoes a rapid and transient depolarization, becoming up to 60 mV more positive inside the cell within 1 to 2 min (Ullrich and Novacky, 1981; McClure et al., 1990a, 1990b; Ullrich and Novacky, 1990; Glass et al., 1992; Tsay et al., 1993b). Application of inhibitors of the plasma membrane  $H^+$ -ATPase and alkalization of the external medium both inhibit nitrate uptake (McClure et al., 1990a, 1990b). Conversely, acidifying the external medium drives the uptake of chlorate, an analog of nitrate, into plasma membrane vesicles preloaded with chlorate (Ruiz-Cristin and Briskin, 1991).

If only one proton were cotransported with each nitrate anion, no depolarization would be observed. Thus, nitrate uptake involves two or more protons cotransported with every nitrate ion. The depolarization is transient, with the membrane potential beginning to recover within 1 to 2 min after exposure to nitrate. The mechanism for this recovery is not known, but it is likely to result from stimulation of the  $H^+$ -ATPase. Similar

depolarizations and proton cotransport mechanisms have been observed for chloride and phosphate uptake (Ullrich-Eberius et al., 1981, 1984; Sakano, 1990; Ullrich and Novacky, 1990; Sakano et al., 1992).

Plants have distinct transport systems with different affinities for nitrate. The high affinity system(s) is responsible for nitrate uptake at low concentrations (below  $\sim 1$  mM). Typically, these systems have saturable kinetics, with  $K_m$  values below 300  $\mu$ M. The lowest reported  $K_m$  values for nitrate uptake are 7  $\mu$ M in barley (Aslam et al., 1992) and 0.2  $\mu$ M in some marine algae (Eppley et al., 1969). These exceptional systems can efficiently scavenge very low levels of nitrate. For example, whereas optimal ryegrass growth requires at least 1.4 mM nitrate, just 14  $\mu$ M nitrate can support growth of ryegrass at 90% optimal levels (Clement et al., 1978).

The cloning of a high affinity nitrate transporter gene has not been reported for higher plants, but three such genes have been isolated from fungi and algae. The *Aspergillus CRNA* gene was first identified in a search for chlorate-resistant fungi (Cove, 1979). *crnA* mutants have a two- to fourfold reduction in high affinity nitrate uptake ( $K_m$  of 200  $\mu$ M) in young mycelia but can still grow normally on nitrate as the sole nitrogen source (Brownlee and Arst, 1983). *CRNA* is part of a gene cluster that includes *NIAD* and *NIIA*, which encode NR and NiR, respectively (Tomsett and Cove, 1979; Johnstone et al., 1990). *CRNA* was cloned and found to encode a membrane protein with 10 to 12 putative membrane-spanning segments, as would be expected for a cotransporter (Unkles et al., 1991). It was concluded that *CRNA* encodes a high affinity nitrate transporter (Unkles et al., 1991). No data on the localization or enzymatic activity of the *CRNA* gene product are available to confirm this conclusion. Presumably, there are additional *Aspergillus* proteins that take up nitrate, but none has been described.

Two related transporter genes have been identified in *Chlamydomonas*. This alga also has a cluster of genes that includes the NR gene, *NIT-1* (Quesada et al., 1993). If three of the five genes in the cluster (*NAR-2*, *NAR-3*, and *NAR-4*) are deleted, nitrate uptake at 50  $\mu$ M is blocked and cells can no longer grow on nitrate (Quesada et al., 1994). Complementation of the triple mutant requires two of the three genes: *NAR-2* and *NAR-3* or *NAR-2* and *NAR-4*. Sequence analysis shows that the *NAR-3* and *NAR-4* proteins are almost 80% identical to each other over the region analyzed (the C-terminal 250 amino acids) and are 27 to 31% identical to the *CRNA* protein of *Aspergillus* over this region. Thus, *Chlamydomonas* appears to have two high affinity nitrate transporter genes that are highly related, are located within the same gene cluster, and share similarity with fungal nitrate transporters. No localization or heterologous expression data for the *NAR-3* and *NAR-4* genes are available. The function of *NAR-2* is not known, but it may play a regulatory role.

That the sequences of these three uptake genes are conserved between fungi and algae suggests that higher plants may also have related genes. Recently, such a gene was cloned from barley (Trueman et al., 1994). The cDNA clone pBCH1 encodes a 55-kD protein with 12 putative membrane-spanning

regions that is 32% identical to CRNA and 50% identical to NAR-3. Transcripts of this gene accumulate in roots and become more abundant upon nitrate treatment. As yet, no functional data have been reported for the pBCH1 protein, but it is likely that a member of this gene family will turn out to encode a high affinity nitrate transporter.

In addition to the high affinity transporters, plants have low affinity systems for uptake of nitrate at high concentrations (above  $\sim 1$  mM). Like the high affinity systems, the low affinity systems are electrogenic and involve proton cotransport (Glass et al., 1992). The low affinity systems, however, often show linear rather than saturable kinetics, have higher  $K_m$  values (often 0.5 mM or above), and can be found in both nitrate-treated and untreated plants (Jackson et al., 1973; Doddema and Telkamp, 1979; Behl et al., 1988; Hole et al., 1990; Siddiqi et al., 1990; Aslam et al., 1992, 1993).

The identification of a low affinity nitrate transporter gene came from studies of the *CHL1* gene of *Arabidopsis*. *chl1* mutants are resistant to chlorate and show reduced nitrate uptake when external nitrate concentrations exceed 1 mM, the low affinity range (Braaksma and Feenstra, 1973; Doddema and Telkamp, 1979). A T-DNA insertion mutant was used to clone the *CHL1* gene, which turned out to encode a membrane protein with 12 putative membrane-spanning segments (Tsay et al., 1993b). Expression of *CHL1* in *Xenopus* oocytes showed that it encodes an electrogenic nitrate transporter whose activity is enhanced by acidifying the external medium. The  $K_m$  for nitrate transport by *CHL1* in oocytes is high ( $\sim 8$  mM; Y.-F. Tsay and N.M. Crawford, unpublished data), which is consistent with *CHL1* encoding a low affinity transporter. *CHL1* mRNA is found predominantly in roots, and its level increases when roots are exposed to nitrate or acidic pH. Analysis of *chl1* mutants suggests that *CHL1* may be involved in the transport of additional ions (Scholten and Feenstra, 1986). The location of *CHL1* in the roots has yet to be determined.

*CHL1* is a member of an interesting gene family that includes at least four additional related genes. Some of these genes may turn out to encode nitrate transporters. Surprisingly, one of the members encodes a peptide transporter. An *Arabidopsis* gene, *AtPTR2*, that was cloned by complementation of a yeast peptide transport mutant (Steiner et al., 1994), encodes a hydrophobic protein with 12 putative membrane-spanning segments that is 25% identical to *CHL1*. *AtPTR2* is also 45% identical to *PTR2*, a yeast di- and tripeptide transporter (Perry et al., 1994). Among other things, peptide transporters are important for seed germination because they transport storage protein degradation products via the scutellum to embryos (Payne and Walker-Smith, 1987).

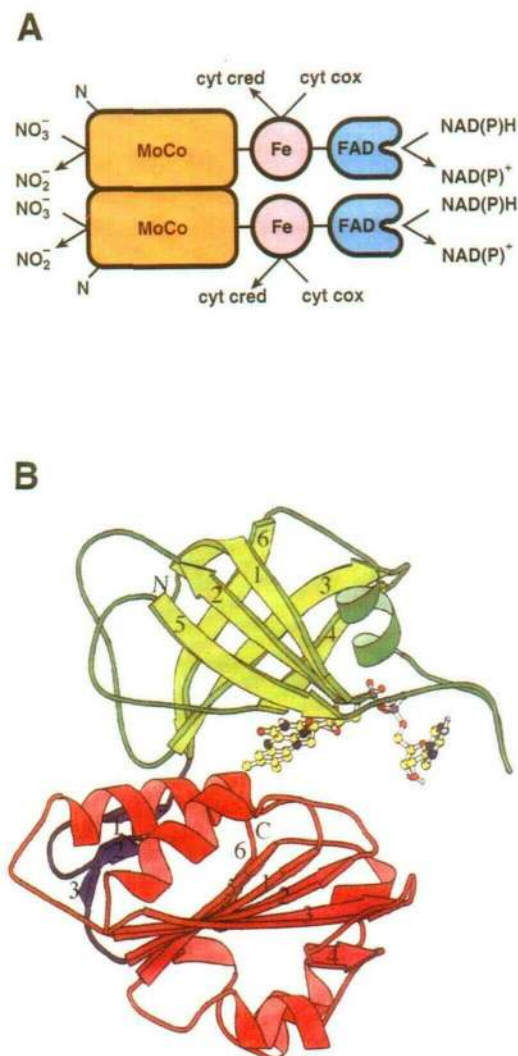
*CHL1*-related proteins are also involved in animal peptide transport, especially in the intestine. The animal peptide transporter gene *Pept1* was isolated by expression cloning in *Xenopus* oocytes using a rabbit intestinal cDNA library (Fei et al., 1994). *Pept1* encodes a protein with 12 putative membrane-spanning segments that is  $\sim 25\%$  identical to *CHL1*. Unlike many animal symporters, protons, not sodium, are used to drive the uptake of peptides by *Pept1*. The *CHL1* gene family

also includes peptide transporters from bacteria and has been named the POT family (proton-dependent oligopeptide transport; Paulsen and Skurray, 1994) and the PTR family (peptide transport; Steiner et al., 1994, 1995). This family is distinct in sequence from the *mdr* (multidrug resistance) P-glycoprotein gene family, whose members have also been shown to transport peptides and many other solutes, although both families encode proteins with 12 membrane-spanning regions (Hyde et al., 1990; Sharma et al., 1992; Sarkadi et al., 1994). It will be interesting to see whether any of the PTR/POT proteins transport other ions, such as nitrate.

## NITRATE REDUCTASE

After the uptake of nitrate into the cell, the next step in the nitrogen assimilation pathway is reduction of nitrate to nitrite. This step competes with both efflux of nitrate from the cell and transport of nitrate into the vacuole (Figure 1). The enzyme that catalyzes the reduction reaction, NR, is located primarily in the cytosols of root epidermal and cortical cells and shoot mesophyll cells (Rufty et al., 1986; Vaughn and Campbell, 1988; Fedorova et al., 1994). NR transfers two electrons from NAD(P)H to nitrate via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor, which is a complex of molybdate and pterin. The complexity of NR is reflected in its size: it is a homodimer or homotetramer of 110-kD subunits. Each redox center is associated with a functional domain of the enzyme that has activity independent of the other domains (Figure 2A) (Redinbaugh and Campbell, 1985; Crawford et al., 1988; Campbell and Kinghorn, 1990; Solomonson and Barber, 1990). For example, just two domains of NR, the heme and FAD domains, are needed to catalyze a reaction in which cytochrome *c* is used instead of nitrate as an alternate electron acceptor. Cytochrome *c* reductase activity involves transfer of electrons from NAD(P)H to the FAD domain, which reduces the heme domain, which in turn reduces cytochrome *c*. The MoCo domain need not be intact for this partial reaction to work.

The FAD domain expressed in *Escherichia coli* has recently been crystallized and found to contain two lobes: an N-terminal lobe containing six antiparallel  $\beta$ -strands that bind directly to the FAD molecule and a C-terminal lobe containing six parallel  $\beta$ -strands that form a cleft with the N-terminal lobe (Figure 2B) (Lu et al., 1994). At the top of the cleft is the FAD, whose isoalloxazine moiety, which accepts the electrons from NAD(P)H, is packed next to the aromatic ring of an invariant tyrosine and is tethered with several hydrogen bonds to the peptide backbone and several side chains. The space underneath the FAD in the cleft is where the NAD(P)H may bind. Comparison of the FAD domain of NR with that of other flavo-proteins, such as ferredoxin reductase, indicates that residues in the loop between the  $\beta 3$  and  $\beta 4$   $\beta$ -strands of the C-terminal lobe may help specify which substrate is bound by the enzyme, NADH or NADPH.



**Figure 2.** Structural Diagrams of Nitrate Reductase.

**(A)** A schematic diagram of the NR homodimer with the functional domains indicated. FAD, flavin domain; Fe, heme domain; MoCo, molybdenum cofactor domain; N, N terminus of NR; cyt c, cytochrome c, which can be used as an alternate electron acceptor in vitro; cyt cred and cyt cox, the reduced and oxidized forms of cytochrome c, respectively.

**(B)** Ribbon model of the structure of the FAD/NADH binding domain of maize NR. This domain contains two lobes, indicated in green and red, that are connected by a short domain shown in purple. The green lobe, which binds the FAD, is N-terminal to the red lobe. The molecule in the cleft is FAD. From Lu et al. (1994) and used with permission of Current Science, Ltd.

The heme domain has also been purified after expression in *E. coli* (Cannons et al., 1993). This isolated domain behaves like the domain in the native enzyme except that it has a more positive "midpoint potential" (+16 mV, compared with -160 mV in the native enzyme). These data indicate that residues outside

the heme domain alter the redox potential of the heme. This potential may be important because transfer of electrons from the heme domain to the MoCo domain may be the rate-limiting step in catalysis (Cannons and Solomonson, 1994). Although the structure of the NR heme domain has not been determined, it bears sequence similarity with mammalian cytochrome *b<sub>5</sub>* and is therefore likely to have a similar structure (Meyer et al., 1991). A mutation in the *Nicotiana plumbaginifolia* NR gene that completely abolishes activity changes a histidine predicted to be one of the axial ligands for the heme to an asparagine (Meyer et al., 1991).

The FAD and heme domains have been expressed as one recombinant protein without the MoCo domain (Campbell, 1992; Cannons and Solomonson, 1994). This protein retains the same activities (NADH-cytochrome c reductase) and enzymatic properties as the corresponding domains in the native enzyme. A crystal structure should be available soon to elucidate how the heme domain interfaces with the FAD domain.

As will be discussed later, NR is tightly regulated at the transcriptional and post-transcriptional levels. Therefore, it is surprising that mutations that reduce NR activity 10-fold have little observable effect on the growth of plants fed only nitrate (Warner et al., 1981; Vaucheret et al., 1990; Wilkinson and Crawford, 1991; Crawford et al., 1992; Dorbe et al., 1992). Why plants produce so much NR protein is a mystery. Overexpression of NR with the cauliflower mosaic virus (CaMV) 35S promoter in transgenic *N. plumbaginifolia* (up to five times the wild-type level) has little effect on growth and metabolism but does reduce leaf nitrate levels (Vincentz and Caboche, 1991; Foyer et al., 1993, 1994; Quillere et al., 1994).

## NITRITE REDUCTASE

The next step in the nitrate assimilation pathway is the reduction of nitrite to ammonia by NiR. NiR is a nuclear-encoded enzyme that is transported into the chloroplast, leaving behind a 30-amino acid transit sequence (Gupta and Beevers, 1987; Back et al., 1988). The holoenzyme is a monomer (60 to 70 kD) with two redox centers: a siroheme-Fe center and an iron-sulfur center. Based on sequence comparisons with other enzymes, the C-terminal half of NiR is thought to contain the redox centers and the N-terminal half is thought to bind the reducing agent ferredoxin (Campbell and Kinghorn, 1990). Ferredoxin reduced by the chloroplast noncyclic electron transport system provides the electrons for reducing nitrite. NiR is also active in roots and is found primarily in proplastids (Suzuki et al., 1985; Bowsher et al., 1989). A ferredoxin-like protein reduced by NADPH from the oxidative pentose phosphate pathway is thought to be the source of reductant.

It has been difficult isolating a NiR mutant in plants because inhibiting nitrite reduction would lead to the accumulation of toxic nitrite (Duncanson et al., 1993). A reduction of NiR levels has been achieved in tobacco by expressing NiR antisense RNA (Vaucheret et al., 1992a). As expected, an antisense

transformant with no detectable NiR activity displayed impaired development and chlorotic leaves when fed nitrate and had to be maintained on ammonia as the sole nitrogen source. Interestingly, this transformant had higher and still inducible levels of NR activity and mRNA, perhaps because the levels of a nitrogen metabolite (such as glutamine) that inhibits NR expression are reduced in the transgenic plant.

## NITRATE AS SIGNAL

One of the especially interesting features of nitrate assimilation is its regulation. Nitrate serves as the primary signal for regulating nitrate assimilation, but light, cytokinin, CO<sub>2</sub> levels, circadian rhythms, carbon, and nitrogen metabolites such as sucrose and glutamine all play regulatory roles as well. A complex regulatory network involving both transcriptional and post-transcriptional mechanisms serves to integrate nitrate assimilation, which consumes energy and carbon skeletons, with photosynthesis and carbon metabolism. The elucidation of the plant regulatory machinery has been guided by the extensive work with fungal systems, primarily *Neurospora* and *Aspergillus*.

One of the universal responses in fungi and plants to nitrate exposure is the induction of gene expression (for review, see Crawford and Arst, 1993; Marzluf, 1993; Hoff et al., 1994). Responding genes encode nitrate transporters, NR, NiR, glutamine synthetase, and ferredoxin-dependent glutamate synthase (Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Redinbaugh and Campbell, 1993; Hoff et al., 1994). In plants, the induction occurs within minutes and in response to very low concentrations of nitrate (down to 10 μM). In fungi and algae but not higher plants, ammonia blocks the induction due to catabolite repression.

The study of nitrate regulation in fungi and algae has led to the identification of regulatory genes that are responsible for both nitrate induction and ammonia repression. In fungi, the genes mediating the nitrate induction (*NIRA* from *Neurospora* and *NIT-4* from *Aspergillus*) encode positive regulators with zinc finger DNA binding domains similar to GAL4 from yeast (Fu et al., 1989; Burger et al., 1991a, 1991b). The genes mediating ammonia repression (*AREA* of *Aspergillus* and *NIT-2* of *Neurospora*) also encode positive regulators with zinc finger DNA binding motifs that are distinct from *NIRA* and *NIT-4* (Fu and Marzluf, 1990a, 1990b; Kudla et al., 1990). In algae, the *Chlamydomonas NIT2* regulatory gene, which was isolated by transposon tagging (Schnell and Lefebvre, 1993), encodes a positive regulator whose expression is repressed by ammonia. Thus, catabolite repression of nitrate assimilatory genes in *Chlamydomonas* may be mediated by the repression of *NIT2*. No nitrate regulatory genes have been identified in higher plants. However, *cis*-acting regulatory regions have been detected in the promoters of both the spinach NiR gene and the Arabidopsis NR gene that confer nitrate-inducible expression

to reporter genes in transgenic tobacco (Rastogi et al., 1993; Lin et al., 1994).

In addition to nitrate, light is an important regulatory signal for NR and NiR expression. In etiolated plants, a phytochrome-mediated pathway increases NR and NiR mRNA levels upon transfer to the light (Rajasekhar et al., 1988; Schuster and Mohr, 1990; Seith et al., 1991; Becker et al., 1992; Mohr et al., 1992). In the phytochrome-deficient *aurea* mutant of tomato, red light induction of NR and NiR genes is reduced (Becker et al., 1992). In *cop1* mutants of Arabidopsis, which display a de-etiolated phenotype in the dark, NR mRNA levels are high in the dark, along with those of many other light-regulated genes (X.-W. Deng et al., 1991). In green tissues, NR mRNA levels also increase in the light, but a constitutive low level persists in the dark when nitrate is present. If green plants are placed in a diurnal cycle, the levels of NR and NiR mRNAs oscillate even if the plants are returned to constant light conditions (Galangau et al., 1988; Deng et al., 1990; Bowsher et al., 1991; Cheng et al., 1991; Becker et al., 1992; Pilgrim et al., 1993; Lillo, 1994). Interestingly, if NR is inactivated by inhibitors or by mutation, NR mRNA levels do not oscillate but stay high during the diurnal cycle, even though mRNA levels of other light-regulated genes, such as *cab*, continue to fluctuate (Deng et al., 1989; Pouteau et al., 1989). Perhaps oscillations in the concentration of a metabolite such as glutamine, which affects NR mRNA levels, cease in NR-deficient plants (M.-D. Deng et al., 1991; Vincentz et al., 1993).

Carbon metabolites signal changes in nitrate assimilation and can supersede the light signal. Carbon metabolites are critical for nitrate reduction because carbon skeletons are required for ammonia fixation and the energy from reduced carbon is needed for nitrate reduction in nongreen tissues. If excised leaves or cells in culture are treated with sucrose in the dark, NR activity and mRNA increase (Aslam and Huffaker, 1984; Cheng et al., 1992; Crawford et al., 1992; Vaucheret et al., 1992b; Vincentz et al., 1993; Lillo, 1994). In addition, NR promoter elements confer on reporter genes sucrose induction in the dark (Cheng et al., 1992; Vincentz et al., 1993). At present, it is not known how sugars induce NR gene expression nor which metabolite is the proximal inducer. Interestingly, NiR mRNA levels do not respond to sugar (Vincentz et al., 1993). Glutamine has a repressive effect on NR mRNA levels (for review, see Hoff et al., 1994).

The regulation discussed previously occurs at the transcriptional level. However, post-transcriptional regulation also occurs, as indicated by the fact that the level of NR mRNA can be uncoupled from the level of NR protein. If tomato plants are starved for nitrogen, for example, the level of NR protein drops to 20% of the induced level within 24 hr, whereas NR mRNA levels remain high for 12 days (Galangau et al., 1988). Similarly, if the NR coding region is constitutively expressed with a CaMV 35S promoter in tobacco, NR protein levels drop four- to fivefold after 72 hr of dark treatment, even though NR mRNA levels remain high (Vincentz and Caboche, 1991). If a mutant form of NR missing 56 amino acids in an acidic region preceding the MoCo domain is constitutively expressed

in the same manner, the levels of the mutant NR do not drop significantly after 72 hr of darkness (Nussaume et al., 1995). These results imply that the structural integrity of the enzyme is needed for post-transcriptional regulation by light and that a sequence in the N-terminal region of the enzyme may play an important role in this regulation.

NR is also regulated by another post-transcriptional mechanism that is both rapid and reversible: phosphorylation. NR activity is rapidly reduced 3- to 10-fold when plants are transferred to the dark or to a low CO<sub>2</sub> environment (Kaiser and Brendle-Behnisch, 1991; Huber et al., 1992; Riens and Heldt, 1992). When the light is turned on or normal CO<sub>2</sub> levels are restored, NR activity is restored. Inactivation of NR is mediated by phosphorylation (Kaiser and Spill, 1991; J.L. Huber et al., 1992, 1994; S.C. Huber et al., 1994; MacKintosh, 1992; Kaiser and Huber, 1994b). Spinach, Arabidopsis, and maize NRs have been shown to be phosphorylated on serines, producing multiple phosphorylated peptides when digested with trypsin or cyanogen bromide (J.L. Huber et al., 1992, 1994; LaBrie and Crawford, 1994). Phosphorylation of two (in spinach) or three (in maize) of these peptides increases when NR is inactivated by the dark treatment (J.L. Huber et al., 1992, 1994). Reactivation of NR correlates with dephosphorylation of these peptides, which can be blocked with phosphatase inhibitors (Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994a). Thus, phosphorylation plays an important role in modulating NR activity rapidly and reversibly in response to carbon and light signals. This regulation of NR may be critical to prevent the accumulation of toxic nitrite when levels of reduced ferredoxin become low enough, especially in the dark, to restrict nitrite reduction in the plastid.

Preliminary evidence suggests that phosphorylation involves the MoCo domain of NR. Partial activity assays using methyl viologen instead of NADH as the electron donor show that electron flow from the heme via the MoCo domain to nitrate is inhibited in phosphorylated NR (Huber et al., 1992). In addition, a mutation in the MoCo domain of NR can drastically impair NR phosphorylation (LaBrie and Crawford, 1994). This mutation converts a glycine to an aspartic acid and reduces phosphate levels to 10 to 20% of the wild-type level. Because the mutant NR is inactive, it has not been possible to determine how reduced phosphorylation affects NR regulation. However, because a single alteration in the MoCo domain can disrupt phosphorylation, possibly at multiple sites, the MoCo domain is likely to be a target of the phosphorylation. More experiments are needed to pinpoint the key regulatory serines that are phosphorylated.

Inactivation of NR involves not only a kinase but also an inhibitory protein. When inactivated NR from dark-treated spinach is partially purified, NR activity is restored even though NR is still phosphorylated (MacKintosh et al., 1995). Other fractions from the purification inactivate NR when mixed with the partially purified, active NR fraction. The inhibitory fractions contain a protein called NIP that stoichiometrically inactivates NR when it is phosphorylated but has no effect on dephosphorylated NR. In another study, two protein fractions,

containing 100-kD and 67-kD proteins, were found to be necessary for inactivating spinach NR in vitro in the presence of ATP (Spill and Kaiser, 1994). The 67-kD fraction appears to contain the NR kinase, and the 100-kD fraction may contain NIP.

## CONCLUSIONS AND FUTURE PROSPECTS

At least one player of each step of the nitrate assimilation pathway has been identified and cloned. In the next year or two, genes that encode plant high affinity nitrate transporters and the plant MoCo enzymes will be isolated and characterized. Already, two MoCo biosynthetic genes from Arabidopsis have been cloned by complementing *E. coli* MoCo mutants (Hoff et al., 1995). Expression systems will be perfected to produce quantities of NR sufficient for crystallization and structure determination. These systems will also allow the production of mutant forms of NR that will help us to better understand how the enzyme functions and is regulated. Progress has been made in our understanding of the regulatory mechanisms in this pathway, especially the role of phosphorylation in controlling NR activity, but regulatory mutants, transcription factors, nitrate receptors, and signal transduction mechanisms still need to be elucidated.

As we learn more about the basic biology and chemistry of nitrate assimilation, this system will continue to provide important tools for both basic and applied research. For example, the NR gene has been used as a selectable marker for fungal and algal transformation (Kindle et al., 1989; Unkles et al., 1989). In higher plants, the NR coding region has been fused to the CaMV 35S promoter (Nussaume et al., 1991) and to the promoter of the light-harvesting chlorophyll *a/b* protein (Heimer et al., 1995) for use in counterselections and mutant isolations. Several nitrate assimilatory genes, including *NIA* and *CHL1*, have been used successfully as transposon traps (Grandbastien et al., 1989; Tsay et al., 1993a). For the future, the NR enzyme holds promise for use in nitrate determination and elimination devices for dealing with nitrate pollution in drinking water. For example, an experimental bioreactor with immobilized NR has been developed that reduces nitrate to dinitrogen gas using electrical currents (Mellor et al., 1992). The hope that major crop plants will be engineered to require less nitrogen fertilizer for optimal yield is still alive because we now have some of the critical genes that control nitrate assimilation and can alter their expression in vivo.

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