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The regulation of nitrate and ammonium transport systems in plants

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Abstract

Inorganic nitrogen concentrations in soil solutions vary across several orders of magnitude among different soils and as a result of seasonal changes. In order to respond to this heterogeneity, plants have evolved mechanisms to regulate NO_3^- and NH_4^+ influx. In addition, efflux analysis using ^{13}N has revealed that there is a co-ordinated regulation of all component fluxes within the root, including biochemical fluxes. Physiological studies have demonstrated the presence of two high-affinity transporter systems (HATS) for NO_3^- and one HATS for NH_4^+ in roots of higher plants. By contrast, in *Arabidopsis thaliana* there exist seven members of the *NRT2* family encoding putative HATS for NO_3^- and five members of the *AMT1* family encoding putative HATS for NH_4^+. The induction of high-affinity NO_3^- transport and *Nrt2.1* and *Nrt2.2* expression occur in response to the provision of NO_3^-, while down-regulation of these genes appear to be due to the effects of glutamine. High-affinity NH_4^+ transport and *AMT1.1* expression also appear to be subject to down-regulation by glutamine. In addition, there is evidence that accumulated NO_3^- and NH_4^+ may act post-transcriptionally on transporter function. The present challenge is to resolve the functions of all of these genes. In *Aspergillus nidulans* and *Chlamydomonas reinhardtii* there are but two high-affinity NO_3^- transporters and these appear to have undergone kinetic differentiation that permits a greater efficiency of NO_3^- absorption over the wide range of concentration normally found in nature. Such kinetic differentiation may also have occurred among higher plant transporters. The characterization of transporter function in higher plants is currently being inferred from patterns of gene expression in roots and shoots, as well as through studies of heterologous expression systems and knockout mutants.

Key words: Ammonium, *AMT1*, flux regulation, nitrate, *Nrt2*.

Introduction

Inorganic ions accumulated in plant cells serve nutritional, osmotic, signalling, and storage functions. Insufficient ion accumulation as well as excess accumulation may therefore compromise these functions. While vacuolar reserves may buffer the cytoplasm against short-term perturbations, in laboratory studies when external sources of ions are removed vacuolar reserves are typically exhausted within a few days (Glass, 1975; Lee *et al.*, 1990; van der Leij *et al.*, 1998). Under field conditions vacuolar reserves may be even more limited. When vacuolar reserves are consumed to sustain cytosolic functions, there is a need to replace their osmotic and charge-balancing function by means of alternative

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Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; Gln, glutamine; Glu, glutamate.

solutes, be they inorganic or organic. Hence vacuolar buffering of cytosolic ion concentrations is not achieved without consequences and, typically, plant roots respond to perturbations of external supply or internal demand long before vacuolar reserves are exhausted. This raises the interesting issue of the signal pathways between vacuole and cytoplasm required to initiate these responses; virtually unexplored territory.

Given that both NO_3^- and NH_4^+ commonly serve as sources of N for plant growth and that they share some metabolic pathways, it is perhaps not surprising to find that they possess features in common: (1) both ions are actively absorbed into root cells at low external concentrations; (2) influx measurements indicate the presence of two high-affinity transport systems (HATS) for NO_3^- (one constitutive and the other inducible) and one HATS for NH_4^+; (3) influx of both ions is responsive to plant N status and subject to diurnal regulation; (4) molecular studies indicate the presence of seven HATS for NO_3^- and five for NH_4^+ in *A. thaliana*; and (5) some of the genes encoding NO_3^- transporters are subject to transcriptional regulation through inductive effects of NO_3^-, while some of those encoding NO_3^- and NH_4^+ transporters are subject to down-regulating effects of glutamine. Notwithstanding these similarities there are also distinct differences in the characteristics of NO_3^- and NH_4^+ uptake, as well as differences among species in the extent of their utilization of these different nitrogen sources.

Soil heterogeneity

Heterogeneity of soil nutrient availability is potentially the most important perturbing effect upon plant nutrient status. In addition, seasonal and diurnal changes in growth rates and plant demand for resources are also substantial. In this paper, the main focus will be upon flux regulation in response to perturbations of external supply and, in particular, the responses of the HATS for NO_3^- and NH_4^+ to these perturbations. In the context of these effects that would displace the plant from steady state, ion fluxes are regulated by feedback from various cellular parameters that serve to counteract such changes.

According to data compiled previously, NO_3^- and NH_4^+ concentrations of agricultural soils range across three to four orders of magnitude (Wolt, 1994). The situation is even more variable in natural soils (Jackson and Caldwell, 1993). In addition, specific habitats (e.g. mature forests, arctic tundra) may be characterized by nitrogen profiles dominated by ammonium or amino acids, rather than NO_3^-. Many species occupying such habitats have become specialists, absorbing NH_4^+ or amino acids in preference to NO_3^- (Kielland, 1994; Kronzucker *et al*., 1997; Nasholm *et al*., 1998, 2000). Even when NO_3^- exceeds NH_4^+ by as much as 10-fold, NH_4^+ uptake may still greatly exceed that of NO_3^- in field and laboratory studies (Gessler *et al*., 1998). In a study of nitrogen absorption by tomato (MY Siddiqi *et al*., unpublished data), it was demonstrated that 50% of plant N was absorbed as NH_4^+, even though this ion represented only 10% of available N, the remaining 90% being NO_3^-. In the context of this variability of N supply plants have evolved numerous mechanisms (physiological/biochemical, developmental and life history-based strategies) that enable them to optimize nitrogen acquisition. Included among the physiological adaptations, are the 'up-regulation' of nitrogen uptake under conditions of N-limitation, but also the restriction of nitrogen uptake under conditions of N excess. The latter presumably serves to minimize potentially harmful osmotic or specific ion effects.

Physiological characterization of NO_3^- and NH_4^+ uptake

Measurements of $^{13}NO_3^-$ influx and net NO_3^- uptake by several groups have revealed the presence of three transport systems for NO_3^- and two for NH_4^+ (reviewed in Glass and Siddiqi, 1995). In roots of species examined for its presence, a low capacity, constitutively expressed, high-affinity transport system (cHATS) allows entry of NO_3^- from low external NO_3^-. The extent of this flux varies among and within species (Siddiqi *et al*., 1989; King *et al*., 1993; Kronzucker *et al*., 1995; Zhuo *et al*., 1999). Following first exposure to NO_3^- there is a rapid increase of an inducible high-affinity influx (iHATS), which is followed (after several h) by an equally rapid down-regulation of this flux (Siddiqi *et al*., 1989; Zhuo *et al*., 1999). There are significant differences in the response time to applied NO_3^- among species. For example, in *Picea glauca*, it was necessary to expose plants to NO_3^- for 3 d in order to induce peak $^{13}NO_3^-$ influx (Kronzucker *et al*., 1995). Both NO_3^- and NO_2^- are capable of inducing this flux (Siddiqi *et al*., 1992; Aslam *et al*., 1993).

Several studies have demonstrated that the provision of NH_4^+ to N-deprived roots may initially increase NH_4^+ uptake prior to down-regulating the flux, and the term induction has also been applied to this initial increase of influx (see Kronzucker *et al*., 1998, for references and discussion). However, in these studies high-affinity NH_4^+ influx was already high (de-repressed) before exposure to NH_4^+, and it has been demonstrated that, in rice, the increase of NH_4^+ influx resulting from NH_4^+ pretreatment was relatively small (25–40%) (Kronzucker *et al*., 1998). By comparison, a 30-fold increase of $^{13}NO_3^-$ influx was recorded in Klondike barley following pretreatment with NO_3^- (Siddiqi *et al*., 1990). Kronzucker *et al*. concluded that the evidence did not support a true inductive effect of NH_4^+ (Kronzucker *et al*., 1998).

At nitrate and ammonium concentrations between ~200 to 500 μM, low-affinity transporter systems (LATS) for these ions become apparent. These were evident in earlier studies (Doddema and Telkamp, 1979; Ullrich *et al.*, 1984), but were largely overlooked, in part because the measurement of NO_3^- and NH_4^+ uptake at high concentration by depletion methods was typically insufficiently sensitive to characterize these transporters. A perplexing feature of these high capacity low-affinity transporters has been their linear concentration responses (Pace and McClure, 1986; Ullrich *et al.*, 1984), that were earlier suggested to result from diffusive fluxes. However, although NH_4^+ fluxes via LATS are typically thermodynamically 'downhill' (Ullrich *et al.*, 1984; Wang *et al.*, 1993), the LATS for NO_3^- was shown to be active even at high external NO_3^- concentration and mediated, like the iHATS, by a proton:nitrate symport (Glass *et al.*, 1992).

Homeostatic processes for nitrogen uptake

As outlined above, the uptake of both NO_3^- and NH_4^+ is subject to down-regulation as tissue N levels approach some upper limit. As early as 1906, Brezeale demonstrated, using hydroponic wheat plants, that withholding K, P, N, Ca or S for 18 h resulted in several-fold increases in rates of absorption of the particular nutrient that had been withheld (Brezeale, 1906). As far as is known, this is the first documented evidence of the physiological regulation of ion uptake by plant roots. Clement *et al.*, using ryegrass as a model system, established that, when available NO_3^- concentrations were maintained from 14.3 μM to 14.3 mM, plant growth was only modestly affected and tissue nitrogen concentration remained essentially constant (Clement *et al.*, 1978*a*). The up-regulation of nitrate fluxes first observed by Brezeale forms an important component of the processes responsible for achieving nitrogen homeostasis (Brezeale, 1906), while adjustments in growth rate may also be critical under some circumstances (Ingestad and Lund, 1979). While NH_4^+ transport shows the same general homeostatic propensity (Wang *et al.*, 1993; Rawat *et al.*, 1999), the potential toxicity of elevated ambient NH_4^+ concentrations severely limits the range of NH_4^+ concentration over which adaptation is possible. In a study of $^{13}NH_4^+$ fluxes across the plasma membranes of barley roots, Britto *et al.* showed that at 10 mM external NH_4^+, active NH_4^+ efflux rose to 76% of the value of influx (Britto *et al.*, 2001). Simultaneously, root respiration increased by 40%, and was not diminished by treatment with the GS inhibitor methionine sulphoximine (MSX), indicating that the respiratory increase was not associated with increased assimilation of NH_4^+, but with active extrusion. In summary, while high-affinity NH_4^+ fluxes are effectively regulated, transport via the low-affinity system is poorly regulated, resulting in considerable futile cycling of NH_4^+ across the plasma membrane as well as toxic effects of excessive NH_4^+ accumulation (Britto *et al.*, 2001).

Studies of the many component NO_3^- and NH_4^+ fluxes that occur in plant cells are severely limited, even in single-celled organisms by cellular compartmentation. In multicellular plants fluxes to and from roots via xylem and phloem further complicate the situation. Therefore, for technical reasons involving the ease of measurement, the emphasis in studies of the mechanisms responsible for ion fluxes and their regulation has been upon the influx step (ϕ_{oc}) across the plasma membrane. Nevertheless, there is evidence to suggest that efflux from cytosol to cell wall (ϕ_{co}), fluxes across the tonoplast (ϕ_{cv} and ϕ_{vc}), from cytosol to xylem (ϕ_{cx}), as well as fluxes to biochemical pathways appear to be co-ordinated. The use of efflux analysis to estimate the half-lives ($t_{0.5}$) for $^{13}NO_3^-$ and $^{13}NH_4^+$ residence within the cytosolic compartment, has revealed that $t_{0.5}$ values are virtually independent of prior nitrogen provision (Siddiqi *et al.*, 1991; Wang *et al.*, 1993; Britto and Kronzucker, 2001). Figure 1 shows data for $^{13}NO_3^-$ efflux from roots of barley grown under steady-state conditions with various concentrations of nitrate for 7 d prior to labelling with $^{13}NO_3^-$ and subsequent measurement of $^{13}NO_3^-$ efflux into non-labelled solutions of the same NO_3^- concentration (Britto and Kronzucker, 2001). Despite the wide range of NO_3^- concentrations used and the substantial changes of measured fluxes, the rate constants for $^{13}NO_3^-$ efflux were essentially identical (0.0408, 0.0400, 0.0417, 0.0418, and 0.04908 min^{-1} for plants grown in 10, 1, 0.1, 0.01, and 0 mM NO_3^-, respectively). In a study of the effect of perturbing external NH_4^+ on $^{13}NH_4^+$ efflux from barley

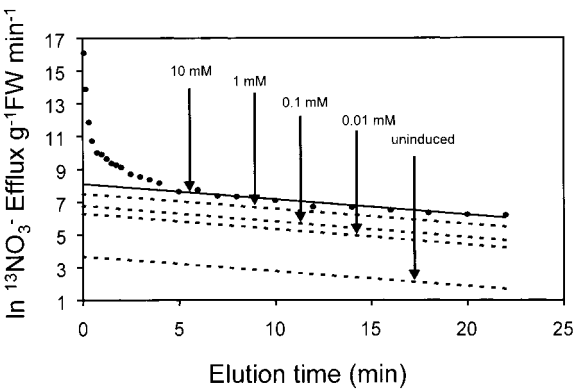

Fig. 1. $^{13}NO_3^-$ efflux from roots of barley plants grown with different concentrations of NO_3^-. Plants were grown for 7 d under steady-state conditions with respect to nitrate provision. Roots were then loaded with $^{13}NO_3^-$ for >5 cytoplasmic half-lives, and subsequently transferred to the same concentration of $^{14}NO_3^-$ for measurement of $^{13}NO_3^-$ efflux. Rate constants for the lines were 0.041 min^{-1} (10 mM), 0.040 min^{-1} (1 mM), 0.042 min^{-1} (0.1 mM), 0.042 min^{-1} (0.01 mM), and 0.039 min^{-1} (uninduced plants), respectively (from Britto and Kronzucker, 2001).

roots, Britto and Kronzucker showed that when external NH_4^+ concentration was increased or decreased, respectively, from 1 mM to either 10 mM or to 100 μM, there was initially a rapid increase or decrease, respectively, of $^{13}NH_4^+$ efflux (Britto and Kronzucker, 2001). Yet, despite this initial perturbation of tracer efflux, rate constants for this flux were restored to their original values within minutes as shown in Fig. 2. Such results point to a precise integration of all component fluxes that impact upon cytosolic ion concentrations.

Several studies using $^{13}NO_3^-$ and $^{13}NH_4^+$ have demonstrated that ϕ_{co} increases as external ion concentration increases (Siddiqi *et al*., 1991; Wang *et al*., 1993) and that net transfer of nitrogen from vacuole to cytosol (ϕ_{vc}–ϕ_{cv}) increases (van der Leij *et al*., 1998), and from cytosol to stele (ϕ_{cx}) decreases (Kronzucker *et al*., 1998), as external ion concentrations decrease. Nevertheless, these fluxes have not been quantified in the same detail that has characterized measurements of ϕ_{oc}, nor have genes yet been cloned that encode these transport systems. Likewise there is a lack of detailed studies of the fluxes of NO_3^- and NH_4^+ into leaf cells. Having noted the paucity of information concerning fluxes other than the root influx step, the remainder of this paper, will focus on the regulation of high-affinity NO_3^- and NH_4^+ influx across the plasma membrane of root cells.

Induction and down-regulation of influx

It is evident from a number of different studies that only NO_3^- or NO_2^- among potential products of nitrogen assimilation are capable of inducing NO_3^- influx by the iHATS (Tompkins *et al*., 1978; Behl *et al*., 1988; Siddiqi *et al*., 1992; Tischner *et al*., 1993; Guy and Heimer, 1993; Henriksen and Spanswick, 1993). Nevertheless, as low-N plants accumulate N, the influxes of both NO_3^- and NH_4^+ are subsequently down-regulated (Lee and Rudge, 1986; Morgan and Jackson, 1988; Siddiqi *et al*., 1989; Kronzucker *et al*., 1995; Glass and Siddiqi, 1995; Forde and Clarkson 1999). Prior to the cloning of genes that encoded NO_3^- and NH_4^+ transporters, two hypotheses emerged to explain this down-regulation. On the one hand it was proposed that accumulated NO_3^- or NH_4^+ themselves, as opposed to their downstream metabolites, were responsible for down-regulation of fluxes. This was based upon inverse correlations between accumulated NO_3^- or NH_4^+ and N fluxes in wild-type plants. This conclusion was supported by the results of experiments in which nitrate reductase (NR) was blocked by tungstate treatment in *Lemna gibba* and *Helianthus annuus* (Ingemarsson *et al*., 1987; De la Haba *et al*., 1990) or by mutation in barley (Warner and Huffaker, 1989; Siddiqi *et al*., 1989; King *et al*., 1993). Incapacitating NR failed to impact upon induction or down-regulation of influx, suggesting that NO_3^- itself was responsible for these effects. Likewise effects of MSX application (Ryan and Walker, 1994; King *et al*., 1993; Feng *et al*., 1994; Glass *et al*., 1997) suggested that NH_4^+ itself was responsible for down-regulating NH_4^+ influx. On the other hand convincing support for effects of down-stream metabolites has been provided by experiments in which exogenously applied amino acids strongly inhibited both NO_3^- and NH_4^+ influx, and by several studies in which MSX application blocked down-regulation (Lee and Rudge, 1986; Morgan and Jackson, 1988; Lee *et al*., 1992; Muller and Touraine, 1992; Rodgers and Barneix, 1993). The contradictory nature of these findings is exemplified by studies on maize and sorghum (Feng *et al*., 1994). While $^{15}NH_4^+$ influx was stimulated by MSX treatment in maize, in sorghum influx was inhibited. Likewise, Glass *et al*. observed that, in low-N rice plants, the effects of MSX were consistent with down-regulation of influx by end-products of NH_4^+ assimilation while in high-N plants NH_4^+ itself appeared to be involved (Glass *et al*., 1997). Unfortunately, given that MSX has been used in so many of these studies, it must be acknowledged that cytosolic NH_4^+ may reach as high as 80 mM when NH_4^+ assimilation is blocked by this compound (Lee and Ratcliffe, 1991). These are clearly abnormal conditions. As will be evident below, the results of molecular studies has provided some clarification of this question at the transcript level.

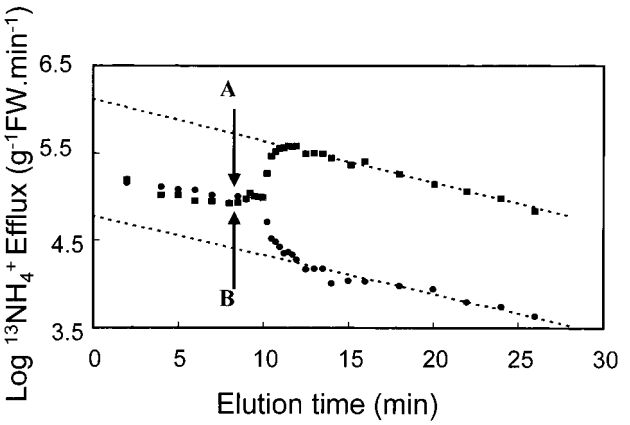

Fig. 2. Efflux of $^{13}NH_4^+$ efflux from roots of barley plants previously grown on 0.1 mM NH_4^+ and loaded with $^{13}NH_4^+$ for 1 h, prior to eluting roots with 0.1 mM $^{14}NH_4^+$ for the first 10 min shown. At this time plant roots were subjected to concentration shifts: (A) from 0.1 to 10 mM NH_4^+ and (B) from 0.1 to 0.01 mM NH_4^+, during elution (from Britto and Kronzucker, 2001).

Genes encoding putative high-affinity NO_3^- and NH_4^+ transporters

The cloning of genes encoding putative high-affinity NO_3^- transporters belonging to the *NRT2* family of genes

(see Forde, 2000, for a recent review) and putative high-affinity NH_4^+ transporters of the *AMT1* family of genes (see Howitt and Udvardi, 2000, for a recent review), has allowed investigations of the regulation of high-affinity NO_3^- and NH_4^+ influx to proceed to the transcript level. As was the case for induction of NO_3^- uptake, only NO_3^- or NO_2^- were capable of inducing the accumulation of *NRT2* transcript. Moreover transcript accumulation followed the same general patterns as had been observed for the induction of NO_3^- uptake/influx, namely induction over a period of up to 3 h or more followed by down-regulation (Trueman *et al*., 1996; Quesada *et al*., 1997; Amarasinghe *et al*., 1998; Filleur *et al*., 1999; Zhuo *et al*., 1999). In NR mutants, high levels of NO_3^- accumulation and increased *NRT2* transcript abundance suggested that while NO_3^- is responsible for inducing gene expression, it is down-stream metabolites that are responsible for down-regulation (Krapp *et al*., 1998; Filleur and Daniel-Vedele, 1999; Lejay *et al*., 1999). Likewise, in barley roots tungstate treatment to block NR caused increased *NRT2* transcript abundance (Vidmar *et al*., 2000). Several reports have documented the down-regulation of *NRT2* transcript abundance in response to pretreatment with NH_4^+ or amino acids (Quesada *et al*., 1997; Krapp *et al*., 1998; Zhuo *et al*., 1999). Unfortunately, exogenous application of amino acids or NH_4^+ provides little information concerning the N pools that might be responsible for these effects. Differences in uptake or assimilation of applied amino acids, as well as their inter-conversion obscure the sources of observed effects. In addition, exogenous application of various amino acids was shown to increase root [NH_4^+] up to 6-fold in rice (Wang, 1994; Kumar *et al*., unpublished results). Another important consideration is whether or not a particular amino acid is a typical/major component of xylem and phloem-translocated N, since cycling/recycling of amino acids within the vascular system has been proposed as the basis of communicating plant N status to roots so that N uptake may be regulated according to plant N demand (Cooper and Clarkson, 1989; Marschner *et al*., 1997; Glass *et al*., 2001). By providing various nitrogen sources (NO_3^-, NH_4^+, and/or amino acids) in the presence and absence of inhibitors of NO_3^- assimilation, for example, tungstate (WO_4^{2-}) to block nitrate reductase, MSX to block glutamine synthetase, and azaserine (AZA) to block glutamate synthase, this confusion can be resolved. In barley, combining results based on the effects of exogenous applications of amino acids with data from inhibitor studies (Fig. 3) demonstrated that *NRT2* transcript abundance was most strongly correlated with root glutamine concentrations (Vidmar *et al*., 2000). Thus, increasing root glutamine by pretreatment with AZA virtually eliminated $^{13}NO_3^-$ influx and *NRT2* transcript in both *A. thaliana* and in *H. vulgare* (Zhuo *et al*., 1999; Vidmar *et al*., 2000).

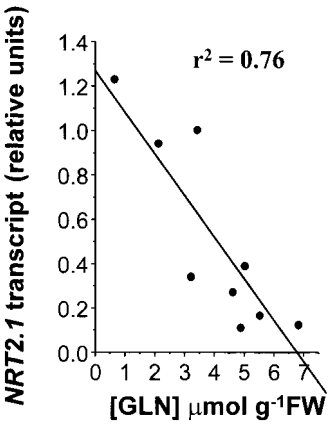

Fig. 3. Correlation between transcript abundance of the barley *HvNrt2* gene and root glutamine concentrations after exogenous application of different amino acids and various inhibitors of nitrate assimilation (from Vidmar *et al*., 2000)

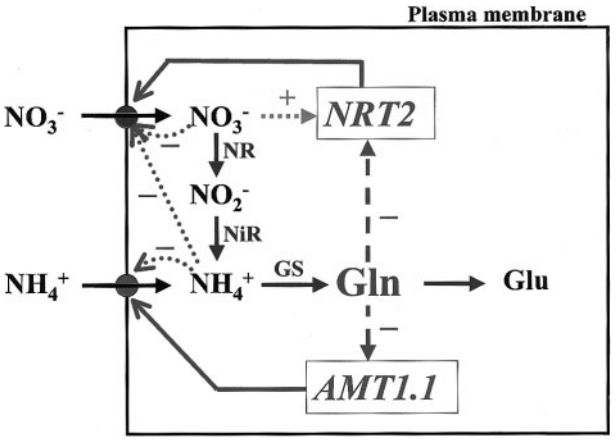

Fig. 4. A model representing proposed feedback processes involved in regulating the abundances of root *Nrt2* and *Amt1* transcripts by root glutamine (- - -), and in direct effects upon the transporters by root cytosolic NO_3^- and NH_4^+ concentrations (.....). Induction of *NRT2* expression by NO_3^- is also indicated. Solid lines from *NRT2* and *Amt1.1* indicate the pathways of transcription and translation leading to high-affinity nitrate and ammonium transporters (circles) in the plasma membrane (outer rectangle). For purposes of simplicity, the diagram makes no attempt to distinguish between plastidic and cytosolic nitrogen pools (from Glass *et al*., 2001).

Using *A. thaliana* as the model system, Rawat *et al*. demonstrated that up-regulation and down-regulation of $^{13}NH_4^+$ influx (following removal and restoration of exogenous N, respectively) was strongly correlated with *AMT1.1* transcript abundance (Rawat *et al*., 1999). In the presence of MSX, NH_4^+ provision caused root [NH_4^+] to increase 27-fold, while root glutamine levels remained at the original (N-deprived) level. Concurrent measurements of $^{13}NH_4^+$ influx and Northern analysis revealed that despite this increase of root [NH_4^+], transcript abundance and influx remained almost at control (N-starved) levels. These results strongly suggest that glutamine is pivotal in regulating *AMT1* transcript abundance.

Multiple members of the *Nrt2* and *Amt1* families

In the study of barley *NRT2* genes by Trueman *et al*., it was suggested that there might be as many as 8–10 homologues in this species (Trueman *et al*., 1996). Following completion of the *Arabidopsis* genome sequencing project, it is now apparent that there are seven homologues in *A. thaliana*. A major task to be resolved is the individual functions of these genes. Work in the senior author's laboratory has been directed toward this goal, using *A. thaliana* as a model system. Under the conditions of this growth system, in which plants are grown hydroponically in open vessels, it has been possible to detect expression of all seven *NRT2* homologues in roots and shoots using RT-PCR (Okamoto *et al*., unpublished data). Based upon the number of PCR cycles required and quantities of template RNA provided, it appears that *AtNRT2.1* and *AtNRT2.2* are the most abundantly expressed genes. In roots these genes are expressed at roughly 10 times the levels of all other genes whether in roots or shoots. The seven genes have been grouped into three categories according to their responses to nitrate feeding in plants previously deprived of NO_3^- for a period of 7 d before resupplying this ion. Category No. 1 includes *AtNRT2.1* and *AtNRT2.2*, genes whose expression in roots increased 3–5-fold following provision of 1 mM NO_3^-. Both genes are subsequently down-regulated, presumably by a gradual increase of tissue glutamine. In shoots expression levels of these genes increased by less than 50% in response to NO_3^- provision, but, as in roots, this increase was followed by substantial down-regulation. Category No. 2 contains genes that are constitutively expressed, showing virtually no response to provision of NO_3^-. In both roots and shoots *AtNRT2.5* and *AtNRT2.6* show this pattern while for *AtNRT2.3* this pattern was restricted to roots. In shoots, *AtNRT2.3* expression levels doubled by 48 h. Category No. 3 contains *AtNRT2.4* and *AtNRT2.7*, genes that are immediately down-regulated following exposure to NO_3^- (Okamoto *et al*., unpublished results). Interestingly, when *AtNRT2.1* and *AtNRT2.2* were first cloned from plants grown for several days with 1 mM KNO_3 (Zhuo *et al*., 1999), it was stated that *AtNRT2.2* was expressed at substantially lower levels than *AtNRT2.1*. However, it is apparent from these time-course studies (Okamoto *et al*., unpublished data) that, following initial exposure to NO_3^-, *AtNRT2.2* transcript abundance is roughly equivalent to that of *AtNRT2.1*, however, by 12 h *AtNRT2.2* transcript abundance is substantially reduced compared to *AtNRT2.1*. Based on the high levels of *AtNRT2.1* and *AtNRT2.2* transcript abundance in roots and the correspondence between the patterns of changes in transcript abundance and high-affinity NO_3^- influx, these genes are good candidates for encoding iHATS. Recently, Filleur *et al*. have isolated a T-DNA insertional mutant of *A. thaliana* disrupted in adjoining *AtNRT2.1* and *AtNRT2.2* genes (Filleur *et al*., 2001). High-affinity NO_3^- transport in this mutant was reduced to 27% of wild-type rates. Thus it can be concluded that *AtNRT2.1* and *AtNRT2.2* make major contributions to the iHATS. The extent to which the remaining transport is due to other *NRT2* genes or to *NRT1* (low-affinity transport) is presently unknown (Wang *et al*., 1998).

If both *AtNRT2.1* and *AtNRT2.2* genes encode iHATS in roots, an important question is what (if any) differential roles these transporters might serve. Some suggestive answers to this question may be provided by comparisons with *NRT2* genes of other organisms. In *Aspergillus nidulans* only two functional *NRT2* genes appear to exist, and all four genotypes (wild type, double mutant and two single mutants) have been characterized with respect to $^{13}NO_3^-$ influx kinetics (Unkles *et al*., 2001). Hoffstee plots of $^{13}NO_3^-$ influx indicate that both transporters contribute to NO_3^- influx in wild-type strains, although the transporters show distinct kinetic differentiation. The NrtA (originally crnA) transporter has a high V_{max} and high K_m (564 nmol mg^{-1} DW h^{-1} and 96.3 μM, respectively) while the second transporter (NrtB) has a low V_{max} and low K_m (141 nmol mg^{-1} DW h^{-1} and 11 μM, respectively). Interestingly the corresponding transporters in *Chlamydomonas reinhardtii* also possess widely different K_m values for NO_3^- uptake (1.6 and 11 μM, respectively), but differ only slightly in V_{max} values (9.0 and 5.6 μmol h^{-1} mg^{-1} chlorophyll, respectively (Galvan *et al*., 1996). This kinetic differentiation presumably enables the organism to access NO_3^- efficiently over a much wider range of concentration than would be possible by means of a single transporter. The *A. nidulans* double mutant is incapable of using NO_3^- as sole source of N at concentrations up to 250 mM NO_3^- or of absorbing $^{13}NO_3^-$ at concentrations up to 500 μM. Continued exposure to NO_3^- leads to down-regulation of $^{13}NO_3^-$ influx in wild-type strains. This is due to down-regulation of NrtA, activity (V_{max} values were 564 ± 67 and 300 ± 71 nmol mg^{-1} DW h^{-1} at 6 h and 16 h, respectively). By contrast, $^{13}NO_3^-$ influx via the NrtB protein was unaffected by duration of exposure to NO_3^- (V_{max} values were 141 ± 6 and 162 ± 26 nmol mg^{-1} DW h^{-1} at 6 and 16 h, respectively). This difference in response to duration of NO_3^- exposure among the strains may be due to slower accumulation of NO_3^- and products of NO_3^- assimilation that would normally down-regulate gene expression in mutant strains expressing only the NrtB protein. Thus, by default, gene mutation is partially compensated for.

The *AMT1* family of high-affinity NH_4^+ transporters contains five members, of which *AtAMT1.1*, *AtAMT1.2* and *AtAMT1.3* have been studied in detail (Gazzarini *et al*., 1999). All three genes are expressed in roots,

while only *AMT1.1* is expressed in significant amounts in leaves. By measuring ^{14}C-methylamine uptake by *Saccharomyces cerevisiae* mutants expressing these genes individually, it was possible to estimate K_m values of ~0.5 μM for the AtAMT1.1, transporter and ~40 μM for the AtAMT1.2 and AtAMT1.3 transporters. During N starvation, transcript abundance of *AtAMT1.1* increased 7-fold during 24 h (Rawat *et al*., 1999). In a comparative study of root *AtAMT1.1*, *AtAMT1.2* and *AtAMT1.3* expression in response to N deprivation, it was shown that *AtAMT1.1* increased 5-fold within 72 h, compared to a 2-fold increase in *AtAMT1.3* and no change in *AtAMT1.2* transcript abundance (Gazzarini *et al*., 1999). In tomato, LeAMT1.1 and LeAMT 1.2 transporters are expressed in roots, while LeAMT1.3 is preferentially expressed in shoots (von Wiren *et al*., 2000). Levels of *LeAMT1.1* transcript in tomato roots also increased over time under conditions of N-deprivation and this was associated with a decline of glutamine and NH_4^+ pool sizes (von Wiren *et al*., 2000). By contrast, and perhaps contrary to expectation, *LeAMT1.2* transcript abundance increased following re-supply of NH_4^+ or NO_3^-. This response may account for the initial stimulation of NH_4^+ influx that was discussed above following resupply of N to N-starved plants (Kronzucker *et al*., 1998). *LeAMT1.3* was not detected in roots.

A T-DNA insertional mutant has recently been isolated from *Arabidopsis* that fails to express *AtAMT1.1* mRNA (Glass *et al*., 2001). Surprisingly, since *AMT1.1* shows the strongest response to N-deprivation and also had the highest affinity for NH_4^+ (at least when expressed heterologously in *S. cerevisiae*) disruption of this gene function reduced $^{13}NH_4^+$ influx by only 20–30% (Glass *et al*., 2001). It is possible that, because of reduced NH_4^+ uptake and thereby reduced negative feedback effects on transcript abundance of other *AMT* genes, there was compensation for the disruption of *AtAMT1.1*. This isssue is currently being explored.

Diurnal effects on NO_3^- and NH_4^+ uptake

There is now abundant evidence to confirm that NO_3^- and NH_4^+ uptake display characteristic diurnal patterns (Clement *et al*., 1978*b*; Macduff *et al*., 1997; Peuke and Jeschke, 1998; Gazzarini *et al*., 1999; Tischner, 2000). In the study by Clement *et al*., peak NO_3^- uptake occurred in the late afternoon while minimum uptake rates occurred at the end of the dark period or even in the first hours of daylight (Clement *et al*., 1978*b*). It is notable that the amplitude of the diurnal pattern and the absolute values of the NO_3^- flux declined substantially during the course of the greenhouse study (Clement *et al*., 1978*b*). This was associated with the onset of poor weather and a 75% reduction of irradiance. This may account for the low amplitude of the diurnal pattern reported in many growth chamber studies where plants are generally maintained under low irradiance. For example, in soybeans maintained on a 9/15 h light/dark regimen, uptake of $^{15}NO_3^-$ was reduced by only 6% in the dark compared to the light period (Rufty *et al*., 1984). It has been suggested that reduced NO_3^- uptake associated with darkness may be countered by exogenously applied carbohydrates (Sehtiya and Goyal, 2000). Thus, in barley and maize, 1% sucrose additions caused 31% and 70% increases of NO_3^- uptake, respectively, in the light, while in dark-grown plants the values were 38% for both barley and maize. Nevertheless, given that dark-grown seedlings should have been substantially more carbohydrate-depleted than light-grown plants, it is surprising that the sucrose effect was actually less (maize) or similar (barley) in dark-grown plants.

NH_4^+ uptake in *Phleum*, *Festuca* and *Arabidopsis* also exhibits a diurnal periodicity, gradually increasing to a peak level toward the end of daylight hours (Macduff *et al*., 1997; Gazzarini *et al*., 1999), and the amplitude of the diurnal pattern of NO_3^-, NH_4^+ and K^+ uptake was highest on high irradiance days (Macduff *et al*., 1997).

Molecular studies have demonstrated that diurnal patterns of N uptake are correlated with diurnal patterns of transcript abundance for the high-affinity *NRT2* and *AMT1* genes (Lejay *et al*., 1999; Ono *et al*., 2000; von Wiren *et al*., 2000; Matt *et al*., 2001). In *A. thaliana*, *NRT2.1* expression in roots increased in daylight hours and declined in the first hours of the dark period, this night-time reduction being prevented by additions of sucrose (Lejay *et al*., 1999). In roots of *A. thaliana*, all three members of the *AMT1* family exhibited diurnal variation, with *AtAMT1.3* expression showing the strongest correlation with diurnal patterns of $^{15}NH_4^+$ uptake. In leaves of tomato, *LeAMT1.2* and *LeAMT1.3* showed a reciprocal diurnal pattern of expression with *LeAMT1.3* transcript being highest in darkness.

The conclusion that C and N metabolism are tightly linked is inescapable (Coruzzi and Bush, 2001). In the study by Matt *et al*., the activities of various enzymes involved in nitrogen metabolism and their transcript abundances, including the high-affinity nitrate transporter, as well as concentrations of various metabolites (NO_3^-, amino acids, sugars and 2-oxoglutarate) were measured during a diurnal cycle in tobacco (Matt *et al*., 2001). Based upon the correspondence between root sugar levels and *NRT2* transcript abundance (and a lack of correspondence with other metabolites) the authors concluded that root sugars were responsible for the diurnal pattern of *NRT2* expression. It is intriguing to consider whether the effects of carbohydrate supply might act directly or indirectly on nitrogen pools and/or transcript abundances. For example, when carbohydrate supply to the root limits N assimilation and/or growth,

accumulation of N metabolites might reduce expression of transporter genes or even act directly upon the transporters. Furthermore, the study by Matt *et al.* acknowledged that the observed correlations between *NRT2* expression and root sugar levels were based upon whole root analyses (Matt *et al.*, 2001). Clearly, cytosolic metabolite concentrations might have provided a different conclusion.

In summary, a high degree of heterogeneity with respect to soil N availability and diurnal and seasonal variation in plant requirements for N impose a need to regulate N fluxes across the plasma membrane of plant roots in order to optimize plant N capture. The need to integrate/co-ordinate N acquisition from several potential soil N sources (NO_3^-, NH_4^+ and amino acids) suggests that regulation might be most effective if a common end-product of NO_3^- assimilation such as glutamine were to serve as the source of negative feedback. Experiments listed above indicate that this may be the case. Nevertheless, there is no reason to assume that, in addition to the clearly demonstrated regulation by transcript abundance, there will not be post-transcriptional regulation by other nitrogen sources. Indeed preliminary evidence for such effects has already been presented (Fraisier *et al.*, 2000; Vidmar *et al.*, 2000; Rawat *et al.*, 1999).

In addition to regulating influx across root plasma membranes, internal redistributions to vacuole and to xylem suggest that there is a need for integration of all component fluxes as well as for the integration of amino acid fluxes involved in nutrient cycling within plants. Thus far, the focus of attention in studies of inorganic N uptake at the physiological and molecular levels has been upon the regulation of root plasma membrane transporters. It is to be anticipated that future physiological and molecular studies will include fluxes to subcellular compartments and between major organs of the plant (such as fluxes from root to xylem, xylem to shoot) and leaf uptake of inorganic N.

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