



# 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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  influx. In addition, efflux analysis using  $^{13}\text{N}$  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  $\text{NO}_3^-$  and one HATS for  $\text{NH}_4^+$  in roots of higher plants. By contrast, in *Arabidopsis thaliana* there exist seven members of the *NRT2* family encoding putative HATS for  $\text{NO}_3^-$  and five members of the *AMT1* family encoding putative HATS for  $\text{NH}_4^+$ . The induction of high-affinity  $\text{NO}_3^-$  transport and *Nrt2.1* and *Nrt2.2* expression occur in response to the provision of  $\text{NO}_3^-$ , while down-regulation of these genes appear to be due to the effects of glutamine. High-affinity  $\text{NH}_4^+$  transport and *AMT1.1* expression also appear to be subject to down-regulation by glutamine. In addition, there is evidence that accumulated  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  transporters and these appear to have undergone kinetic differentiation that

permits a greater efficiency of  $\text{NO}_3^-$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  (one constitutive and the other inducible) and one HATS for  $\text{NH}_4^+$ ; (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  $\text{NO}_3^-$  and five for  $\text{NH}_4^+$  in *A. thaliana*; and (5) some of the genes encoding  $\text{NO}_3^-$  transporters are subject to transcriptional regulation through inductive effects of  $\text{NO}_3^-$ , while some of those encoding  $\text{NO}_3^-$  and  $\text{NH}_4^+$  transporters are subject to down-regulating effects of glutamine. Notwithstanding these similarities there are also distinct differences in the characteristics of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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,  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$ . Many species occupying such habitats have become specialists, absorbing  $\text{NH}_4^+$  or amino acids in preference to  $\text{NO}_3^-$  (Kielland, 1994; Kronzucker *et al.*, 1997; Nasholm *et al.*, 1998, 2000). Even when  $\text{NO}_3^-$  exceeds  $\text{NH}_4^+$  by as much as 10-fold,

$\text{NH}_4^+$  uptake may still greatly exceed that of  $\text{NO}_3^-$  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  $\text{NH}_4^+$ , even though this ion represented only 10% of available N, the remaining 90% being  $\text{NO}_3^-$ . 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 $\text{NO}_3^-$ and $\text{NH}_4^+$ uptake

Measurements of  $^{13}\text{NO}_3^-$  influx and net  $\text{NO}_3^-$  uptake by several groups have revealed the presence of three transport systems for  $\text{NO}_3^-$  and two for  $\text{NH}_4^+$  (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  $\text{NO}_3^-$  from low external  $\text{NO}_3^-$ . 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  $\text{NO}_3^-$  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  $\text{NO}_3^-$  among species. For example, in *Picea glauca*, it was necessary to expose plants to  $\text{NO}_3^-$  for 3 d in order to induce peak  $^{13}\text{NO}_3^-$  influx (Kronzucker *et al.*, 1995). Both  $\text{NO}_3^-$  and  $\text{NO}_2^-$  are capable of inducing this flux (Siddiqi *et al.*, 1992; Aslam *et al.*, 1993).

Several studies have demonstrated that the provision of  $\text{NH}_4^+$  to N-deprived roots may initially increase  $\text{NH}_4^+$  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  $\text{NH}_4^+$  influx was already high (de-repressed) before exposure to  $\text{NH}_4^+$ , and it has been demonstrated that, in rice, the increase of  $\text{NH}_4^+$  influx resulting from  $\text{NH}_4^+$  pretreatment was relatively small (25–40%) (Kronzucker *et al.*, 1998). By comparison, a 30-fold increase of  $^{13}\text{NO}_3^-$  influx was recorded in Klondike barley following pretreatment with  $\text{NO}_3^-$  (Siddiqi *et al.*, 1990). Kronzucker *et al.* concluded that the evidence did not support a true inductive effect of  $\text{NH}_4^+$  (Kronzucker *et al.*, 1998).

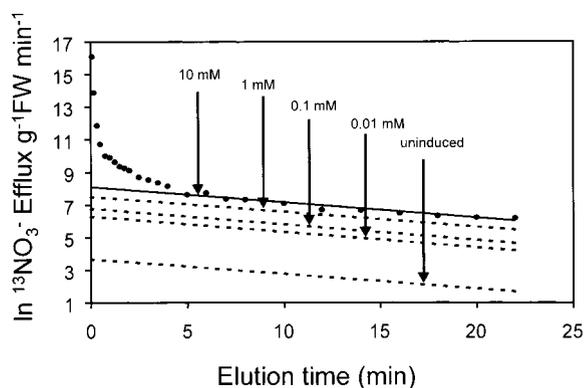
At nitrate and ammonium concentrations between  $\sim 200$  to  $500 \mu\text{M}$ , low-affinity transporter systems (LATS) for these ions become apparent. These were evident in earlier studies (Doddema and Telkamp, 1979; Ullrich *et al.*, 1984), but were largely overlooked, in part because the measurement of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NH}_4^+$  fluxes via LATS are typically thermodynamically 'downhill' (Ullrich *et al.*, 1984; Wang *et al.*, 1993), the LATS for  $\text{NO}_3^-$  was shown to be active even at high external  $\text{NO}_3^-$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  concentrations were maintained from  $14.3 \mu\text{M}$  to  $14.3 \text{ mM}$ , plant growth was only modestly affected and tissue nitrogen concentration remained essentially constant (Clement *et al.*, 1978a). 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  $\text{NH}_4^+$  transport shows the same general homeostatic propensity (Wang *et al.*, 1993; Rawat *et al.*, 1999), the potential toxicity of elevated ambient  $\text{NH}_4^+$  concentrations severely limits the range of  $\text{NH}_4^+$  concentration over which adaptation is possible. In a study of  $^{13}\text{NH}_4^+$  fluxes across the plasma membranes of barley roots, Britto *et al.* showed that at  $10 \text{ mM}$  external  $\text{NH}_4^+$ , active  $\text{NH}_4^+$  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  $\text{NH}_4^+$ , but with active extrusion. In summary, while high-affinity  $\text{NH}_4^+$  fluxes are effectively regulated, transport via the low-affinity

system is poorly regulated, resulting in considerable futile cycling of  $\text{NH}_4^+$  across the plasma membrane as well as toxic effects of excessive  $\text{NH}_4^+$  accumulation (Britto *et al.*, 2001).

Studies of the many component  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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 ( $\phi_{oc}$ ) across the plasma membrane. Nevertheless, there is evidence to suggest that efflux from cytosol to cell wall ( $\phi_{co}$ ), fluxes across the tonoplast ( $\phi_{cv}$  and  $\phi_{vc}$ ), from cytosol to xylem ( $\phi_{cx}$ ), 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  $^{13}\text{NO}_3^-$  and  $^{13}\text{NH}_4^+$  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  $^{13}\text{NO}_3^-$  efflux from roots of barley grown under steady-state conditions with various concentrations of nitrate for 7 d prior to labelling with  $^{13}\text{NO}_3^-$  and subsequent measurement of  $^{13}\text{NO}_3^-$  efflux into non-labelled solutions of the same  $\text{NO}_3^-$  concentration (Britto and Kronzucker, 2001). Despite the wide range of  $\text{NO}_3^-$  concentrations used and the substantial changes of measured fluxes, the rate constants for  $^{13}\text{NO}_3^-$  efflux were essentially identical ( $0.0408$ ,  $0.0400$ ,  $0.0417$ ,  $0.0418$ , and  $0.04908 \text{ min}^{-1}$  for plants grown in  $10$ ,  $1$ ,  $0.1$ ,  $0.01$ , and  $0 \text{ mM}$   $\text{NO}_3^-$ , respectively). In a study of the effect of perturbing external  $\text{NH}_4^+$  on  $^{13}\text{NH}_4^+$  efflux from barley



**Fig. 1.**  $^{13}\text{NO}_3^-$  efflux from roots of barley plants grown with different concentrations of  $\text{NO}_3^-$ . Plants were grown for 7 d under steady-state conditions with respect to nitrate provision. Roots were then loaded with  $^{13}\text{NO}_3^-$  for  $>5$  cytoplasmic half-lives, and subsequently transferred to the same concentration of  $^{14}\text{NO}_3^-$  for measurement of  $^{13}\text{NO}_3^-$  efflux. Rate constants for the lines were  $0.041 \text{ min}^{-1}$  ( $10 \text{ mM}$ ),  $0.040 \text{ min}^{-1}$  ( $1 \text{ mM}$ ),  $0.042 \text{ min}^{-1}$  ( $0.1 \text{ mM}$ ),  $0.042 \text{ min}^{-1}$  ( $0.01 \text{ mM}$ ), and  $0.039 \text{ min}^{-1}$  (uninduced plants), respectively (from Britto and Kronzucker, 2001).

roots, Britto and Kronzucker showed that when external  $\text{NH}_4^+$  concentration was increased or decreased, respectively, from 1 mM to either 10 mM or to 100  $\mu\text{M}$ , there was initially a rapid increase or decrease, respectively, of  $^{13}\text{NH}_4^+$  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  $^{13}\text{NO}_3^-$  and  $^{13}\text{NH}_4^+$  have demonstrated that  $\phi_{\text{co}}$  increases as external ion concentration increases (Siddiqi *et al.*, 1991; Wang *et al.*, 1993) and that net transfer of nitrogen from vacuole to cytosol ( $\phi_{\text{vc}} - \phi_{\text{cv}}$ ) increases (van der Leij *et al.*, 1998), and from cytosol to stele ( $\phi_{\text{cx}}$ ) 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  $\phi_{\text{oc}}$ , nor have genes yet been cloned that encode these transport systems. Likewise there is a lack of detailed studies of the fluxes of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  or  $\text{NO}_2^-$  among potential products of nitrogen assimilation are capable of inducing  $\text{NO}_3^-$  influx by the iHATS (Tompkins *et al.*, 1978; Behl *et al.*, 1988; Siddiqi *et al.*, 1992; Tischner *et al.*, 1993; Guy and Heimer, 1993;

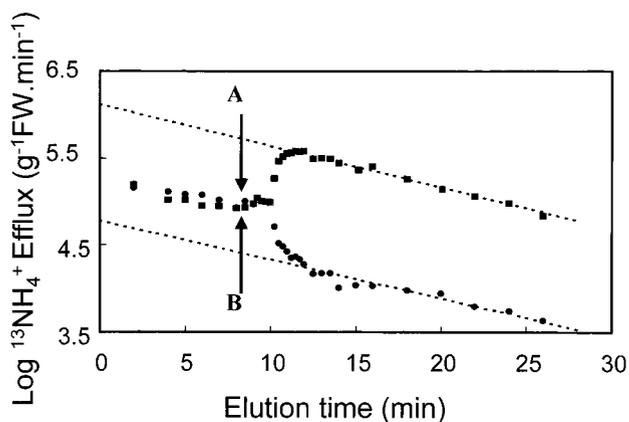


Fig. 2. Efflux of  $^{13}\text{NH}_4^+$  from roots of barley plants previously grown on 0.1 mM  $\text{NH}_4^+$  and loaded with  $^{13}\text{NH}_4^+$  for 1 h, prior to eluting roots with 0.1 mM  $^{14}\text{NH}_4^+$  for the first 10 min shown. At this time plant roots were subjected to concentration shifts: (A) from 0.1 to 10 mM  $\text{NH}_4^+$  and (B) from 0.1 to 0.01 mM  $\text{NH}_4^+$ , during elution (from Britto and Kronzucker, 2001).

Henriksen and Spanswick, 1993). Nevertheless, as low-N plants accumulate N, the influxes of both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  transporters, two hypotheses emerged to explain this down-regulation. On the one hand it was proposed that accumulated  $\text{NO}_3^-$  or  $\text{NH}_4^+$  themselves, as opposed to their downstream metabolites, were responsible for down-regulation of fluxes. This was based upon inverse correlations between accumulated  $\text{NO}_3^-$  or  $\text{NH}_4^+$  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  $\text{NO}_3^-$  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  $\text{NH}_4^+$  itself was responsible for down-regulating  $\text{NH}_4^+$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $^{15}\text{NH}_4^+$  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  $\text{NH}_4^+$  assimilation while in high-N plants  $\text{NH}_4^+$  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  $\text{NH}_4^+$  may reach as high as 80 mM when  $\text{NH}_4^+$  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.

### Genes encoding putative high-affinity $\text{NO}_3^-$ and $\text{NH}_4^+$ transporters

The cloning of genes encoding putative high-affinity  $\text{NO}_3^-$  transporters belonging to the *NRT2* family of genes

(see Forde, 2000, for a recent review) and putative high-affinity  $\text{NH}_4^+$  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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  influx to proceed to the transcript level. As was the case for induction of  $\text{NO}_3^-$  uptake, only  $\text{NO}_3^-$  or  $\text{NO}_2^-$  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  $\text{NO}_3^-$  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  $\text{NO}_3^-$  accumulation and increased *NRT2* transcript abundance suggested that while  $\text{NO}_3^-$  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  $\text{NH}_4^+$  or amino acids (Quesada *et al.*, 1997; Krapp *et al.*, 1998; Zhuo *et al.*, 1999). Unfortunately, exogenous application of amino acids or  $\text{NH}_4^+$  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  $[\text{NH}_4^+]$  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 ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and/or amino acids) in the presence and absence of inhibitors of  $\text{NO}_3^-$  assimilation, for example, tungstate ( $\text{WO}_4^{2-}$ ) 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  $^{13}\text{NO}_3^-$  influx and *NRT2* transcript in both *A. thaliana* and in *H. vulgare* (Zhuo *et al.*, 1999; Vidmar *et al.*, 2000).

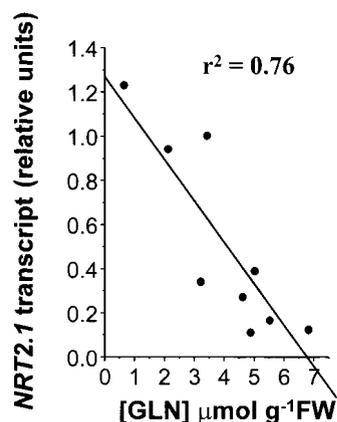


Fig. 3. Correlation between transcript abundance of the barley *HvNrt2* gene and root glutamine concentrations after exogenous application of different amino acids and various inhibitors of nitrate assimilation (from Vidmar *et al.*, 2000)

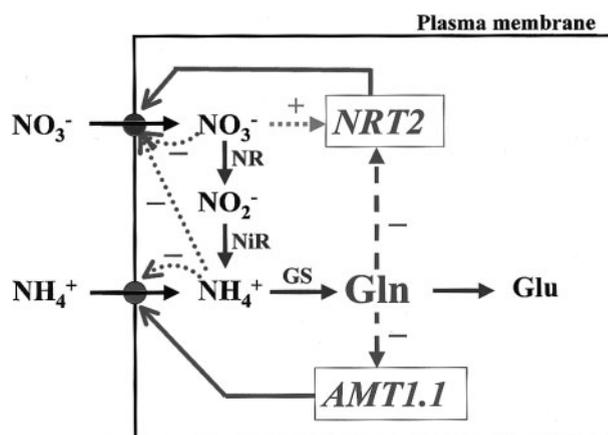


Fig. 4. A model representing proposed feedback processes involved in regulating the abundances of root *Nrt2* and *Amt1* transcripts by root glutamine (---), and in direct effects upon the transporters by root cytosolic  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations (.....). Induction of *NRT2* expression by  $\text{NO}_3^-$  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  $^{13}\text{NH}_4^+$  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,  $\text{NH}_4^+$  provision caused root  $[\text{NH}_4^+]$  to increase 27-fold, while root glutamine levels remained at the original (N-deprived) level. Concurrent measurements of  $^{13}\text{NH}_4^+$  influx and Northern analysis revealed that despite this increase of root  $[\text{NH}_4^+]$ , 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  $\text{NO}_3^-$  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  $\text{NO}_3^-$ . 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  $\text{NO}_3^-$  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  $\text{NO}_3^-$ . 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  $\text{NO}_3^-$  (Okamoto *et al.*, unpublished results). Interestingly, when *AtNRT2.1* and *AtNRT2.2* were first cloned from plants grown for several days with 1 mM  $\text{KNO}_3$  (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  $\text{NO}_3^-$ , *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  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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  $^{13}\text{NO}_3^-$  influx kinetics (Unkles *et al.*, 2001). Hoffstee plots of  $^{13}\text{NO}_3^-$  influx indicate that both transporters contribute to  $\text{NO}_3^-$  influx in wild-type strains, although the transporters show distinct kinetic differentiation. The NrtA (originally crnA) transporter has a high  $V_{\text{max}}$  and high  $K_m$  (564 nmol  $\text{mg}^{-1}$  DW  $\text{h}^{-1}$  and 96.3  $\mu\text{M}$ , respectively) while the second transporter (NrtB) has a low  $V_{\text{max}}$  and low  $K_m$  (141 nmol  $\text{mg}^{-1}$  DW  $\text{h}^{-1}$  and 11  $\mu\text{M}$ , respectively). Interestingly the corresponding transporters in *Chlamydomonas reinhardtii* also possess widely different  $K_m$  values for  $\text{NO}_3^-$  uptake (1.6 and 11  $\mu\text{M}$ , respectively), but differ only slightly in  $V_{\text{max}}$  values (9.0 and 5.6  $\mu\text{mol h}^{-1} \text{mg}^{-1}$  chlorophyll, respectively (Galvan *et al.*, 1996). This kinetic differentiation presumably enables the organism to access  $\text{NO}_3^-$  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  $\text{NO}_3^-$  as sole source of N at concentrations up to 250 mM  $\text{NO}_3^-$  or of absorbing  $^{13}\text{NO}_3^-$  at concentrations up to 500  $\mu\text{M}$ . Continued exposure to  $\text{NO}_3^-$  leads to down-regulation of  $^{13}\text{NO}_3^-$  influx in wild-type strains. This is due to down-regulation of NrtA, activity ( $V_{\text{max}}$  values were  $564 \pm 67$  and  $300 \pm 71$  nmol  $\text{mg}^{-1}$  DW  $\text{h}^{-1}$  at 6 h and 16 h, respectively). By contrast,  $^{13}\text{NO}_3^-$  influx via the NrtB protein was unaffected by duration of exposure to  $\text{NO}_3^-$  ( $V_{\text{max}}$  values were  $141 \pm 6$  and  $162 \pm 26$  nmol  $\text{mg}^{-1}$  DW  $\text{h}^{-1}$  at 6 and 16 h, respectively). This difference in response to duration of  $\text{NO}_3^-$  exposure among the strains may be due to slower accumulation of  $\text{NO}_3^-$  and products of  $\text{NO}_3^-$  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  $\text{NH}_4^+$  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  $^{14}\text{C}$ -methylamine uptake by *Saccharomyces cerevisiae* mutants expressing these genes individually, it was possible to estimate  $K_m$  values of  $\sim 0.5 \mu\text{M}$  for the *AtAMT1.1*, transporter and  $\sim 40 \mu\text{M}$  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 *LeAMT1.2* 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  $\text{NH}_4^+$  pool sizes (von Wiren *et al.*, 2000). By contrast, and perhaps contrary to expectation, *LeAMT1.2* transcript abundance increased following re-supply of  $\text{NH}_4^+$  or  $\text{NO}_3^-$ . This response may account for the initial stimulation of  $\text{NH}_4^+$  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  $\text{NH}_4^+$  (at least when expressed heterologously in *S. cerevisiae*) disruption of this gene function reduced  $^{13}\text{NH}_4^+$  influx by only 20–30% (Glass *et al.*, 2001). It is possible that, because of reduced  $\text{NH}_4^+$  uptake and thereby reduced negative feedback effects on transcript abundance of other *AMT* genes, there was compensation for the disruption of *AtAMT1.1*. This issue is currently being explored.

### Diurnal effects on $\text{NO}_3^-$ and $\text{NH}_4^+$ uptake

There is now abundant evidence to confirm that  $\text{NO}_3^-$  and  $\text{NH}_4^+$  uptake display characteristic diurnal patterns (Clement *et al.*, 1978b; Macduff *et al.*, 1997; Peuke and Jeschke, 1998; Gazzarini *et al.*, 1999; Tischner, 2000). In the study by Clement *et al.*, peak  $\text{NO}_3^-$  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.*, 1978b). It is notable that the amplitude of the diurnal pattern and the absolute values of the  $\text{NO}_3^-$  flux declined substantially during the course of the greenhouse study (Clement *et al.*, 1978b). 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  $^{15}\text{NO}_3^-$  was reduced by only 6% in the dark compared to the light period (Rufty *et al.*, 1984). It has been suggested that reduced  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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.

$\text{NH}_4^+$  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  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and  $\text{K}^+$  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  $^{15}\text{NH}_4^+$  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 ( $\text{NO}_3^-$ , 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 ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and amino acids) suggests that regulation might be most effective if a common end-product of  $\text{NO}_3^-$  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 sub-cellular 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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