The regulation of nitrate and ammonium transport systems in plants

Anthony D.M. Glass¹, Dev T. Britto², Brent N. Kaiser³, James R. Kinghorn⁴, Herbert J. Kronzucker², Anshuman Kumar¹, Mamoru Okamoto¹, Suman Rawat¹, M.Y. Siddiqi¹, Shiela E. Unkles⁴ and Joseph J. Vidmar⁵

¹ University of British Columbia, 6270 University Blvd, Vancouver, V6T1Z4, Canada
² Division of Life Sciences, University Of Toronto, 1265 Military Trail, Scarborough, Ontario, M1C 1A4 Canada
³ Environmental Biology, RSBS, Australian National University, GPO Box 475, Canberra ACT 2601, Australia
⁴ School of Biology, University of St Andrews, St Andrews KY16 9TH, UK

Received 21 August 2001; Accepted 3 December 2001

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 NO₃⁻ and NH₄⁺ influx. In addition, efflux analysis using ¹³N 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 NO₃⁻ and one HATS for NH₄⁺ in roots of higher plants. By contrast, in Arabidopsis thaliana there exist seven members of the NRT2 family encoding putative HATS for NO₃⁻ and five members of the AMT1 family encoding putative HATS for NH₄⁺. The induction of high-affinity NO₃⁻ transport and Nrt2.1 and Nrt2.2 expression occur in response to the provision of NO₃⁻, while down-regulation of these genes appear to be due to the effects of glutamine. High-affinity NH₄⁺ transport and AMT1.1 expression also appear to be subject to down-regulation by glutamine. In addition, there is evidence that accumulated NO₃⁻ and NH₄⁺ may act post-transcriptionally on transporter function. The present challenge is to resolve the functions of all of these genes.

Permits a greater efficiency of NO₃⁻ 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

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

© Society for Experimental Biology 2002
solute, 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 $^{15}$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 NO₃⁻ and NH₄⁺ 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 NH₄⁺ fluxes via LATS are typically thermodynamically ‘downhill’ (Ullrich et al., 1984; Wang et al., 1993), the LATS for NO₃⁻ was shown to be active even at high external NO₃⁻ 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 NO₃⁻ and NH₄⁺ 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 NO₃⁻ 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., 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 NH₄⁺ transport shows the same general homeostatic propensity (Wang et al., 1993; Rawat et al., 1999), the potential toxicity of elevated ambient NH₄⁺ concentrations severely limits the range of NH₄⁺ concentration over which adaptation is possible. In a study of ¹³NH₄⁺ fluxes across the plasma membranes of barley roots, Britto et al. showed that at 10 mM external NH₄⁺, active NH₄⁺ 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 sulfoximine (MSX), indicating that the respiratory increase was not associated with increased assimilation of NH₄⁺, but with active extrusion. In summary, while high-affinity NH₄⁺ fluxes are effectively regulated, transport via the low-affinity system is poorly regulated, resulting in considerable futile cycling of NH₄⁺ across the plasma membrane as well as toxic effects of excessive NH₄⁺ accumulation (Britto et al., 2001).

Studies of the many component NO₃⁻ and NH₄⁺ fluxes that occur in plant cells are severely limited, even in single-celled organisms by cellular compartmentation. In multi-cellular 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 (φᵢᵣ) across the plasma membrane. Nevertheless, there is evidence to suggest that efflux from cytosol to cell wall (φₓᵢ), fluxes across the tonoplast (φᵢₜ), from cytosol to xylem (φᵣₓ), as well as fluxes to biochemical pathways appear to be co-ordinated. The use of efflux analysis to estimate the half-lives (t₀.₅) for ¹³NO₃⁻ and ¹³NH₄⁺ residence within the cytosolic compartment, has revealed that t₀.₅ 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 ¹³NO₃⁻ efflux from roots of barley grown under steady-state conditions with various concentrations of nitrate for 7 d prior to labelling with ¹³NO₃⁻ and subsequent measurement of ¹³NO₃⁻ efflux into non-labelled solutions of the same NO₃⁻ concentration (Britto and Kronzucker, 2001). Despite the wide range of NO₃⁻ concentrations used and the substantial changes of measured fluxes, the rate constants for ¹³NO₃⁻ 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 NO₃⁻, respectively). In a study of the effect of perturbing external NH₄⁺ on ¹³NH₄⁺ efflux from barley roots for 7 d under steady-state conditions with respect to nitrate provision. Roots were then loaded with ¹¹NO₃⁻ for >5 cytoplasmic half-lives, and subsequently transferred to the same concentration of ¹⁴NO₃⁻ for measurement of ¹³NO₃⁻ 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).

![Fig. 1. ¹⁴NO₃⁻ efflux from roots of barley plants grown with different concentrations of NO₃⁻. Plants were grown for 7 d under steady-state conditions with respect to nitrate provision. Roots were then loaded with ¹¹NO₃⁻ for >5 cytoplasmic half-lives, and subsequently transferred to the same concentration of ¹⁴NO₃⁻ for measurement of ¹³NO₃⁻ 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).](https://academic.oup.com/jxb/article-abstract/53/370/855/2908384)
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 $^{14}$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 $\phi_{oc}$ increases as external ion concentration increases (Siddiqi et al., 1991; Wang et al., 1993) and that net transfer of nitrogen from vacuole to cytosol ($\phi_{oc}$-$\phi_{cv}$) increases (van der Leij et al., 1998), and from cytosol to stele ($\phi_{cs}$) 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 $\phi_{oc}$, 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 Ratcliff, 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 NO$_3^-$ and NH$_4^+$ transporters

The cloning of genes encoding putative high-affinity NO$_3^-$ transporters belonging to the *NRT2* family of genes...
application of amino acids or NH₄⁺ 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 NO₃⁻ and NH₄⁺ influx to proceed to the transcript level. As was the case for induction of NO₃⁻ uptake, only NO₃⁻ or NO₂⁻ 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 NO₃⁻ 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; Amarasinge et al., 1998; Filleur et al., 1999; Zhuo et al., 1999). In NR mutants, high levels of NO₃⁻ accumulation and increased NRT2 transcript abundance suggested that while NO₃⁻ is responsible for inducing gene expression, it is downstream 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 NH₄⁺ or amino acids (Quesada et al., 1997; Krapp et al., 1998; Zhuo et al., 1999). Unfortunately, exogenous application of amino acids or NH₄⁺ 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 interconversion obscure the sources of observed effects. In addition, exogenous application of various amino acids was shown to increase root [NH₄⁺] 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; Marshner et al., 1997; Glass et al., 2001). By providing various nitrogen sources (NO₃⁻, NH₄⁺, and/or amino acids) in the presence and absence of inhibitors of NO₃⁻ assimilation, for example, tungstate (WO₄²⁻) 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 glutamate concentrations (Vidmar et al., 2000). Thus, increasing root glutamate by pretreatment with AZA virtually eliminated ¹³NO₃⁻ influx and NRT2 transcript in both A. thaliana and in H. vulgare (Zhuo et al., 1999; Vidmar et al., 2000).

Using A. thaliana as the model system, Rawat et al. demonstrated that up-regulation and down-regulation of ¹¹NH₄⁺ 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, NH₄⁺ provision caused root [NH₄⁺] to increase 27-fold, while root glutamine levels remained at the original (N-deprived) level. Concurrent measurements of ¹¹NH₄⁺ influx and Northern analysis revealed that despite this increase of root [NH₄⁺], 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 \textit{Nrt2} and \textit{Amt1} families

In the study of barley \textit{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 \textit{Arabidopsis} genome sequencing project, it is now apparent that there are seven homologues in \textit{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 \textit{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 \textit{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 \textit{AtNRT2.1} and \textit{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\textsubscript{3} for a period of 7 d before resupplying this ion. Category No. 1 includes \textit{AtNRT2.1} and \textit{AtNRT2.2}, genes whose expression in roots increased 3–5-fold following provision of 1 mM NO\textsubscript{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\textsubscript{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\textsubscript{3}. In both roots and shoots \textit{AtNRT2.5} and \textit{AtNRT2.6} show this pattern while for \textit{AtNRT2.3} this pattern was restricted to roots. In shoots, \textit{AtNRT2.3} expression levels doubled by 48 h. Category No. 3 contains \textit{AtNRT2.4} and \textit{AtNRT2.7}, genes that are immediately down-regulated following exposure to NO\textsubscript{3} (Okamoto et al., unpublished results). Interestingly, when \textit{AtNRT2.1} and \textit{AtNRT2.2} were first cloned from plants grown for several days with 1 mM KNO\textsubscript{3} (Zhao et al., 1999), it was stated that \textit{AtNRT2.2} was expressed at substantially lower levels than \textit{AtNRT2.1}. However, it is apparent from these time-course studies (Okamoto et al., unpublished data) that, following initial exposure to NO\textsubscript{3}, \textit{AtNRT2.2} transcript abundance is roughly equivalent to that of \textit{AtNRT2.1}, however, by 12 h \textit{AtNRT2.2} transcript abundance is substantially reduced compared to \textit{AtNRT2.1}. Based on the high levels of \textit{AtNRT2.1} and \textit{AtNRT2.2} transcript abundance in roots and the correspondence between the patterns of changes in transcript abundance and high-affinity NO\textsubscript{3} influx, these genes are good candidates for encoding iHATS. Recently, Filleur et al. have isolated a T-DNA insertional mutant of \textit{A. thaliana} disrupted in adjoining \textit{AtNRT2.1} and \textit{AtNRT2.2} genes (Filleur et al., 2001). High-affinity NO\textsubscript{3} transport in this mutant was reduced to 27% of wild-type rates. Thus it can be concluded that \textit{AtNRT2.1} and \textit{AtNRT2.2} make major contributions to the iHATS. The extent to which the remaining transport is due to other \textit{NRT2} genes or to \textit{NRT1} (low-affinity transport) is presently unknown (Wang et al., 1998).

If both \textit{AtNRT2.1} and \textit{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 \textit{NRT2} genes of other organisms. In \textit{Aspergillus nidulans} only two functional \textit{NRT2} genes appear to exist, and all four genotypes (wild type, double mutant and two single mutants) have been characterized with respect to \textsuperscript{15}NO\textsubscript{3} influx kinetics (Unkles et al., 2001). Hoffstee plots of \textsuperscript{15}NO\textsubscript{3} influx indicate that both transporters contribute to NO\textsubscript{3} influx in wild-type strains, although the transporters show distinct kinetic differentiation. The NrtA (originally \textit{crnA}) transporter has a high \(V_{\text{max}}\) and high \(K_m\) values of 564 nmol m\textsuperscript{-1} h\textsuperscript{-1} and 96.3 \(\mu\text{M}\), respectively while the second transporter (NrtB) has a low \(V_{\text{max}}\) and low \(K_m\) of 141 nmol m\textsuperscript{-1} h\textsuperscript{-1} and 11 \(\mu\text{M}\), respectively. Interestingly the corresponding transporters in \textit{Chlamydomonas reinhardtii} also possess widely different \(K_m\) values for NO\textsubscript{3} uptake (1.6 and 11 \(\mu\text{M}\), respectively), but differ only slightly in \(V_{\text{max}}\) values (9.0 and 5.6 \(\mu\text{mol} \text{ h}^{-1} \text{ mg}^{-1}\) chlorophyll, respectively (Galvan et al., 1996). This kinetic differentiation presumably enables the organism to access NO\textsubscript{3} efficiently over a much wider range of concentration than would be possible by means of a single transporter. The \textit{A. nidulans} double mutant is incapable of using NO\textsubscript{3} as sole source of N at concentrations up to 250 mM NO\textsubscript{3} or of absorbing \textsuperscript{15}NO\textsubscript{3} at concentrations up to 500 \(\mu\text{M}\). Continued exposure to NO\textsubscript{3} leads to down-regulation of \textsuperscript{15}NO\textsubscript{3} influx in wild-type strains. This is due to down-regulation of NrtA, activity (\(V_{\text{max}}\) values were 564 ± 67 and 300 ± 71 nmol mg\textsuperscript{-1} h\textsuperscript{-1} at 6 h and 16 h, respectively). By contrast, \textsuperscript{15}NO\textsubscript{3} influx via the NrtB protein was unaffected by duration of exposure to NO\textsubscript{3} (\(V_{\text{max}}\) values were 141 ± 6 and 162 ± 26 nmol mg\textsuperscript{-1} h\textsuperscript{-1} at 6 and 16 h, respectively). This difference in response to duration of NO\textsubscript{3} exposure among the strains may be due to slower accumulation of NO\textsubscript{3} and products of NO\textsubscript{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 \textit{AMT1} family of high-affinity NH\textsubscript{4}\textsuperscript{+} transporters contains five members, of which \textit{AtAMT1.1}, \textit{AtAMT1.2} and \textit{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 $\sim 0.5 \text{ mM}$ for the AtAMT1.1 transporter and $\sim 40 \text{ mM}$ 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 Wirén 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 Wirén et al., 2000). By contrast, and perhaps contrary to expectation, LeAMT1.2 transcript abundance increased following re-supply of NH$_4^+$ or NO$_3^-$, which 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 insertion 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 issue 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., 1978b; 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., 1978b). 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., 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 soya beans 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 (Seathiya 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 Wirén 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\textsubscript{3}\textsuperscript{-}, NH\textsubscript{4}\textsuperscript{+} and amino acids) suggests that regulation might be most effective if a common end-product of NO\textsubscript{3}\textsuperscript{-} 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 (Fraiser 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.

Acknowledgements

The work undertaken by the authors was financed by grants from NSERC to ADM Glass, who gratefully acknowledges this support. In addition we gratefully acknowledge the provision of \textsuperscript{15}N by the UBC TRIUMF cyclotron.

References


