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AtAMT1 gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels

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Summary

The mechanisms involved in regulating high-affinity ammonium (NH_4^+) uptake and the expression of the *AtAMT1* gene encoding a putative high-affinity NH_4^+ transporter were investigated in the roots of *Arabidopsis thaliana*. Under conditions of steady-state nitrogen (N) supply, transcript levels of the *AtAMT1* gene and V_{max} values for high-affinity $^{13}NH_4^+$ influx were inversely correlated with levels of N provision. Following re-supply of NH_4NO_3 to N-starved plants, *AtAMT1* mRNA levels and $^{13}NH_4^+$ influx declined rapidly but remained high when the conversion of NH_4^+ to glutamine (Gln) was blocked with methionine sulfoximine (MSX). This result demonstrates that end products of NH_4^+ assimilation, rather than NH_4^+ itself, are responsible for regulating *AtAMT1* gene expression. Consistent with this hypothesis, *AtAMT1* gene expression and NH_4^+ influx were suppressed by provision of Gln alone, or together with NH_4NO_3 plus MSX. Furthermore, *AtAMT1* transcript levels and $^{13}NH_4^+$ influx were negatively correlated with root Gln concentrations, following re-supply of N to N-starved plants. In addition to this level of control, the data suggest that high cytoplasmic [NH_4^+] may inhibit NH_4^+ influx.

Introduction

Inorganic nitrogen (N) uptake by plant roots is subject to strict regulation according to whole plant demand (Crawford and Glass, 1998). There is widespread agreement that rates of NO_3^- and/or NH_4^+ uptake are determined by negative feedback from accumulated N (Glass and Siddiqi, 1995; Imsande and Touraine, 1994; Lee and Rudge, 1986; Morgan and Jackson, 1988). However, there is a lack of consensus regarding the identity of the cellular N pool(s) responsible for initiating this control. A co-ordinated regulation of all forms of nitrogen transport by feedback from downstream metabolites of inorganic N was proposed by Lee and Rudge (1986). The operation of a carbon/nitrogen sensor in microorganisms, the so-called PII protein, that regulates both glutamine synthetase and NH_4^+ transport (Arcondeguy *et al.*, 1997; Magasanik, 1988) is consistent with this proposal. Recently, Hsieh *et al.* (1998) have demonstrated the presence of a putative PII homologue in *Arabidopsis thaliana*.

Cooper and Clarkson (1989) proposed that the cycling of amino acids between shoots and roots was responsible for integrating and regulating N uptake. However, others have proposed that NO_3^-, NH_4^+ and/or various amino acids may regulate inorganic N influx (see Glass and Siddiqi, 1995 for review). Furthermore, details of the underlying molecular mechanisms responsible for these putative feedback loops are rudimentary. Such details will facilitate attempts to improve nutrient uptake efficiency in transgenic crop plants, overexpressing particular ion transporters.

The cloning of genes that encode the NO_3^- and NH_4^+ transporters (Ninnemann *et al.*, 1994; Trueman *et al.*, 1996) now makes it possible to evaluate the mechanism(s) responsible for these control systems at the molecular level. Here we focus on a putative high-affinity NH_4^+ transporter. The importance of NH_4^+ as a direct source of N for plant growth has been substantially underestimated. In many soils, NO_3^- is undetectable, and NH_4^+ and amino acids represent the main sources of N for plant nutrition (Glass and Siddiqi, 1995; Kielland, 1994; Stark and Hart, 1997). Furthermore, in agricultural soils where both NO_3^- and NH_4^+ are present, NH_4^+ strongly inhibits NO_3^- uptake (Aslam *et al.*, 1996; Lee and Drew, 1989). Thus, the uptake of NH_4^+ and particularly the regulation of this uptake at the physiological and molecular levels warrants much greater attention.

Genes encoding NH_4^+ transport systems have been isolated and characterized in *Saccharomyces cerevisiae* and other microorganisms (Arcondeguy *et al.*, 1997; Dubois and Grenson, 1979; Marini *et al.*, 1994; Marini *et al.*, 1997). Three such genes, namely *MEP1, MEP2* and *MEP3,* which are thought to encode two high-affinity transport systems and a low affinity-system, respectively, have been cloned from *S. cerevisiae* (Dubois and Grenson, 1979; Marini *et al.*, 1994; Marini *et al.*, 1997). In higher plants, a putative high-affinity NH_4^+ transporter gene (*AtAMT1*) was

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cloned from *A. thaliana* by heterologous complementation of the *mep1-1 mep2-1* NH_4^+ transport mutant of *S. cerevisiae* (Ninneman *et al.*, 1994). The predicted sequence of the AtAmt1 transporter was homologous with the Mep1 NH_4^+ transport protein from *S. cerevisiae.* Furthermore, when expressed heterologously in the *mep1-1 mep2-1* double mutant, it functioned as a saturable high-affinity transporter capable of restoring ^{14}C-methylamine uptake in the yeast mutant.

A root-specific homologue (*LeAMT1*) has also been isolated from *Lycopersicon esculentum* (Lauter *et al.*, 1996). In addition to the *AMT1* gene homologues, a peribacteroid NH_4^+ channel encoded by the *GmSAT1* gene transports NH_4^+ across the peribacteroid membrane from the bacteroid to the host plant in soybean (Kaiser *et al.*, 1998). This gene has no homology with the *AtAMT1* gene.

The paucity of information concerning the regulation of *AtAMT1* gene expression, together with the lack of agreement regarding the control of NH_4^+ influx from physiological studies, prompted our investigation of the putative regulatory mechanism(s). Preliminary results using heterologous expression of the *AtAMT1* gene in *S. cerevisiae* indicated that NH_4^+ transport in cells grown in the presence of NH_4^+ was not subject to down-regulation, and the authors proposed the measurement of *AMT1* expression directly in plants subjected to different nutritional conditions (Ninnemann *et al.*, 1994). Here we report the results of such a study of *AtAMT1* gene expression integrated with physiological studies of $^{13}NH_4^+$ influx and biochemical analyses of tissue N pools in the roots of *A. thaliana*. These results demonstrate that *AtAMT1* transcript levels are not regulated by tissue NH_4^+ concentrations, but rather by Gln. Nevertheless, high levels of accumulated NH_4^+ may exert inhibitory effects, possibly post-translational or allosteric effects, at the level of the high-affinity transport protein.

Results

Transmembrane fluxes and compartmentation of NH_4^+

Standard compartmental analyses (Kronzucker *et al.*, 1995; Wang *et al.*, 1993a) were undertaken using intact roots to develop protocols for estimating plasma membrane $^{13}NH_4^+$ influx and cellular turnover rates for NH_4^+. Typically, approximately 98% of ^{13}N effluxing from the cytoplasmic compartment of $^{13}NH_4^+$-labeled *Arabidopsis* roots consisted of $^{13}NH_4^+$. Semi-log plots of this $^{13}NH_4^+$ efflux against time generated $t_{0.5}$ values for cell wall and cytoplasmic $^{13}NH_4^+$ exchange of 39 ± 3 sec and 12.6 ± 0.5 min, respectively, in roots of plants provided with 100 μM NH_4NO_3. Corresponding values for 1 mM NH_4NO_3-grown plants were not significantly different, as shown in Table 1. These values are similar to those obtained from previous studies (Kronzucker *et al.*, 1995; Presland and McNaughton, 1986; Wang *et al.*, 1993a). Flux values (Table 1) calculated from efflux analysis indicated a high value for plasma membrane NH_4^+ influx (5.2 ± 1.6 μmol g^{-1} FW h^{-1}) and net uptake (4.5 ± 1.37 μmol g^{-1} FW h^{-1}) into roots of plants acclimated in 100 μM NH_4NO_3. Efflux values (0.7 ± 0.2 μmol g^{-1} FW h^{-1}) were relatively low, while cytoplasmic NH_4^+ concentrations were estimated to be 22 ± 4 mM. Cytoplasmic values for $[NH_4^+]$ were in the same range as those estimated by NMR using maize roots (Lee *et al.*, 1992). Steady state growth at 1 mM NH_4NO_3 increased influx, efflux, cytoplasmic $[NH_4^+]$ and the ratio of efflux to influx, as observed in previous studies (Kronzucker *et al.*, 1995; Wang *et al.*, 1993a).

Table 1. NH_4^+ fluxes (μmol g^{-1} FW h^{-1}) and cytoplasmic $[NH_4^+]$ (μmol g^{-1} FW) derived from compartmental analysis using roots of *A. thaliana*

Parameter	100 μM plants	1 mM plants
φ_{oc}	5.2 ± 1.6	9.5 ± 2.1
φ_{co}	0.7 ± 0.2	3.3 ± 1.3
φ_{net}	4.5 ± 1.4	6.2 ± 1.3
$t_{0.5}$ (cell wall)	39 ± 3 s	36 ± 5 s
$t_{0.5}$ (cytoplasm)	12.6 ± 0.5 min	10.8 ± 1.1 min
$[NH_4^+]_{cyt}$	22 ± 4 mM	43 ± 8 mM

Plants were grown in 100 μM ($n = 6$) or 1 mM ($n = 11$) NH_4NO_3. φ_{oc}: influx of NH_4^+ across the plasma membrane from cell wall to cytosol, φ_{co}: efflux across the plasma membrane from cytosol to cell wall; φ_{net}: net flux across the plasma membrane from cell wall to cytosol, $[NH_4^+]_{cyt}$: cytoplasmic $[NH_4^+]$. Fluxes were measured under steady state conditions.

The high-affinity NH_4^+ transport system (HATS): regulation of AtAMT1 mRNA levels and NH_4^+ influx by N supply

Northern analyses of root *AtAMT1* gene expression in plants maintained in 100 μM NH_4NO_3 revealed an abundant 1.8 kb transcript (Figure 1a), whose intensity was reduced to approximately 7% of this value by provision of 1 mM or 10 mM NH_4NO_3. Short-term measures of high-affinity $^{13}NH_4^+$ influx revealed a hyperbolic pattern (Figure 1b), with K_m and V_{max} values (derived from direct fits to the Michaelis–Menten equation) of 168 μM and 17 μmol g^{-1} FW h^{-1}, respectively, for plants previously grown in 100 μM NH_4NO_3. V_{max} values were reduced to 6.1 and 3.1 μmol g^{-1} FW h^{-1} in plants maintained in 1 mM and 10 mM NH_4NO_3. Statistical testing of the differences among K_m and V_{max} values by *F*-tests of the slopes of Hoffstee and Hanes plots,

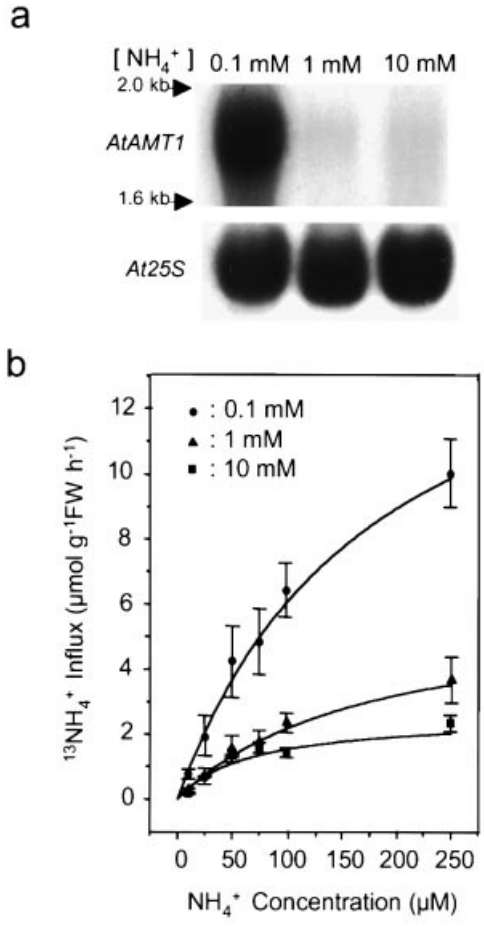

Figure 1. Accumulation of *AtAMT1* mRNA and short-term $^{13}NH_4^+$ influx (µmol g^{-1} FW h^{-1}) into roots of *A. thaliana* as a function of prior N provision.
(a) Northern blot analysis of root total RNA, probed with the *AtAMT1* cDNA. RNA molecular size standards are indicated on the left. The same Northern blot was reprobed with *At25S rDNA*, used as internal standard.
(b) $^{13}NH_4^+$ influx into roots of plants grown in 0.1 mM, 1 mM and 10 mM NH_4NO_3. $^{13}NH_4^+$ influx values are means ± SEM (n = 4). Each flux experiment was repeated three times.

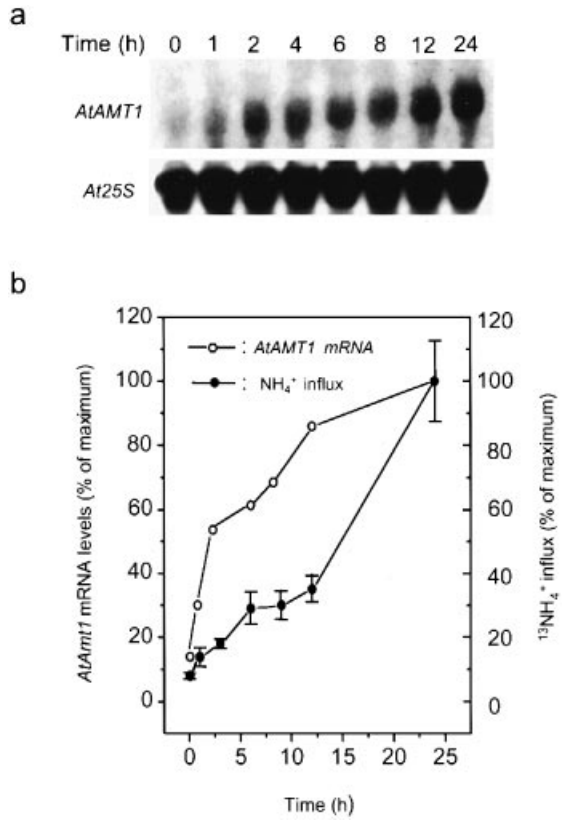

Figure 2. Time-dependent up-regulation of *AtAMT1* mRNA levels and $^{13}NH_4^+$ influx (µmol g^{-1} FW h^{-1}) in roots of *A. thaliana*, following the transfer of 3-week-old plants from 1 mM NH_4NO_3 to 0.1 mM NH_4NO_3.
(a) Northern blot analysis of root RNA, probed with the *AtAMT1* cDNA. Internal standard was shown in Figure 1.
(b) *AtAMT1* expression levels (○) and $^{13}NH_4^+$ influx (●), expressed as percentages of their maximum values.

respectively, revealed significant differences only among V_{max} values.

Time-dependent up-regulation and down-regulation of AtAMT1 mRNAs and high-affinity NH_4^+ influx on withdrawal or resupply of NH_4NO_3

Transfer of plants from 1 mM to 100 µM NH_4NO_3 solutions resulted in rapid increases of *AtAMT1* mRNA expression levels (Figure 2a,b), which by 24 h had increased 7.5-fold relative to those at time 0. High-affinity $^{13}NH_4^+$ influx (Figure 2b) also increased continuously, although lagging behind the changes of *AtAMT1* mRNA levels. By 24 h, $^{13}NH_4^+$ influx had increased 12-fold from 0.36 µmol g^{-1} FW h^{-1} at the time of transfer to 4.47 µmol g^{-1} FW h^{-1}. For purposes of comparison, Figure 2(b) shows the changes of *AtAMT1* mRNA levels and $^{13}NH_4^+$ influx during the 24 h treatment as percentages of their values at 24 h.

Down-regulation of $^{13}NH_4^+$ influx was examined by transferring plants, previously acclimated in 1 mM NH_4NO_3, to N-free media for 2 days to minimize internal N reserves and increase NH_4^+ influx. These plants were then exposed to 5 mM NH_4NO_3 for intervals of up to 24 h. Northern analysis of root *AtAMT1* transcript abundance following resupply of N revealed a rapid decline during the first 6 h of down-regulation (Figure 3a,b). High-affinity $^{13}NH_4^+$ influx, measured at 100 µM NH_4NO_3, also declined rapidly during this time (Figure 3b). Thus, by 12 h, $^{13}NH_4^+$ influx had decreased by 93% from 4.82 to 0.34 µmol g^{-1} FW h^{-1}. Expressing *AtAMT1* transcript abundance and NH_4^+ influx as percentages of their maximum values (Figure 3b) reveals that NH_4^+ influx declined more rapidly than the decline of root *AtAMT1* transcript abundance. This pattern was consistently observed during down-regulation.

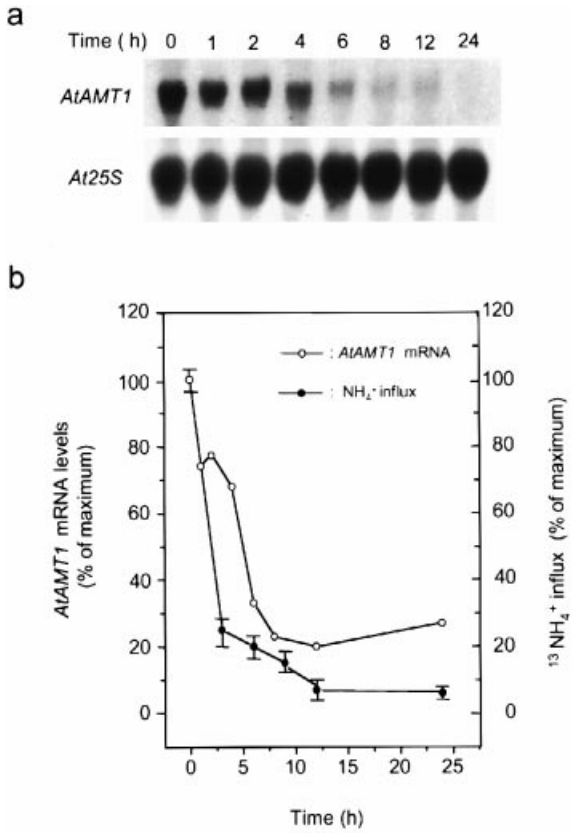

Figure 3. Time-dependent down-regulation of *AtAMT1* mRNA levels and $^{13}NH_4^+$ influx in roots of *A. thaliana*, following re-supply of NH_4NO_3.
Plants were supplied with 1 mM NH_4NO_3 for 19 days, then deprived of N for 2 days, prior to transfer to 5 mM NH_4NO_3.
(a) Northern blot analysis of root RNA, probed with the *AtAMT1* cDNA. Internal standard was as in Figure 1.
(b) *AtAMT1* mRNA levels (○) and $^{13}NH_4^+$ influx (●) during down-regulation, expressed as percentages of their maximum values.

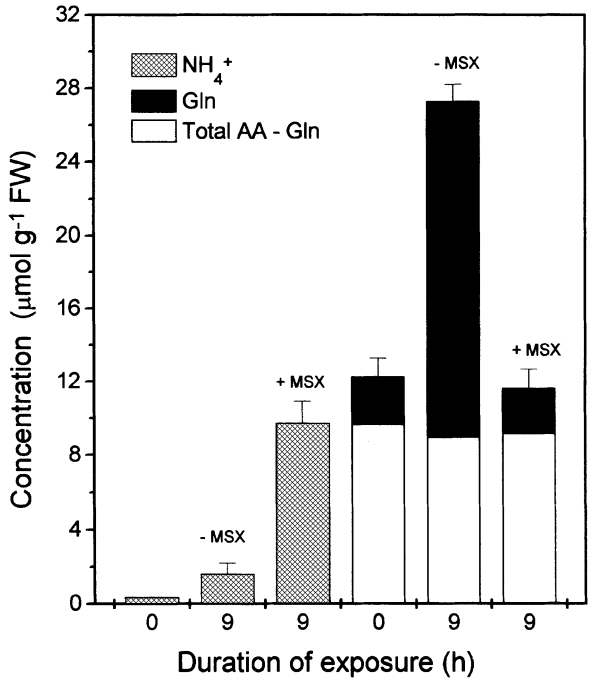

Figure 4. Root NH_4^+, glutamine and total amino acid concentrations of *A. thaliana* plants (μmol g^{-1} FW), at 0 and 9 h after resupply of 5 mM NH_4NO_3 to N-limited plants, in the presence and absence of 1 mM MSX.

Changes of cellular N pools during down-regulation of AtAMT1 transcript levels and NH_4^+ influx

In order to identify the N pools (NH_4^+ or assimilation products) responsible for the observed changes of NH_4^+ influx and *AtAMT1* expression during down-regulation, we measured root and shoot concentrations of NH_4^+ and individual amino acids, patterns of $^{13}NH_4^+$ influx, and *AtAMT1* expression levels, in the presence and absence of 1 mM methionine sulfoximine (MSX) following re-supply of 5 mM NH_4NO_3. The inhibitor MSX blocks the action of the enzyme glutamine synthetase (GS) resulting in elevated [NH_4^+] and reduced [Gln] (King *et al.*, 1993; Lee *et al.*, 1992). As shown in Figure 3(a,b), the *AtAMT1* transcript level was reduced to about 20% of its initial (time 0) level, 9 h after the transfer to 5 mM NH_4NO_3. Therefore, to minimize the duration of exposures to MSX in subsequent experiments, 9 h treatments were employed as the standard for experiments using MSX.

The most prominent change in root N pools during down-regulation was that of Gln (Figure 4), which increased ninefold, from 2.58 μmol g^{-1} FW at time 0 to 23.2 μmol g^{-1} FW at 24 h. No other amino acid showed such a substantial change in concentration, while root [NH_4^+] increased 3.8-fold. Table 2 provides r^2 values for the regressions of *AtAMT1* transcript levels (expressing transcript level at time 0 as 100%), against root or shoot amino acid concentrations during the 24 h following resupply of NH_4NO_3. Only Gln, aspartate and the sum of all amino acids gave statistically significant r^2 values (0.92, 0.85 and 0.86, respectively). Figure 5 reveals the strong negative correlations between *AtAMT1* levels and root [Gln], and $^{13}NH_4^+$ influx and root [Gln], throughout the 24 h of exposure to 5 mM NH_4NO_3. In leaf tissue, like roots, the largest absolute and relative change was in Gln concentrations.

When MSX was provided together with 5 mM NH_4NO_3, the *AtAMT1* transcript level at 9 h remained at 88% of its original value (Figure 6a,b). During this time, root [NH_4^+] increased 27-fold from 0.35 μmol g^{-1} FW at time 0 to 9.7 μmol g^{-1} FW at 9 h (Figure 4), while root Gln concentration remained relatively unchanged (declining from 2.58 to 2.41 μmol g^{-1} FW). When plants were treated for 9 h with 1 mM MSX, together with 5 mM NH_4NO_3 and 5 mM Gln, *AtAMT1* transcript expression was reduced by 76% (Figure 6a), confirming the capacity of Gln to

Table 2. Coefficients of determination (r^2) for the relationships between *AtAMT1* transcript levels and tissue amino acid concentrations ([A.A.]), and changes in tissue [A.A.] (μmol g^{-1} FW) 24 h after supplying 5 mM NH_4NO_3

	r^2 †		Changes in [A.A.] ‡	
Amino acid	Root	Shoot	Root	Shoot
Ammonium	0.50	0.46	0.98 (3.8)	0.80 (3.6)
Alanine	0.03	0.72*	0.63 (1.4)	0.82 (2.7)
Arginine	0.23	0.49	0.15 (1.9)	0.97 (5.7)
Aspartate	0.84**	0.54	0.57 (6.5)	0.47 (3.2)
Asparagine	0.60	0.39	0.54 (2.1)	1.83 (2.9)
Gaba	0.01	0.52	nc	0.63 (1.9)
Glutamate	0.04	0.28	nc	0.54 (3.0)
Glutamine	0.92**	0.79*	20.6 (9.0)	14.3 (6.5)
Glycine	0.58	0.22	0.18 (1.7)	nc
Histidine	0.24	0.53	nc	0.05 (4.6)
Isoleucine	0.36	0.35	0.05 (0.5)	0.02 (1.9)
Leucine	0.23	0.35	nc	0.07 (4.4)
Lysine	0.18	0.54	nc	0.09 (3.8)
Methionine	0.01	0.06	nc	nc
Phenylalanine	0.01	0.37	nc	0.03 (2.0)
Proline	0.40	0.8*	–0.98 (0.6)	1.00 (1.9)
Serine	0.13	0.52	nc	0.55 (1.8)
Threonine	0.08	0.78*	nc	0.28 (3.1)
Tyrosine	0.07	0.16	nc	nc
Valine	0.07	0.35	nc	0.08 (2.2)
Total A.A.	0.86**	0.74*	21.8 (2.8)	21.7 (3.8)
Total A.A. – Gln	0.24	0.59	nc	7.41 (2.4)

In parentheses: $[A.A.]_{t=24\,h}/[A.A.]_{t=0\,h}$ ratio.
†Regressions were based upon measurements at six time intervals (four replicates each) within a 24 h period.‡Changes in [A.A.] are shown only for the longest interval (0–24 h).
*$P<0.05$, **$P<0.01$, nc: no change.

circumvent the MSX blockage of NH_4NO_3 assimilation. Glutamine, either alone or together with 5 mM NH_4NO_3, also strongly reduced *AtAMT1* mRNA expression levels (Figure 6a). Corresponding measurements of NH_4^+ influx (Figure 6b) revealed that the presence of MSX, together with NH_4NO_3, had reduced influx by <10% by 3 h, compared to 90% in the absence of MSX. By 9 h, however, influx was reduced by approximately 35%. When N-deprived plants (control treatment) were supplied with 5 mM Gln in place of 5 mM NH_4NO_3, $^{13}NH_4^+$ influx was reduced from 8.67 ± 1.42 μmol g^{-1} FW h^{-1} (control) to 1.33 ± 0.23 μmol g^{-1} FW h^{-1} by 9 h.

The low-affinity NH_4^+ transport system (LATS): NH_4^+ influx is independent of N supply

As shown in Figure 7, at NH_4^+ concentrations from 1 to 10 mM, a low-affinity (LATS) transport system was evident. In plants grown in 100 μM NH_4NO_3, this transport system showed no indication of saturation and, in contrast to the down-regulation of high-affinity NH_4^+ influx, low-affinity influx was not reduced by growth at 1 mM or 10 mM NH_4NO_3. Since measured $^{13}NH_4^+$ fluxes at high external NH_4^+ concentration are the resultant of the two transport systems (HATS plus LATS), the V_{max} values for $^{13}NH_4^+$ were subtracted from measured $^{13}NH_4^+$ influxes to generate the LATS contribution to measured $^{13}NH_4^+$ fluxes. Figure 7 shows the subtracted values for LATS fluxes in plants grown at 100 μM, 1 mM and 10 mM when $^{13}NH_4^+$ fluxes were measured at 1–10 mM external $[NH_4^+]$. The regression lines for $^{13}NH_4^+$ influx against external $[NH_4^+]$ were so similar that a single regression line provided a good fit of the data ($r^2 = 0.94$) at all values except perhaps at 10 mM external $[NH_4^+]$.

Discussion

Regulation of NH_4^+ influx in response to N supply

The results of the present compartmental analyses confirm that roots of *Arabidopsis* plants behave essentially like those of rice and white spruce, with respect to half-lives of $^{13}NH_4^+$ exchange and accumulation of NH_4^+ in cytoplasmic and cell wall compartments (Kronzucker *et al.*, 1995; Wang *et al.*, 1993a). We therefore settled on influx and desorption times of 10 and 3 min, respectively, to estimate plasma

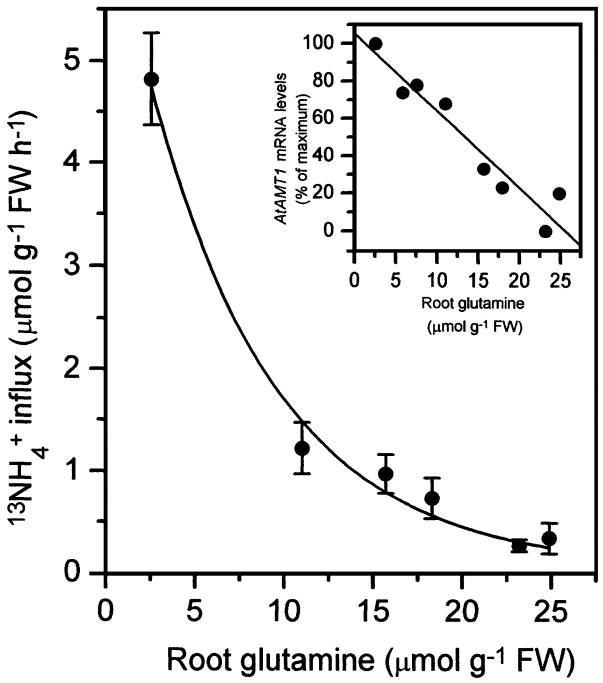

Figure 5. *AtAMT1* expression levels (main figure) expressed as a percentage of maximum expression levels at time 0, and $^{13}NH_4^+$ influx in μmol g^{-1} FW h^{-1} (inset) during a 24 h period of down-regulation (data from Figure 3) as functions of root glutamine concentrations in μmol g^{-1} FW (from Figure 4).
Regression lines for *AtAMT1* expression levels and $^{13}NH_4^+$ influx are given by a linear regression ($r^2 = 0.92$), and a polynomial regression $Y = 5.55–0.46\ X + 0.01\ X^2$ ($r^2 = 0.89$), respectively.

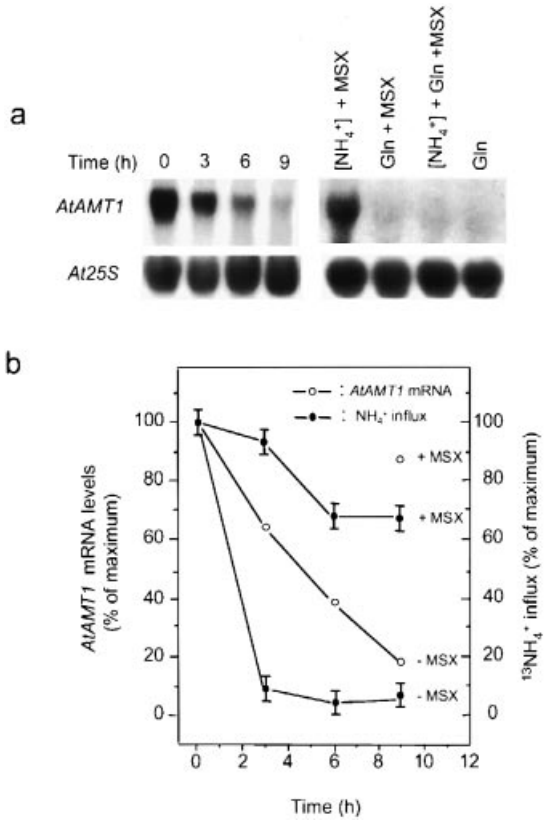

Figure 6. Effects of NH_4NO_3, MSX and Gln on *AtAMT1* mRNA levels and $^{13}NH_4^+$ influx in roots of *A. thaliana*.
(a) Down-regulation of *AtAMT1* mRNA levels by 5 mM NH_4NO_3 (left panel) and effects of MSX and Gln (right panel). Internal standard as shown in Figure 1.
(b) *AtAMT1* mRNA levels (○) and $^{13}NH_4^+$ influx (●) in the presence and absence of MSX, expressed as percentages of their maximum values.

membrane NH_4^+ influx. Varying the ambient N supply to *A. thaliana* plants prior to influx determinations resulted in a strong down-regulation of the high-affinity NH_4^+ transport system (HATS). This is consistent with several earlier studies using several crop species (Becking, 1956; Causin and Barneix, 1993; Morgan and Jackson, 1988; Wang *et al.*, 1993a; Wang *et al.*, 1993b). The results of these studies established that NH_4^+ uptake was negatively correlated with prior N supply but failed to provide consensus regarding the underlying mechanism(s) of this feedback, particularly which N pool, NH_4^+ or products of its assimilation were responsible for the feedback.

High V_{max} values and a saturable concentration response for $^{13}NH_4^+$ influx in roots of *A thaliana* validate the designation of this transport system as a high-affinity (HATS) NH_4^+ transport system. Furthermore, while V_{max} values responded to the NH_4NO_3 supply under steady-state conditions, as shown in previous reports (Kronzucker *et al.*, 1995; Wang *et al.*, 1993b), K_m values were not correlated with N supply. Absolute values for K_m varied from 85 to 168 μM, but failed to correlate with N supply as was reported by Wang *et al.* (1993a) for rice. Literature reports for K_m values vary considerably (see Glass and Siddiqi, 1995), ranging from approximately 20–167 μM. Lycklama (1963) reported values from 40 to 200 μM according to plant age, using $^{14}NH_4^+$ depletion to determine K_m values for net NH_4^+ uptake by roots of ryegrass.

AtAMT1 gene expression and N supply

The high levels of *AtAMT1* gene expression in roots of low-N plants, which showed high HATS activity, provide correlative evidence that this gene encodes a HATS for NH_4^+ influx in *A. thaliana*. Likewise, the demonstrated changes of V_{max} for $^{13}NH_4^+$ influx, and the short-term changes of influx resulting from manipulating the N supply, were correlated with changes of *AtAMT1* gene expression. These results provide evidence that this feedback loop operates, at a minimum, through effects at the level of *AtAMT1* mRNA. However, while changes of *AtAMT1* expression consistently preceded changes of $^{13}NH_4^+$ influx during up-regulation (Figure 2), $^{13}NH_4^+$ influx was reduced more rapidly than *AtAMT1* transcript levels during down-regulation (Figures 3 and 6). We interpret this

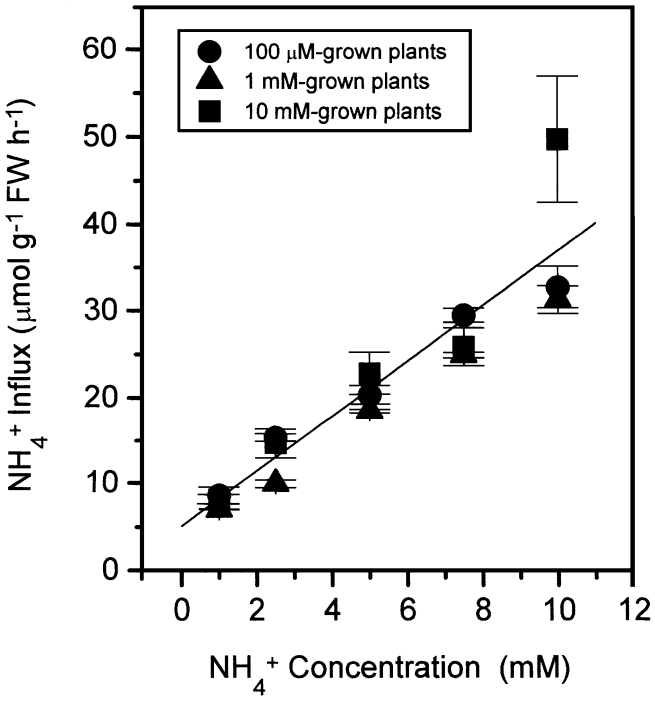

Figure 7. $^{13}NH_4^+$ influx (μmol g^{-1} FW h^{-1}) due to the LATS activity (calculated by subtracting the HATS fluxes from measured fluxes) at 1–10 mM external [NH_4^+].

anomaly as the result of direct inhibitory effects of cytosolic NH_4^+ on membrane transport activity (as discussed below).

Regulation of AtAMT1 gene expression depends upon tissue Gln concentrations

The results of the short-term experiments in which N supply was withheld or resupplied provide the context in which to identify which cellular N pool(s) are responsible for regulating *AtAMT1* gene expression. The outcome of the experiments using MSX, with or without added Gln, generate a convincing argument that tissue NH_4^+ is not responsible for the observed effects on *AtAMT1* gene expression. Rather, the correlations between *AtAMT1* gene expression and root [Gln], and between $^{13}NH_4^+$ influx and root [Gln] (Figure 5 and Table 2), provide strong evidence that Gln is the controlling agent. Although aspartate and total amino acid concentrations were significantly correlated with *AtAMT1* gene expression (Table 2), root aspartate concentrations were typically <4% of Gln concentrations at all time points and, therefore, represented only a minor component of the increased tissue N associated with N resupply. The data from Figure 4 and Table 2 establish that the major part of absorbed NH_4^+ was accounted for by conversion to Gln within the roots. Moreover, the high r^2 for the sum of all amino acids (Table 2) was reduced from 0.86 to 0.24 when Gln was removed from the regression. Thus the evidence points to root Gln concentrations as responsible for the down-regulation of *AtAMT1* transcript levels. These conclusions are consistent with earlier physiological studies using wheat, barley and squash which demonstrated that exogenous application of Gln reduced NH_4^+ uptake (Causin and Barneix, 1993; Lee *et al.*, 1992; Wieneke and Roeb, 1998).

Although our analyses of root and shoot Gln concentrations were average tissue concentrations, it has been demonstrated in earlier studies (Winter *et al.*, 1992) that cellular amino acids are mainly localized within the cytosol. The rapidity of the observed changes of $^{13}NH^+$ influx and *AtAMT1* gene expression, resulting from perturbation of the N supply, argue for control via cytoplasmic N pools. While we have no data on rates of amino acid turnover in the cytoplasm, the present compartmental analyses indicate that the $t_{0.5}$ for cytoplasmic NH_4^+ exchange is about 12 min. Thus, changes of gene expression and NH_4^+ transport might be evident within hours of perturbing the N supply.

Effects of cellular NH_4^+ on the NH_4^+ transporter

The application of MSX plus 5 mM NH_4NO_3 caused virtually no down-regulation of *AtAMT1* gene expression, which remained at 88% of the control (0 time) value after 9 h (Figure 6). By contrast, $^{13}NH_4^+$ influx was consistently reduced by 30–40% under the same conditions (Figure 6). Given that the [NH_4^+] of MSX-treated roots had increased 27-fold by 9 h (Figure 4), and that Gln levels were essentially unchanged, increased efflux of NH_4^+ during influx measurement might have reduced the specific activities of $^{13}NH_4^+$ in the influx media, resulting in an artificial lowering of the calculated influx values. By measuring the [NH_4^+] of influx media after the 10 min influx period, we demonstrated that there were no changes to the $^{13}NH_4^+$ specific activity. Thus, the reduced values for $^{13}NH_4^+$ influx associated with high cytosolic [NH_4^+] in the presence of MSX are real and may result from inhibitory effects of cytosolic NH_4^+ on the activity of the HATS protein. Previous experiments have demonstrated that NH_4^+ is able to inhibit plasma membrane influx and efflux of NO_3^- within minutes of its application (Aslam *et al.*, 1996; Glass and Siddiqi, 1995; Lee and Drew, 1989).

Differential response of the HATS and LATS to N status

Ammonium transport in *A. thaliana* showed biphasic uptake kinetics as previously reported for roots of *Lemna*, rice and white spruce (Kronzucker *et al.*, 1996; Ullrich *et al.*, 1984; Wang *et al.*, 1993b). At low external [NH_4^+], the saturable high-affinity transporter is capable of active NH_4^+

absorption, while at higher concentrations (> 1 mM NH_4^+) transport is passive (Ullrich *et al.*, 1984; Wang *et al.*, 1993b). In contrast to the observed down-regulation of HATS activity and *AtAMT1* transcript levels, expression of the low-affinity transport system in roots of *A. thaliana* failed to demonstrate down-regulation when plants were grown at 1 mM and 10 mM external [NH_4^+] (Figure 7). This phenomenon whereby internal ion concentrations regulate the activity of high-affinity transporters but not the corresponding low-affinity systems, was first observed for K^+($^{86}Rb^+$) influx in barley and ryegrass (Glass and Dunlop, 1978) and subsequently in corn (Kochian and Lucas, 1982). It was also evident in studies of NH_4^+ influx in rice (Wang *et al.*, 1993b). It is perplexing that only the high-affinity systems should be regulated in this way. However, the apparent absence of LATS regulation may account for the toxic effects associated with elevated NH_4^+ provision in many species (Bloom, 1988; Gill and Reisenauer, 1993; Magalhaes and Wilcox, 1991). It might be argued that the elevated concentrations employed to measure low-affinity transport systems are representative only of agricultural soils where high levels of fertilizer are applied. Hence, the conditions required to measure the operation of the low-affinity transporters could be considered somewhat unnatural. Nevertheless, the existence of biphasic (high- and low-affinity) transporters for a wide range of inorganic and organic nutrients appears to be universal, suggesting functional operation outside the agricultural context.

In summary, both high- and low-affinity NH_4^+ transporters are expressed in roots of *A. thaliana*. Expression levels of the *AtAMT1* gene were strongly correlated with high-affinity NH_4^+ influx, providing further evidence for the physiological function of this gene in high-affinity NH_4^+ transport. Transcript levels of the *AtAMT1* gene appear to be regulated by root Gln concentrations, but not by NH_4^+. Since Gln is the first product of NH_4^+ assimilation in roots (and in shoots), this compound probably serves as the primary signal of cellular N-status. Nevertheless, high levels of cytoplasmic NH_4^+ act upon the transport protein, either through direct (e.g. allosteric effects) or via post-translational events. A model summarizing the findings of the present study is shown in Figure 8.

Experimental procedures

Growth of plants

A. thaliana (L.) plants (ecotype Columbia) were grown in a controlled environment chamber at 20 ± 2°C, in 16 h light/8 h dark cycles, 70% RH and 250 μmol photons $m^{-2}s^{-1}$ at plant level (Vitalite fluorescent tubes, Durotest, North Bergen, NJ, USA). Twenty seeds were germinated in 5 ml of sterile culture solution: 29 mM sucrose, 2.6 mM MES, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM NH_4NO_3, 1 mM $CaCl_2$, 20 μM NaFeEDTA, 25 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuCl_2$, and 0.2 μM Na_2MoO_4 at pH 5.7. The choice of NH_4NO_3 as the N source for plant growth was based upon the relatively poor growth of *A. thaliana* on NH_4Cl or $(NH_4)_2SO_4$ (S,R. Rawat *et al.*, unpublished observations).

After 3 days, seedlings were transferred to sterile culture vessels (Magenta Corporation, Chicago, IL, USA), containing 60 ml of fresh culture solution and were gently agitated on a gyrotory shaker. Nutrient solutions were replaced frequently to maintain steady state with respect to plant N status. In order to generate low-, intermediate-and high-N plants, respectively, 2-week-old plants were transferred from media containing 1 mM NH_4NO_3 to fresh media containing 100 μM NH_4NO_3, 1 mM NH_4NO_3, or 10 mM NH_4NO_3 which were replaced regularly. In short-term perturbation experiments, low-N plants were transferred to solutions containing 5 mM NH_4NO_3, and high-N plants were transferred to solutions containing 100 μM NH_4NO_3. During

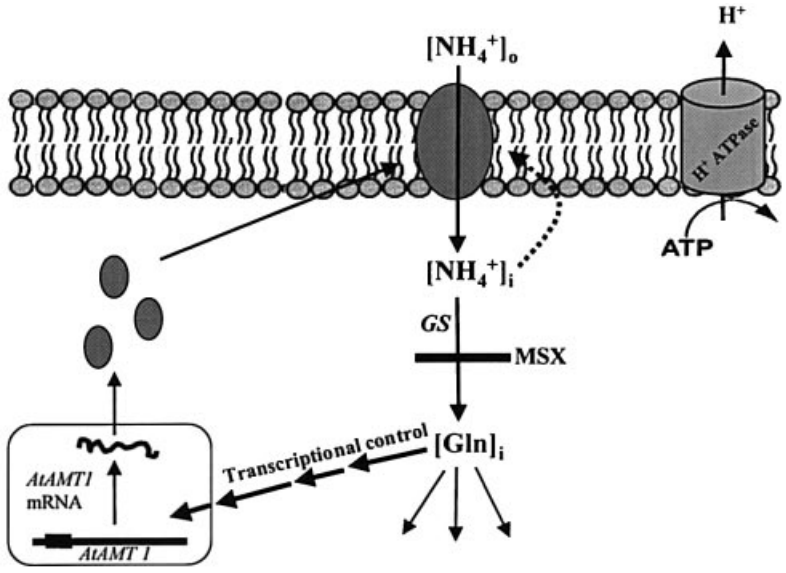

Figure 8. A model describing the regulation of high-affinity NH_4^+ transport, at the level of *AtAMT1* transcription and at the level of the NH_4^+ transporter.
AtAMT1 gene transcript level is controlled through effects of cellular Gln (solid lines), while NH_4^+ may act upon the pAtAMT1 transport protein (dotted lines) through direct (e.g. allosteric or post-translational) effects.

experiments lasting up to 24 h, NH_4^+ influx values, tissue N levels and *AtAMT1* expression levels were determined.

Efflux analysis

In order to develop an appropriate protocol for influx measurements, efflux analyses were undertaken using 21-day-old plants maintained at 100 μM or 1 mM NH_4NO_3. Efflux analysis was undertaken according to previously published methods (Kronzucker *et al.*, 1995). Roots were equilibrated for 5 min in fresh media and then exposed to $^{13}NH_4^+$-labeled culture medium containing 100 μM or 1 mM NH_4NO_3 for 45 min ^{13}N eluting from the superficial solution, the cell wall and the cytoplasmic compartments was counted in a Packard gamma-counter (Minaxi γ, Auto-gamma 5000 series, Downer's Grove, IL, USA), and the half-lives for $^{13}NH_4^+$ exchange of the above compartments, NH_4^+ flux values between compartments, and cytoplasmic [NH_4^+] were estimated (see Kronzucker *et al.*, 1995; Lee and Clarkson, 1986). To ensure that the label effluxing from roots consisted of $^{13}NH_4^+$ rather than products of $^{13}NH_4^+$ assimilation, the difference between counts before and after alkalization and boiling was determined. Typically, about 98% of ^{13}N contained in the original eluates were volatilized by 6 min of boiling.

$^{13}NH_4^+$ influx determinations

$^{13}NH_4^+$ influx was determined from efflux analysis (see above) and by direct measurement of the accumulation of ^{13}N from nutrient solutions during a 10 min influx period followed by 3 min desorption in identical non-labeled solution to remove tracer from the cell wall. These times were based upon the calculated half-lives for tracer exchange of the cell wall (0.56–0.66 min) and cytoplasmic compartments (10.7–12.6 min) as a compromise which optimized removal of tracer from the cell wall while minimizing loss of tracer from the cytoplasm. Total ^{13}N accumulated in roots and shoots was determined by gamma-counting.

Time-dependent analysis of $^{13}NH_4^+$ influx, AtAMT1 mRNA levels and root [NH_4^+] and [Gln]

For up-regulation, plants previously acclimated in 1 mM NH_4NO_3 for 21 days were transferred to 100 μM NH_4NO_3. AtAMT1 mRNA levels and $^{13}NH_4^+$ influx (from 100 μM NH_4NO_3) were subsequently measured at intervals up to 24 h. To investigate the pattern of down-regulation, plants were acclimated in 1 mM NH_4NO_3 for 19 days and then transferred to solutions without N for 2 days to reduce tissue N and generate plants with high initial rates of NH_4^+ influx. These were then transferred to 5 mM NH_4NO_3, and AtAMT1 mRNA levels and $^{13}NH_4^+$influx (from 100 μM NH_4NO_3) were measured for up to 24 h. All transfers were staggered so that AtAMT1 mRNA levels and $^{13}NH_4^+$ fluxes were determined at the same time so as to eliminate diurnal effects. This was also essential because of the short half-life (9.98 min) of the ^{13}N tracer. To investigate the down-regulation of NH_4^+ influx, 1 mM methionine sulfoximine (MSX), an inhibitor of the enzyme glutamine synthetase, was used to block the conversion of NH_4^+ to Gln. This treatment increased tissue [NH_4^+] and reduced Gln and amino acid levels (King *et al.*, 1993; Lee *et al.*, 1992). In separate experiments, MSX with or without 5 mM Gln, was added to the pre-treatment solutions containing 5 mM NH_4NO_3. AtAMT1 expression and $^{13}NH_4^+$ influx were then measured at intervals up to 9 h. All flux data presented are the means of at least three, typically four replicates. All experiments were repeated at least three times. Analysis of root [NH_4^+] and individual [amino acid] were analyzed by HPLC. Plant tissues from 30 plants were ground to powder in liquid nitrogen and extracted in cold 10 mM sodium acetate buffer (pH 6.5). The extracts were centrifuged at 16 000 ***g***, filtered through a 0.45 μm, filter and then derivatized using the AccQ.Fluor reagent (Waters Chromatography, Milford, MA, USA). Separations were carried out using a 3.9 × 150 mm AccQ.Tag Column (Waters Chromatography, Milford, MA, USA), according to published methods (Van Wandelen and Cohen, 1997) on a Waters 600 LC system and detected with a Waters 474 scanning fluorescence detector (Waters Chromatography, Milford, MA, USA).

RNA extraction and Northern blot analysis

For expression studies, each treatment unit consisted of a single culture vessel containing 30 *Arabidopsis* seedlings. Each treatment was replicated and all experiments were repeated. Total RNAs were isolated from root and shoot tissues using TRIzol Reagent (GIBCO BRL) according to the manufacturer's instructions. Total RNA (15 μg) were fractionated on a 1.2% MOPS-formaldehyde agarose gel and transferred to nylon membrane (Amersham, Oakville, Canada). Northern hybridizations were carried out according to standard protocols (Sambrook *et al.*, 1989). A 1.75 kb *Not*1 fragment of p*AtAMT1* representing a full-length cDNA was purified, ^{32}P-labeled using random priming, and used as a probe. Final washes of Northern blots were done once at 65°C in 0.1× SSPE and 0.1% SDS and subjected to autoradiography using X-OMAT film at –70°C overnight. All EtBr stained gels (for loading corrections) and autoradiographs were normalized by reprobing the same Northern blots with an *At25S* rDNA probe, and analyzed by densitometric scanning using the Molecular Analyst computer software program (Bio-Rad, Hercules, CA, USA).

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