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Characterization of *Arabidopsis* AtAMT2, a novel ammonium transporter in plants

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Abstract **We have cloned and characterized the first member of a novel family of ammonium transporters in plants: AtAMT2 from *Arabidopsis thaliana*. AtAMT2 is more closely related to bacterial ammonium transporters than to plant transporters of the AMT1 family. The protein was expressed and functionally characterized in yeast. AtAMT2 transported ammonium in an energy-dependent manner. In contrast to transporters of the AMT1 family, however, AtAMT2 did not transport the ammonium analogue, methylammonium. *AtAMT2* was expressed more highly in shoots than roots and was subject to nitrogen regulation.**

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Key words: Ammonium transporter; Methylammonium; Nitrogen regulation; *Arabidopsis thaliana*

1. Introduction

Ammonium is one of the major sources of nitrogen for plants. It is taken up from the soil by one or more transporters in the plasma membrane of root cells. Physiological studies of ammonium transport into roots have revealed biphasic kinetics in different species [1–3]. One component of ammonium uptake, the so-called high-affinity ammonium transport system (HATS), is predominant at low (submillimolar) external ammonium concentrations and exhibits saturation kinetics. Electrophysiological experiments have shown that the substrate of the HATS is NH_4^+, and that the membrane electrical potential, $\Delta\psi$, is the driving force for ammonium uptake at low concentrations of the ion [3–7]. The second component of ammonium uptake is a low-affinity system (LATS) that becomes significant at higher external ammonium concentrations (above 1 mM) and exhibits non-saturation kinetics [3,8]. It has been suggested that the LATS may represent either a broad-specificity cation channel that can transport NH_4^+, or simple diffusion of NH_3 through the plasma membrane [9].

Ammonium transporters were first cloned from yeast (MEP1 from *Saccharomyces cerevisiae*; [10]) and higher plants (AMT1 from *Arabidopsis*; [11]). MEP1 and AMT1 are related proteins, homologues of which have been found in animals (e.g. *Caenorhabditis elegans* cDNA yk1g3.5, protein accession number AAA96190), as well as prokaryotes [12–14]. Thus, they represent an ancient family of proteins. The biochemical properties of several plant members of this family, including AtAMT1;1, AtAMT1;2 and AtAMT1;3 from *Arabidopsis* [15], have been studied in yeast. All plant AMT1 proteins examined to date are saturable, high-affinity NH_4^+ transporters that can also transport methylammonium (MA). In fact, [^{14}C]MA has often been used as an analogue of ammonium for transport studies [11].

AMT1 proteins are encoded by multigene families in plants. For example, at least three functional *AtAMT1* genes have been found in *Arabidopsis* [15]. The *Arabidopsis* AtAMT1 proteins are closely related to each other (at least 71% identical) and to homologues in other plant species (at least 62% identical). The expression patterns of the *AMT1* genes in plants suggest that they have different physiological roles, one of which appears to be ammonium uptake from the soil [15,16].

In this paper, we describe the isolation and characterization of AtAMT2, the first member of a novel family of ammonium transporters in plants. AtAMT2 is very distantly related to plant AMT1 proteins but more closely related to some bacterial ammonium transporters. The *AtAMT2* gene exhibits a different pattern of expression in plants than genes of the *AtAMT1* family, and the protein displays distinct biochemical features.

2. Materials and methods

2.1. Construction of cDNA library

A cDNA library was made from polyadenylated RNA extracted from roots of 3 week old *Arabidopsis* (C24), using a Gibco BRL Superscript cDNA synthesis kit. Plants were grown in complete Murashige and Skoog (MS) [17] medium (Sigma), then deprived of nitrogen for 24 h prior to RNA extraction. Total RNA was extracted as described by Logemann et al. [18] and polyadenylated RNA was prepared using oligo dT columns (AMRAD Pharmacia).

2.2. Isolation of AtAMT2 cDNA

Full-length *AtAMT2* cDNA was isolated in two ways. First, PCR was used to generate a full-length cDNA with 5′ *Bam*HI and 3′ *Xba*I sites for directional cloning into the yeast expression vector pYES3 [19]. PCR reactions were performed using *Pfu* polymerase (Stratagene), the *Arabidopsis* root cDNA library as a template, and the following primers: 5′-ACGTGGATCCCAAACCCAACAAAAATTTCATC-3′ (5′ end) and 5′-ACGTTCTAGAATCATAGAACAATGGTGACACCTC-3′ (3′ end).

A second, full-length *AtAMT2* clone was isolated by high-stringency screening of the λZipLox cDNA library, using the *AtAMT2* PCR product as probe. In vivo excision of the phagemid containing *AtAMT2* was carried out prior to sequencing. Sequencing of both the PCR product (cloned in pBluescript) and the phagemid cDNA was

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Abbreviations: HATS, high-affinity ammonium transporter; LATS, low-affinity ammonium transporter; MA, methylammonium; YNB-N, yeast nitrogen base without amino acids and ammonium sulfate

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done using a Perkin Elmer ABI 377 HT sequencer and Big Dye™ chemistry.

2.3. Yeast growth and mutant complementation

Yeast strains were grown at 28°C in yeast nitrogen base without amino acids and ammonium sulfate (YNB-N) supplemented with 2% galactose and 0.1% proline. For growth on solid media, 2% agar was added. Strain MLY131a/α (Δ*mep1* Δ*mep2* Δ*mep3 ura3*; [20]) was transformed with pYES3 and with pYES3 harboring *AtAMT1;1* cDNA [11] or *AtAMT2* cDNA, using a lithium acetate protocol [21]. Yeast transformants were selected on YNB-N supplemented with 2% glucose and 0.1% proline. Complementation of the AMT^- phenotype of MLY131a/α was demonstrated by growth on YNB-N agar supplemented with 2% galactose and 0.5 mM $(NH_4)_2SO_4$.

2.4. Ammonium uptake assay

For ammonium uptake studies, yeast cells were grown to an OD_{600} of 0.7–0.9, washed once in 50 mM sodium acetate buffer, pH 5.0, and resuspended in the same buffer. Yeast cells were resuspended to an OD_{600} of 16 or to a concentration of 20 mg fresh weight per ml, depending on the experiment. Cells were preincubated while stirring for 60 min at room temperature. To start the reaction, an equal volume of 50 mM sodium acetate buffer, pH 5.0, containing 100 μM $(NH_4)_2SO_4$ and 100 mM glucose was added to the cell suspension. Other cations were added in a 5-fold molar excess. Samples were taken and cells pelleted rapidly by centrifugation. The supernatant was filtered through a 0.45 μm pore membrane filter prior to determination of ammonium concentration using a glutamate dehydrogenase (GDH) assay [22]. GDH was purchased from Roche. MA was shown not to interfere with the GDH assay.

2.5. Plant growth for RNA isolation

Arabidopsis thaliana (C24) seedlings were grown in axenic cultures at 22°C with 16 h light (140 μE) per day in a modified 0.5×MS [17] medium in which glycine and MES were omitted. After 16 days, one batch of plants was cultivated for a further 3 days in K-replacement/N-starvation medium (20.6 mM NH_4NO_3 and 18.8 mM KNO_3 were replaced by 9.4 mM KCl and 4.7 mM K_2SO_4), whereas control plants were further cultivated in the modified 0.5×MS medium. Plants were harvested and roots and green plant parts were separated and stored at −80°C until required.

2.6. Northern analysis

RNA was isolated by the GTC method [23]. 20 μg samples of total RNA were separated on formaldehyde gels, transferred to nylon membranes and probed with a 1.5 kb fragment of *AtAMT2* cDNA, as described previously [24]. As a loading control, translation initiation factor elF4a [25] was used.

3. Results and discussion

3.1. Isolation of AtAMT2 and functional complementation of MLY131a/α, a yeast mutant deficient in ammonium uptake

The starting point for this work was an *Arabidopsis* genomic sequence (AC003028, sequence F16M14.22) that encoded a hypothetical protein with weak homology to plant ammonium transporters of the AMT1 family. To determine whether the gene was expressed in *Arabidopsis*, we performed a PCR, using the enzyme *Pfu*, to amplify a full-length cDNA corresponding to the putative gene. An amplicon of the expected size (1.5 kb) was obtained from a root cDNA library and preliminary sequence confirmed that it was derived from a transcript of the gene of interest. Thus, the gene was transcribed in roots of nitrogen-deprived *Arabidopsis* plants.

We used a yeast mutant complementation approach to determine whether the protein encoded by the cDNA was a functional ammonium transporter. Several ammonium transporters have been isolated by complementing yeast mutants deficient in ammonium uptake [10,11,26]. *S. cerevisiae* strain MLY131a/α [20] is defective in all three endogenous ammonium transporters (Δ*mep1* Δ*mep2* Δ*mep3*) and, therefore, unable to grow on medium containing less than 5 mM ammonium as sole nitrogen source [20]. The 1.5 kb PCR amplicon described above was cloned into the *Bam*HI and *Xba*I sites of yeast expression vector pYES3 [19], where it was under the control of the galactose-inducible GAL1 promoter. Transformation of yeast strain MLY131a/α [20] with this construct resulted in complementation of the AMT^- phenotype of the strain (Fig. 1A). A similar result was obtained using a full-length *AtAMT1;1* cDNA cloned into pYES3 as a positive control. In contrast, transformation of MLY131a/α with pYES3 alone (labelled mutant in Fig. 1A) did not enable the strain to grow on media containing 1 mM ammonium. Therefore, the novel cDNA encoded a functional ammonium transporter. We have called the transporter AtAMT2 to distinguish it from the distantly related ammonium transporters of the AMT1 family in plants (see below).

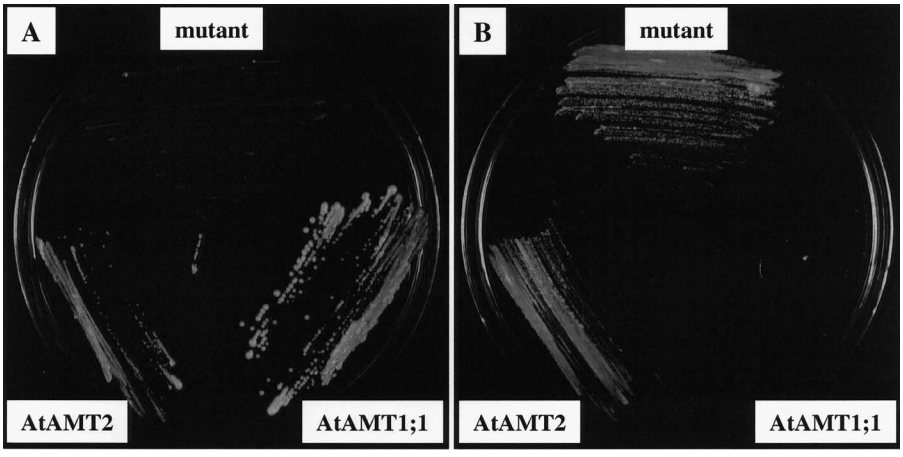

Fig. 1. Growth of the yeast mutant MLY131a/α expressing *AtAMT2*. Controls are MLY131a/α expressing *AtAMT1;1* and MLY131a/α harboring the empty vector pYES3 (labelled mutant). Growth on selective YNB-N minimal media containing (A) 0.5 mM $(NH_4)_2SO_4$ and 2% (w/v) galactose, (B) 100 mM MA, 0.1% (w/v) proline and 2% (w/v) galactose.

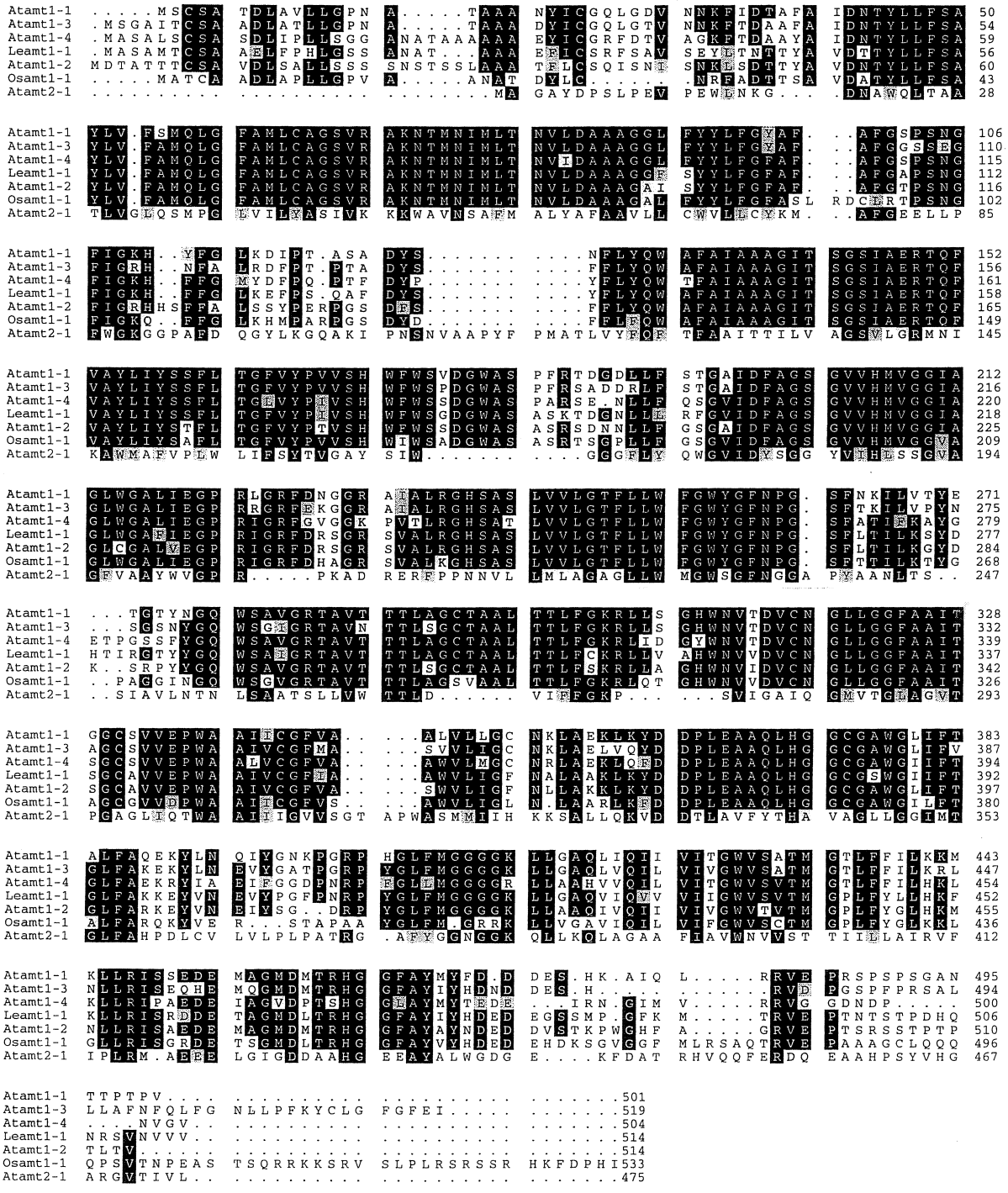

Fig. 2. Alignment of the deduced amino acid sequences of different plant ammonium transporters. Identical residues are boxed. At: *A. thaliana*, Le: *Lycopersicum esculentum*, Os: *Oryza sativa*. The accession numbers are: AtAMT1;1: P54144, AtAMT1;2: AAD17001, AtAMT1;3: AAD54638, AtAMT1;4: CAA22982, AtAMT2: AF182039, LeAMT1;1: O04161, OsAMT1;1: AAB58937.

3.2. Sequence and phylogenetic analysis of AtAMT2

The full-length *AtAMT2* PCR product from *Arabidopsis* ecotype C24 was completely sequenced and found to possess several polymorphisms when compared to the genomic sequence from ecotype Columbia (AC003028, F16M14.22). To verify the sequence of the PCR product, we isolated and completely sequenced a full-length *AtAMT2* clone from a λZipLox cDNA library representing transcripts from N-deprived roots of ecotype C24. The sequence of the cDNA (GenBank accession number AF182039) was identical to that of the PCR product. Thus, there are slight differences between the *AtAMT2* sequence from *Arabidopsis* ecotypes C24 and Columbia. These polymorphisms translate into one difference in the amino acid sequence (N95D) of the encoded protein (Fig. 2).

The *AtAMT2* cDNA from C24 encodes a 50.7 kDa polypeptide of 475 amino acid residues. The protein is smaller than ammonium transporters of the AMT1 family which

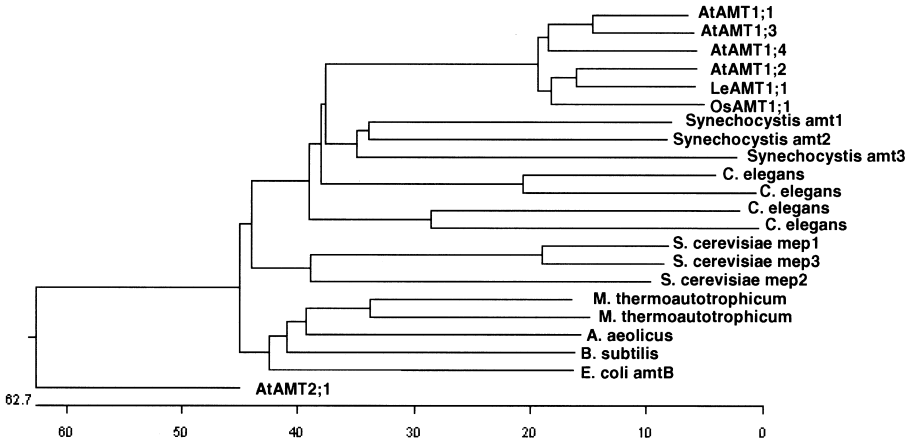

Fig. 3. A phylogenetic tree for selected eukaryotic and prokaryotic members of the AMT family. The tree was made using the program DNA Star™ by aligning the complete amino acid sequences of the transport proteins. The scale indicates percent divergence.

range in mass from 53.0 to 55.7 kDa (Fig. 2). Interestingly, AtAMT2 lacks approximately 20–30 amino acids at the N-terminal end compared to AMT1 proteins.

AtAMT2 shares only 23–25% sequence identity with proteins of the AtAMT1 family (Fig. 2). In contrast, AMT1 proteins from different species share between 62% and 73% sequence identity with each other. Phylogenetic analysis showed that AtAMT2 is more closely related to ammonium transporters in some prokaryotes than to AMT1 proteins in plants (Fig. 3). However, AtAMT2 is clearly on a branch of its own, a result that supports its classification in a novel AMT family.

3.3. Biochemical analysis of ammonium uptake by AtAMT2

Many ammonium transporters, including those of the AMT1 family in plants, are able to transport MA as well as ammonium [11,12,14]. This has proven useful because it allows [^{14}C]MA to be used to investigate the kinetics of ammonium transporters. Intriguingly, MA appeared not to be transported by AtAMT2. When presented with up to 10 mM [^{14}C]MA, there was no significant difference in the rate of MA uptake by yeast mutant MLY131a/α cells expressing AtAMT2, and control cells containing the pYES3 vector alone (results not shown). This result was supported by growth experiments in the presence of MA. Wild-type yeast cells with functional ammonium transporters are unable to grow in the presence of high concentrations of MA. However, yeast mutant strains that are impaired in ammonium (and MA) transport are able to grow on media containing 100 mM MA [27,28]. Thus, yeast mutant strain MLY131a/α was able to grow on 100 mM MA, but the same strain expressing AtAMT1;1 was not able to do so (Fig. 1B). Cells expressing AtAMT2 retained the ability to grow on MA (Fig. 1B). In other words, MA was not taken up by these cells. Although all plant AMT1 family members studied to date are able to transport MA [11,15,16], this appears not to be the case in yeast. *S. cerevisiae* encodes three ammonium transporters of the AMT1 family: Mep1p, Mep2p and Mep3p. Only the first two of these are also able to transport MA. Apparent lack of affinity for MA in the case of Mep3p is accompanied by a relatively low affinity for ammonium (K_m of 1.4–2.1 mM; compared to 1–2 μM and 5–10 μM for Mep2p and Mep1p, respectively [27]. This does not seem to be the case for AtAMT2 (see below and Fig. 4).

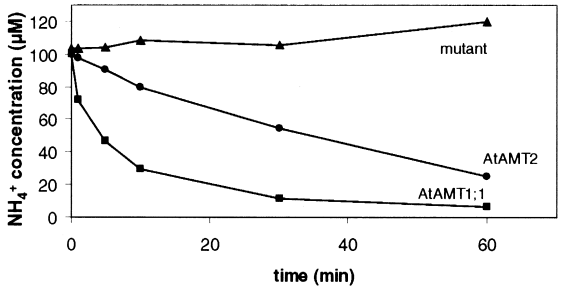

Fig. 4. Uptake of ammonium by MLY131 expressing *AtAMT1;1* and *AtAMT2*. MLY131a/α harboring the empty vector pYES3 (labelled mutant) was used as a control. Net uptake resulted in depletion of ammonium from the medium which is shown. Yeast cells were grown in YNB-N minimal medium containing 0.1% (w/v) proline and 2% (w/v) galactose. Cells were resuspended in 50 mM sodium acetate, pH 5.0, and uptake was started by adding $(NH_4)_2SO_4$ to a final concentration of 50 μM and glucose to a final concentration of 50 mM. The results shown are the data from a typical experiment.

To study the transport properties of AtAMT2 expressed in yeast cells, we resorted to a method that measures ammonium directly. Net uptake of ammonium by yeast cells was monitored by measuring the depletion of ammonium from the incubation buffer. When yeast strain MLY131a/α was incubated in the presence of 100 μM ammonium, no net uptake of ammonium was observed. In fact, a net loss of ammonium from these cells was observed (Fig. 4). Apparently, ammonium present in the yeast prior to uptake assays, or derived from catabolism of organic N during the assays, was lost from the cells to the buffer, probably by simple diffusion. As no functional ammonium transporter was expressed in these cells, the lost ammonium could not be recovered. In contrast, MLY131a/α cells expressing either AtAMT1;1 or AtAMT2 removed ammonium rapidly from the incubation medium (Fig. 4). Yeast cells expressing AtAMT1;1 took up ammonium faster than those expressing AtAMT2. This may explain the better growth of the former on low concentrations of ammonium in both solid media (Fig. 1A) and liquid media. Cells expressing AtAMT1;1 had a doubling time (180 min) less than half that of cells expressing AtAMT2 (420 min) in

liquid YNB-N containing 2% galactose and 0.5 mM ammonium sulfate.

To learn more about the substrate specificity of AtAMT2, we tested different cations, including MA, for their effects on ammonium transport by AtAMT2 in yeast. Measurements were confined to the first 5 min of uptake to avoid, as much as possible, complications due to ammonium metabolism. Ammonium uptake by AtAMT2-expressing cells was always linear during this period (Fig. 4). A 5-fold molar excess of cesium or rubidium did not inhibit ammonium transport by AtAMT2. A 5-fold excess of potassium inhibited ammonium uptake slightly (inhibition of 7 ± 2% S.E.M.) whilst MA had a somewhat greater effect (inhibition of 12 ± 4% S.E.M.). The latter result was interesting in view of the fact that MA was apparently not transported by AtAMT2 (see above). Taken together, our results suggest that AtAMT2 is able to bind MA but is unable to transport it.

Several inhibitors of energy metabolism were used to investigate the energetics of ammonium transport by AtAMT2. Once again, assays were confined to the first 5 min of ammonium uptake. The protonophores 2,4-dinitrophenol (DNP) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) severely inhibited ammonium uptake by yeast strain MLY131a/α expressing AtAMT2: 100 μM DNP led to 83% inhibition, whilst 10 μM CCCP inhibited ammonium uptake by 64%. DCCD (*N,N′*-dicyclohexylcarbodiimide; at 200 μM), an inhibitor of plasma membrane H^+-ATPase activity, inhibited ammonium uptake via AtAMT2 by 58%. Although we cannot exclude the possibility that one or more of the inhibitors effected ammonium metabolism rather than transport per se, similar results have been reported previously for AtAMT1;1, using the non-metabolizable ammonium analog, MA [11]. Therefore, it is most likely that ammonium uptake via AtAMT2 is an energy-dependent process that is driven by the membrane electrical potential, $\Delta\psi$. Confirmation of this will require electrophysiological studies which are underway.

3.4. Expression pattern of *AtAMT2* under different growth conditions

To gain insight into the possible physiological roles of AtAMT2 in *Arabidopsis*, we studied the expression of *AtAMT2* under different growth conditions. Transcripts of *AtAMT2* were found in both roots and shoots, although expression was always higher in shoots (Fig. 5). The level of the *AtAMT2* transcript increased in roots in response to nitrogen depletion. Similar nitrogen regulation of gene expression has been observed for *AtAMT1;1* in the past [15]. A shorter exposure of the blot depicted in Fig. 5 showed that *AtAMT2* transcript levels also increased in leaves following nitrogen depletion, although the increase was not as marked as in roots. In the root tissues of both nitrogen-deprived and nitrogen-replete plants, a second, smaller transcript (approximately 1.2 kb) hybridized to the *AtAMT2* probe. It is not clear if this transcript was derived from *AtAMT2* or a related gene. High stringency Southern analysis, together with the known genomic sequence of *AtAMT2*, indicated the presence of another closely related gene in *Arabidopsis* (data not shown).

The expression pattern of *AtAMT2* is different from all *Arabidopsis AtAMT1* genes studied to date [15] in that higher transcript levels were seen in shoots than in roots. It has been suggested that the primary role of the various AtAMT1 proteins may be to acquire ammonium from the soil [15]. The transporters may also play a secondary role in recovering ammonium lost from root cells, and shoot cells in the case of AtAMT1;1 which appears to be the only member of this family expressed in shoots. Such roles require a plasma membrane location for these transporters which has not yet been demonstrated. Higher expression of *AtAMT2* in shoots compared to roots suggests that this protein performs a physiological role(s) distinct from those of AtAMT1 family members. It is interesting to speculate that AtAMT2 may play a role in photorespiratory ammonium metabolism. Photorespiration in leaves of C3 plants generates ammonium at a rate that may exceed primary nitrogen assimilation by 10-fold [29]. Ammonium liberated from glycine in mitochondria and reassimilated in chloroplasts must be transported across the membranes of both organelles. It will be interesting to determine the intracellular location of AtAMT2 and whether or not it plays a role in ammonium recycling during photorespiration.

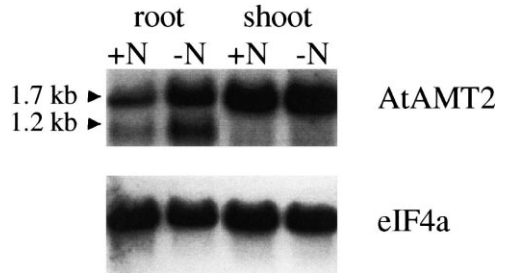

Fig. 5. Northern blot analysis of *AtAMT2* expression in *Arabidopsis*. Plants were grown in medium containing 20.6 mM NH_4NO_3 and 18.8 mM KNO_3 for 19 days (+N), or for 16 days followed by transfer to a similar medium lacking nitrogen for 3 days (−N). 20 μg of total RNA from roots or shoots was loaded in each lane. The lower panel shows the loading control, elF4a.

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