The Organization of High-Affinity Ammonium Uptake in Arabidopsis Roots Depends on the Spatial Arrangement and Biochemical Properties of AMT1-Type Transporters

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The AMMONIUM TRANSPORTER (AMT) family comprises six isoforms in Arabidopsis thaliana. Here, we describe the complete functional organization of root-expressed AMTs for high-affinity ammonium uptake. High-affinity influx of 15N-labeled ammonium in two transposon-tagged amt1;2 lines was reduced by 18 to 26% compared with wild-type plants. Enrichment of the AMT1;2 protein in the plasma membrane and localization of AMT1;2 promoter activity in the endodermis and root cortex indicated that AMT1;2 mediates the uptake of ammonium entering the root via the apoplastic transport route. An amt1;1 amt1;2 amt1;3 amt2;1 quadruple mutant (qko) showed severe growth depression under ammonium supply and maintained only 5 to 10% of wild-type high-affinity ammonium uptake capacity. Transcriptional upregulation of AMT1;5 in nitrogen-deficient rhizodermal and root hair cells and the ability of AMT1;5 to transport ammonium in yeast suggested that AMT1;5 accounts for the remaining uptake capacity in qko. Triple and quadruple amt insertion lines revealed in vivo ammonium substrate affinities of 50, 234, 61, and 4.5 μM for AMT1;1, AMT1;2, AMT1;3, and AMT1;5, respectively, but no ammonium influx activity for AMT2;1. These data suggest that two principle means of achieving effective ammonium uptake in Arabidopsis roots are the spatial arrangement of AMT1-type ammonium transporters and the distribution of their transport capacities at different substrate affinities.

INTRODUCTION

In plants, transport of nutrients, water, and metabolites is mostly facilitated by families of membrane transporters. Heterologous expression, tissue and subcellular localization, and physiological analysis of mutants have contributed much to our understanding of the function of individual transporters. Knockout lines have revealed functional redundancy and specialization of transporter family members. Severe growth phenotypes have been obtained by mutating individual family members in the presence of other members that show similar expression or location patterns (Hirsch et al., 1998; Javot et al., 2003; Hirner et al., 2006; Takano et al., 2006). However, most single-gene insertion mutants from multi-gene families have no recognizable phenotype (Sohlenkamp et al., 2002; Hussain et al., 2004; Kataoka et al., 2004; Shin et al., 2004; Lanquart et al., 2005). The scarcity of viable transporter mutants that are defective in several genes of one transporter family has somewhat hampered the characterization of transporter functions. A deeper understanding of the principles underlying a coordinated substrate transport via multiple family members in plants requires not only a consideration of the biochemical properties, cell type–specific expression patterns, and the regulation of individual transporter homologs, but also a dissection of the physiological contribution of each member. Knowledge of the molecular and physiological basis of ammonium transport in Arabidopsis thaliana plants is growing and points to the involvement of multiple members in defined transport functions (Gazzarrini et al., 1999; Rawat et al., 1999; Kaiser et al., 2002; Sohlenkamp et al., 2002; Lejay et al., 2003; Loqué et al., 2006). Based on these findings, uptake of ammonium by roots would appear to be a suitable process to learn how members of protein families can coordinate transport of a substrate in planta.

In a wide range of organisms, transport of ammonium across membranes is mediated by proteins of the AMMONIUM TRANSPORTER/METHYLAMMONIUM PERMEASE/RHESUS (AMT/MEP/Rh) family (von Wirén and Merrick, 2004). Plant members of this family belong either to the AMT subfamily and permeate ammonium via NH4+ uniport or NH3/H+ cotransport (Ludewig, 2006) or to the MEP subfamily that also includes AmtB from Escherichia coli shown to channel NH3 across the cell membrane (Khademi et al., 2004; Zheng et al., 2004; Javelle et al., 2005). In Arabidopsis, AMT2;1 is the only member of the MEP subfamily, while five homologs, AMT1;1 to AMT1;5, constitute the AMT clade (Ludewig et al., 2001). At the level of individual AMT-type transporters, several mechanisms have been shown to regulate root ammonium fluxes in response to the cellular and/or whole-plant demand for nitrogen. At the transcriptional level, AMT gene expression in Arabidopsis roots is generally repressed by high...
nitrogen, most likely by the internal pool of Gin, and derepressed under nitrogen deficiency or supply of sugars (Gazzarrini et al., 1999; Rawat et al., 1999; Lejay et al., 2003). The nitrogen nutrition status of plants may specifically affect transcript stability, as has been observed in Arabidopsis for AMT1;1 but not for AMT1;3 (Yuan et al., 2007). At the posttranslational level, AMT1;1 can be inactivated by C-terminal phosphorylation. Most likely, AMT1;1 assembles as a trimer and the phosphorylation signal transinhibits the two neighboring subunits, representing an example of cooperative transporter regulation (Loqué et al., 2007). The tightly controlled transport of ammonium is not only essential for maintaining the cation-anion balance and plant growth (Bloom et al., 1993; Marschner, 1995) but also for adjusting levels of phytohormones regulating leaf development (Walch-Liu et al., 2000; Rahayu et al., 2005) and for preventing overaccumulation of ammonium that may otherwise cause membrane depolarization and cellular damage (Britto and Kronzucker, 2002).

Transcriptome and RNA gel blot analyses have shown that four of the six AMT/MEP homologs in Arabidopsis are expressed in roots and upregulated under nitrogen deficiency (Gazzarrini et al., 1999; Sohlenkamp et al., 2002; Birnbaum et al., 2003; Schmid et al., 2005). RNA interference (RNAi)-mediated repression of AMT2;1 provided no evidence for a contribution of AMT2;1 to overall ammonium uptake (Sohlenkamp et al., 2002), whereas influx measurements in a T-DNA insertion line showed that AMT1;1 may confer approximately one-third of the overall high-affinity transport capacity in nitrogen-deficient Arabidopsis roots (Kaiser et al., 2002). AMT1;3 was shown to confer also a third of the high-affinity ammonium transport capacity in roots. A double insertion mutant for AMT1;1 and AMT1;3 had 60 to 70% reduced transport capacity, indicating an additive contribution of these transporters under nitrogen deficiency, consistent with the observation that these proteins both localize preferentially to the plasma membrane of rhizodermal cells of the root hair zone (Loqué et al., 2006). Despite the substantial reduction in high-affinity ammonium uptake in an amt1;1 amt1;3 double mutant and the absence of any evidence for an accompanying compensatory upregulation of AMT2;1 or AMT1;2, the double insertion line showed no recognizable growth phenotype even under purely ammonium-based nitrogen nutrition. These observations suggest that other root-expressed ammonium transporters have functions redundant to AMT1;1 or AMT1;3 in root ammonium uptake and raised the question of how individual transporters are arranged to coordinate ammonium influx into roots.

We have used reverse genetics to investigate the functional organization of ammonium transporters in Arabidopsis. In this and a previous study (Loqué et al., 2006), we measured ammonium influx in single T-DNA or transposon insertion lines to determine the in vivo contribution of individual AMT members to overall ammonium uptake in roots. In this study, we constructed a quadruple amt1;1 amt1;2 amt1;3 amt2;1 insertion mutant line in which 90 to 95% of the high-affinity ammonium uptake capacity was lost. Analysis of triple insertion lines in which only one of these four AMT transporter genes was functional allowed the determination of individual transporter capacities against a background of low ammonium uptake. The results indicate that high-affinity uptake of ammonium in Arabidopsis roots is mostly conferred by four AMT proteins. The capacities of the individual proteins are additive and enhanced under nitrogen deficiency but may vary according to the presence of the other homologs. Furthermore, we provide evidence that the biochemical transport properties of individual AMT-type proteins in roots are suited to the respective root cell types in which they are expressed, providing a potential explanation of how specialized AMTs cooperate together to achieve effective uptake of ammonium into the plant.

RESULTS

amt1;2 Transposon Insertion Lines Have a Reduced High-Affinity Ammonium Uptake Capacity in Roots

To evaluate the contribution of AMT1;2 to ammonium uptake in roots, two lines containing independent transposon insertions in this gene were obtained from the Jonathan Jones En/Spm collection (Tissier et al., 1999) via the Nottingham Arabidopsis Stock Centre (NASC; http://www.nasc.nott.ac.uk). The transposons in the lines amt1;2-1 and amt1;2-2 were found to be inserted 983 and 1375 bp downstream of the start codon of AMT1;2, respectively (Figure 1A). RNA gel blot analysis using the 3′-end of AMT1;2 as probe failed to detect any AMT1;2 transcripts in roots of amt1;2 homozygotes for either insertion (Figure 1B). However, a full-length AMT1;2 open reading frame (ORF) probe detected a signal in the amt1;2-2 mutant, suggesting expression of a truncated AMT1;2-ORF (see Supplemental Figure 1A online). The cDNA corresponding to this transcript was cloned from amt1;2-2 genomic DNA consisting of a fusion between the 5′-end of the AMT1;2-ORF and the transposon. Its sequence revealed that the DNA fusion could be translated into a chimeric protein containing the first 458 amino acids of AMT1;2 and a short translated part of the transposon. When the corresponding cDNA was heterologously expressed in an ammonium uptake-defective yeast mutant (Jmep1,2,3; Marini et al., 1997), it failed to complement the defect in ammonium uptake, indicating that the translation product was either inactive or unstable (see Supplemental Figure 1B online). To evaluate plants for the presence of AMT1;2 protein, we subjected microsomal membrane fractions from roots of hydroponically grown Arabidopsis to protein gel blot analysis using an antibody raised against a 15–amino acid stretch in the C terminus of AMT1;2. A specific band at ~40 kD was detected in wild-type plants, which is somewhat smaller than the calculated molecular weight of AMT1;2 (55 kD), although a faster gel migration would be consistent with the hydrophobic nature of the protein (Figure 1C). An additional specific band was detected at ~80 to 90 kD, representing a possible dimer of AMT1;2 (Ludwig et al., 2003) or a stable complex with other unknown proteins. The antibody revealed no signal for membrane protein preparations from roots of the amt1;2-2 and amt1;2-2 insertion lines (Figure 1C). The higher molecular weight band was then evaluated for possible variation in plants deprived of nitrogen for up to 4 d. In contrast with AMT1;1 and AMT1;3, neither the 40- nor 80-kD protein band of AMT1;2 considerably changed under nitrogen deficiency (Figure 1D), indicating that the signal ratio of these two forms is not subject to nitrogen-dependent regulation.
Neither mutant line showed any visible phenotype when grown on soil or on agar medium supplemented with different concentrations of ammonium or ammonium nitrate. In the presence of methylammonium (MeA), however, both insertion lines grew slightly better than the wild type (see Supplemental Figure 2 online). At 5 to 15 mM MeA, leaves of \textit{amt1;2-1} and \textit{amt1;2-2} were greener and larger, suggesting a lower uptake of the toxic ammonium analog. Short-term influx of 15N-labeled ammonium was determined in hydroponically grown plants precultured under different nitrogen regimes. In plants presupplied with adequate nitrogen, high-affinity ammonium uptake into the roots of both insertion lines was 28 or 23% lower than in the wild type, while in plants presupplied with insufficient nitrogen, high-affinity uptake rates decreased by 26 or 18% in \textit{amt1;2-1} or \textit{amt1;2-2}, respectively (Figure 2A). The mutants showed significant reductions of influx at 5 mM external substrate only after pregrowth under nitrogen deficiency (Figure 2B). However, calculation of low-affinity ammonium transport activity as the difference between influxes measured at 5 mM and 200 \(\mu\)M indicated that AMT1;2 did not significantly contribute to low-affinity ammonium transport activity. It was concluded that AMT1;2 represents primarily a high-affinity ammonium transporter. Gene and protein expression levels of other root-expressed AMTs in the roots of nitrogen-sufficient and -deficient plants were investigated by RNA and protein gel blot analyses. No differences in mRNA or protein levels of AMT1;1, AMT1;3, and AMT2;1 relative to the wild type were detected in the \textit{amt1;2-1} or \textit{amt1;2-2} mutants (Figures 2C and 2D), indicating the absence of any regulatory compensation in these three AMTs resulting from the loss of AMT1;2.

**Subcellular Localization of AMT1;2**

The location of AMT1;2 was investigated in membrane fractions of \textit{Arabidopsis} roots and shoots prepared by two-phase partitioning. Enrichment of plasma membrane proteins in the upper phase was verified by protein gel blot analysis using an antibody against the plasma membrane ATPase AHA2 (DeWitt et al., 1996), while enrichment of endosomal membrane proteins in the lower fraction was confirmed by detection of DET3, a subunit of the vacuolar ATPase (Schumacher et al., 1999) and of the vacuolar pyrophosphatase (VPPase; Takasu et al., 1997). Probing of these fractions with the AMT1;2 antibody showed that in both cases, AMT1;2 was enriched in the upper phase (Figure 3). This
confirmed a mainly plasma membrane location for AMT1;2 in root and shoot tissues.

**Localization of AMT1;2 in Endodermis and Cortex Cells**

Ammonium influx studies in single insertion lines had indicated that AMT1;2 contributes a somewhat lower absolute ammonium uptake capacity than AMT1;1 or AMT1;3 (Loqué et al., 2006). We investigated whether the tissue location of AMT1;2 transcription could help explain the different contribution of AMT1;2. To localize AMT1;2 transcription, we examined roots of Arabidopsis plants expressing an AMT1;2:green fluorescent protein (GFP) fusion protein from a transgene driven by the native AMT1;2 promoter. In plants grown in nitrogen-deficient conditions, green fluorescence was not evident in the root tip but was strong in the root hair zone and in regions further up the root (Figure 4A). In the root hair differentiation zone, longitudinal and radial sections showed the AMT1;2:GFP protein to be confined mainly to endodermal cells (Figures 4B and 4E). Higher up in the root hair zone, AMT1;2 expression of the fusion protein also extended to cortical cells (Figures 4C and 4F), while in the zone of emerging lateral roots, GFP-dependent fluorescence became exclusively restricted to cortical cells (Figures 4D and 4G). This localization clearly differed from that of AMT1;1 and AMT1;3, which had been shown to be expressed mainly in cortical and rhizodermal cells (Loqué et al., 2006).

To investigate whether the location of AMT1;2 in inner root cells might favor root-to-shoot translocation of ammonium, xylem sap was collected from wild-type and amt1;2 insertion lines. Ammonium concentrations in the xylem sap were in the millimolar range, confirming that this form of nitrogen is translocated in substantial amounts from roots to the shoot as in other cruciferous plant species (Finnemann and Schjoerring, 1999). A difference between wild-type and amt1;2 insertion lines, however, was not observed, irrespective of whether plants were nitrogen deficient, resupplied with ammonium or nitrogen-sufficient, or assayed at different time points during a diurnal cycle (see Supplemental Figure 3 online).

Since AMT1;2 is expressed in inner root cell layers and its loss of function reduced ammonium uptake capacity, the lack of AMT1;2 might also reduce depletion of apoplastic ammonium...
pools in roots. To verify this hypothesis, apoplastic fluid was extracted for ammonium analysis from wild-type and \textit{amt1;2} plants that were nitrogen deficient or ammonium resupplied. However, no differences in apoplastic ammonium concentrations among these lines were found irrespective of whether plants were nitrogen deficient or resupplied with ammonium (Figure 5), suggesting that the size of this pool was not significantly affected by \textit{amt1;2}-mediated ammonium uptake. Unexpectedly, ammonium concentrations in the root apoplast of nitrogen-deficient plants were between 0.7 and 1 mM and increased up to 1.7 mM after resupply of 250 ClM ammonium. Thus, apoplastic ammonium concentrations were several times higher than the external supply, consistent with those illustrated in Figure 1D, emphasizing little or no nitrogen-dependent regulation of \textit{AMT1;2} protein levels in Arabidopsis roots.

**Generation of a Quadruple (\textit{qko}) and Triple Insertion Lines**

Using a double insertion mutant line, we had shown that the \textit{AMT1;1} and \textit{AMT1;3} plasma membrane–localized ammonium transporters contribute in an additive manner to high-affinity ammonium uptake in nitrogen-deficient \textit{Arabidopsis} roots (Loquè et al., 2006). Since these AMTs, as well as \textit{AMT1;2} and \textit{AMT2;1}, are all expressed in the roots (Figures 2C and 2D; Sohlenkamp et al., 2002), we made a quadruple \textit{amt1;1-1 amt1;3-1 amt2;1-1} mutant to investigate the total contribution of these four AMTs to ammonium uptake. The source of the \textit{amt2;1} mutant allele was the \textit{amt2;1-1} insertion mutant line (kindly provided by Doris Rentsch, University of Bern, Switzerland, who in turn obtained it from the enhancer trap collection of Thomas Jack, Fairmount College, NH, which was shown to contain a T-DNA insertion located 680 bp downstream of the start codon, resulting in a loss of \textit{AMT2;1} protein; see Supplemental Figure 4 online). To begin, we crossed the \textit{amt1;1} \textit{amt1;3} double mutant with the \textit{amt2;1-1} line and selected F2 progeny homozygous for all three mutant alleles. The \textit{amt1;1-1 amt1;3-1 amt2;1-1} triple insertion line was then crossed to the transposon insertion line \textit{amt1;2-1} (Figure 1) to generate a quadruple mutant \textit{amt1;1-1 amt1;3-1 amt2;1-1 amt1;2-1} line (\textit{qko}).

The insertion mutant alleles of the four AMT genes were derived from three different ecotype backgrounds (see Methods). To obtain lines containing individual wild-type endogenous AMT genes in a genetic background that was relatively homogeneous, we added individual wild-type AMT genes from a single ecotype back to the quadruple knockout line (\textit{qko}) by crossing. The \textit{qko} was crossed to wild-type Columbia (Col-0), and PCR was used to identify F3 progeny plants homozygous for insertions in three of the four AMT genes and homozygous wild type for the remaining AMT gene. The lines containing wild-type \textit{AMT1;1}, \textit{AMT1;2}, \textit{AMT1;3}, or \textit{AMT2;1} genes were named \textit{qko+11}, \textit{qko+12}, \textit{qko+13}, and \textit{qko+21}, respectively. In parallel, a homozygous \textit{qko} line was isolated from the same F3 progeny to serve as a reference line for the triple insertion lines. Each F3 plant was allowed to self, and the F4 plants were used to conduct experiments.

Plants of these lines were cultivated in nutrient solution under nitrogen-sufficient and -deficient conditions and evaluated for expression of AMT transcript and protein. RNA and protein gel blot analyses confirmed that none of the four AMTs were detectable in \textit{qko} and that \textit{AMT1;1}, \textit{AMT1;2}, \textit{AMT1;3}, and \textit{AMT2;1} were expressed in the \textit{qko+11}, \textit{qko+12}, \textit{qko+13}, and \textit{qko+21} lines, respectively (Figures 6A and 6B). RNA gel blot analyses showed upregulation of wild-type \textit{AMT1;1}, \textit{AMT1;2}, \textit{AMT1;3}, and \textit{AMT2;1} transcripts under nitrogen deficiency. While protein levels of \textit{AMT1;1}, \textit{AMT1;3}, and possibly \textit{AMT2;1} increased under nitrogen deficiency, those of \textit{AMT1;2} did not. These results are consistent with those illustrated in Figure 1D, emphasizing little or no nitrogen-dependent regulation of \textit{AMT1;2} protein levels in Arabidopsis roots.

**Phenotypic Analysis of \textit{qko} and Triple Insertion Lines**

When grown in a nitrate-fertilized peat-based substrate or in nutrient solution supplemented with 2 mM ammonium nitrate, neither the insertion lines nor the \textit{qko} developed any visible phenotype. However, on sterile agar medium and in the presence of 20 or 50 mM MeA, \textit{qko} grew dramatically better than Col-0, which was severely inhibited in root growth and shoot biomass production (Figures 7A and 7B). As expected from a lack of MeA transport activity by \textit{AMT2;1} when expressed in yeast (Sohlenkamp et al., 2000), the \textit{qko+21} triple insertion line showed no increase in MeA sensitivity relative to the \textit{qko}, whereas \textit{qko+11}, \textit{qko+12}, and \textit{qko+13} displayed intermediate MeA sensitivities.

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**Figure 3.** Subcellular Localization of AMT1;2 in \textit{Arabidopsis}.

Microsomal fractions (M) from roots (A) and shoots (B) of nitrogen-sufficient wild-type (Col-0) plants were separated by aqueous two-phase partitioning into a plasma membrane–enriched upper phase (U) and an endosomal membrane–enriched lower phase (L). Protein gel blot analysis was conducted with antibodies against AMT1;2, an \textit{Arabidopsis} plasma membrane H$^+$-ATPase (AHA2), a vacuolar H$^+$-ATPase subunit (DET3), and vacuolar pyrophosphatase (VPPase). Six-week-old plants were precultured hydroponically under continuous supply of 2 mM ammonium nitrate.

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Out of the AMT1;1, AMT1;2, and AMT1;3 wild-type genes, AMT1;2 seemed to confer the least amount of MeA sensitivity. Phenotypes were then assessed on sterile agar medium, in which ammonium could be readily maintained as the sole nitrogen source over a few weeks. At 500 to 750 μM ammonium, on which Col-0 grew best, the qko produced only 50% of the shoot biomass of Col-0 (Figure 8A). Despite the lack of all four AMTs, qko was able to significantly increase shoot biomass at 0 to 200 μM ammonium, indicating that qko was still able to use ammonium supplied at low micromolar concentrations. A comparative growth analysis including the triple insertion lines on 500 μM ammonium showed that the presence of AMT1;1, AMT1;2, or AMT1;3 alone was sufficient to restore ammonium-dependent growth to the wild-type level (Figures 8B and 8C). Although shoot biomass of qko +12 was similar to that of qko +11 and qko +13, qko +12 shoot nitrogen concentrations were lower under pure ammonium nutrition or ammonium nitrate nutrition, suggesting that AMT1;2 has a comparatively lower ammonium transport capacity. In contrast with the three AMT1 transporters, the presence of AMT2;1 in the qko +21 triple insertion line failed to increase shoot nitrogen accumulation or biomass production relative to qko (Figures 8C and 8D). This failure of AMT2;1 to contribute to ammonium-based nitrogen nutrition was also reflected under supply of ammonium nitrate, in which growth of qko +21 was repressed to similar levels as the qko (Figures 8B to 8D). When plants were supplied with ammonium as a sole nitrogen source at concentrations above 4 mM, growth suppression in qko relative to the wild type was absent, suggesting that low-affinity ammonium transport then recovered growth of qko (see Supplemental Figure 5 online).

**Comparative Analysis of AMT Transport Properties in Plants**

Short-term influx of 15N-labeled ammonium into roots of qko and the triple insertion line at 200 μM external ammonium was examined as a measure of high-affinity transport capacity. In plants presupplied with sufficient nitrogen, ammonium influx into the roots of the qko or qko +21 lines was almost ten-fold lower than in Col-0. Therefore, despite transcriptional upregulation under nitrogen deficiency, AMT2;1 was unable to contribute to short-term ammonium influx into roots. By contrast, the qko +12 line reached 18% and the qko +11 and qko +13 lines reached 45 to 50% of wild-type capacity after presupply with nitrogen (Figure 9A). After pregrowth in nitrogen-deficient conditions, the qko +11 line reached 18% and the qko +11 and qko +13 lines reached 45 to 50% of wild-type capacity after presupply with nitrogen (Figure 9A). After pregrowth in nitrogen-deficient conditions, the qko +11 and qko +13 lines showed improved transport capacities of 64 and 70% of that of the wild type, respectively. However, the qko +12 line continued to show relatively low capacity (22% of the wild type) after growth in nitrogen-deficient conditions. The lower contribution of AMT1;2 relative to AMT1;1 and AMT1;3 was in agreement with the relatively low MeA sensitivity and lower nitrogen accumulation of the qko +12 line when grown on ammonium (Figures 7 and 8). Notably, ammonium influx in nitrogen-deficient qko plants increased approximately threefold to fourfold.
Evidence for a Role of AMT1;5 in Residual Ammonium Influx Present in the qko Line

Ammonium influx in the qko line still increased under nitrogen deficiency. To quantify this remaining transport activity, the concentration-dependent ammonium influx into nitrogen-sufficient and -deficient qko plants was determined. Ammonium influx into roots of plants pregrown on adequate nitrogen followed a linear concentration dependency, while influx into nitrogen-deficient roots steeply increased between 0 and 50 μM ammonium and became saturated above 50 μM (Figure 10A). To calculate the kinetic properties of the remaining nitrogen-dependent transport system in qko, uptake rates determined in nitrogen-sufficient qko plants were subtracted from those of nitrogen-deficient plants. Plotting of the subtracted values against the external concentrations up to 500 μM ammonium did not allow the fitting of a saturation curve, while use of values up to 200 μM ammonium yielded a saturation curve with a correlation coefficient of $R^2 = 0.70$. The calculated $V_{\text{max}}$ of the remaining nitrogen-dependent transport system in qko amounted to $24.3 \pm 1.7 \, \mu\text{mol h}^{-1} \, \text{g}^{-1}$ at an affinity constant of $4.5 \pm 1.7 \, \mu\text{M}$. This indicated the existence of an as yet unidentified ammonium transport system of low capacity but very high affinity.

Prior transcriptome analyses failed to reveal expression of the two remaining AMT genes AMT1;4 and AMT1;5 in roots (Birnbaum et al., 2003; Schmid et al., 2005). Since these experiments were conducted with RNA from roots of wild-type plants grown in sufficient levels of nitrogen, we reexamined the expression of these two genes by quantitative RT-PCR in nitrogen-deficient roots of wild-type and amt mutant plants. Consistent with the previous studies, no expression of either of the two compared with nitrogen-sufficient plants, indicating an unidentified nitrogen-dependent uptake system still operates in qko.

Nitrogen-deficient plants were subjected to a concentration-dependent ammonium influx analysis. Ammonium uptake rates in the qko+11 and qko+13 lines became saturated between 150 and 500 μM ammonium, with a $V_{\text{max}}$ of 344.7 ± 14.3 and 286.6 ± 10.4 μmol h$^{-1}$ g$^{-1}$, respectively, while qko+12 only reached a capacity of $141.9 \pm 11.4 \, \mu\text{mol h}^{-1} \, \text{g}^{-1}$ (Figure 9B). Reassuringly, the uptake capacities of AMT1;2 determined by comparing the single amt1;2 insertion lines with the wild type were in close agreement with those determined by comparing the qko+12 triple insertion line with the qko line (Figures 2 and 8). Differences in uptake rates between the qko and triple insertion lines were direct fitted to the Michaelis-Menten equation to give estimates of in planta affinity constants for the individual ammonium transporters. These were $50.0 \pm 9.6 \, \mu\text{M}$ for AMT1;1, $60.5 \pm 13.7 \, \mu\text{M}$ for AMT1;3, and $233.9 \pm 72.9 \, \mu\text{M}$ for AMT1;2.

Figure 5. Ammonium Concentrations in the Apoplastic Washing Fluid from Roots.

Ammonium concentrations in the apoplastic washing fluid from roots of wild-type (Col-0) plants or roots of the insertion lines amt1;2-1 and amt1;2-2. Bars indicate means ± SD ($n = 8$ to 10), and significant differences at $P < 0.05$ are indicated by different letters. Six-week-old plants were precultured hydroponically under continuous supply of 2 mM ammonium nitrate and then for 2 d under nitrogen deficiency (~N) and then resupplied with 250 μM NH$_4^+$ for 30 min. Root apoplastic washing fluid was extracted by centrifugation. Determination of phosphoenolpyruvate carboxylase activity (EC4.1.1.31) indicated <1% of contamination of apoplastic washing fluid by symplastic constituents. n.d., not determined.

Figure 6. Characterization of a Quadruple (qko) and Triple Insertion Lines.

RNA gel blot analysis of root RNA (A) and protein gel blot analysis of microsomal membrane fractions (B) from roots of the wild-type (Col-0), the quadruple insertion line qko (amt1;1 amt1;2 amt1;3 amt2;1), and triple insertion lines expressing AMT1;1 (qko+11), AMT1;2 (qko+12), AMT1;3 (qko+13), or AMT2;1 (qko+21) using the 3’-ends of AMT1;1, AMT1;2, or AMT1;3 or the ORF of AMT2;1 as a probe or antibodies directed against AMT1;1, AMT1;2, or AMT1;3, respectively. Ethidium bromide (EtBr)-stained rRNA and protein levels of DET3 served as loading controls. Six-week-old plants were precultured hydroponically under continuous supply of 2 mM ammonium nitrate (+N) or under nitrogen deficiency for 4 d (~N).
genes was detected in nitrogen-sufficient roots (Figure 10B). However, AMT1;5 transcripts accumulated considerably in roots of wild-type plants grown under nitrogen-insufficient conditions (Figure 10B, bottom panel). Interestingly, AMT1;5 transcript accumulation was highest in the qko and qko+21 lines (Figure 10B, bottom panel), suggesting the existence of a possible compensatory upregulation of this homolog in the absence of other AMT genes. However, in a time-course study of plants after they were moved to nitrogen-deficient conditions, AMT1;5 transcript accumulated in roots earlier in the qko and qko+21 lines (Figure 10B, bottom panel), suggesting the existence of a possible compensatory upregulation of this homolog in the absence of other AMT genes. Root mRNA levels of AMT1;4 were much lower than those of AMT1;5, and in contrast with AMT1;5, nitrogen-deficient conditions resulted in little or no increase in AMT1;4 transcript abundance (Figure 10B, top panel). Thus, AMT1;5 appeared to be the only AMT-type protein that could be responsible for the remaining nitrogen-dependent high-affinity ammonium influx observed in qko (Figure 10A).

Characterization of AMT1;5

No Arabidopsis line with a transposon or T-DNA insertion in AMT1;5 was available from public Arabidopsis seed collections, preventing a functional analysis of the endogenous gene in planta. We therefore took other approaches to address whether AMT1;5 encodes a functional ammonium transporter. Due to the genetic linkage between AMT1;5 (At3g24290) and AMT1;3 (At3g24300) and the Wassilewskija (Ws) ecotype origin of the amt1;3-1 insertion line, two AMT1;5 ORFs from the qko and Col-0 lines were PCR amplified and cloned independently. Sequence comparison showed only one nucleotide change that generated an Arg-to-Thr substitution at position 9 in the encoded protein from Ws. Functionality of both protein variants was confirmed by their ability to restore growth of the ammonium uptake-defective yeast cells when subjected to low external ammonium concentration as a sole nitrogen source. Cells transformed with
the empty vector did not restore growth (Figure 11A). Relative to cells expressing AMT1;3, however, growth complementation of those expressing AMT1;5 was weaker, suggesting that AMT1;5 conferred a smaller ammonium transport capacity to this yeast strain.

We then localized AMT1;5 promoter activity using transgenic Arabidopsis plants expressing a GFP reporter gene driven by the native AMT1;5 promoter. In roots of plants grown under nitrogen-deficient conditions, GFP-dependent fluorescence was high at the root tip and in the root hair zone where it localized to rhizodermal...
cells, including root hairs (Figure 11B). The nitrogen-dependent expression of AMT1;5 in outer root cells and the ammonium transport activity of AMT1;5 demonstrated in yeast support the notion that AMT1;5 contributes to overall ammonium uptake capacity of nitrogen-deficient Arabidopsis roots.

DISCUSSION

In most plant species, ammonium transporters belong to the AMT1- and AMT2/MEP-type classes of proteins and are encoded by gene families (Ludewig et al., 2001). Of the sequenced plant genomes, Arabidopsis contains six homologs, Chlamydomonas has eight, rice (Oryza sativa) has 10, and poplar (Populus spp) has 14 (Sonoda et al., 2003; Suenaga et al., 2003; Gonzalez-Ballester et al., 2004; Loqué and von Wirén, 2004; Couturier et al., 2007). How AMT genes might contribute to adaptation of plant species to particular growth conditions and how the number and properties of individual AMT homologs are arranged to achieve productive transport remain important unsolved questions. With the growing knowledge of individual AMT transporters from Arabidopsis, this multigene family may serve as a model for how other membrane transporter families are coordinated to achieve controlled substrate transport within plants. Here, we show that biochemical transport properties and the root tissue-specific expression patterns of AMT transporters are important factors that allow them to coordinate effective high-affinity ammonium uptake in Arabidopsis roots.

Of the six Arabidopsis AMT genes, AMT1;1, AMT1;2, AMT1;3, and AMT2;1 generally show a high mRNA abundance in roots (Gazzarrini et al., 1999; Birnbaum et al., 2003; Schmid et al., 2005). All four genes are transcriptionally upregulated in roots under nitrogen deficiency or sucrose supply, encode transport proteins that localize to the plasma membrane in planta, and can confer ammonium uptake in yeast and in planta (Gazzarrini et al., 1999; Lejay et al., 2003; Loqué et al., 2006; Engineer and Kranz, 2007; Figures 3, 8, and 9). Ammonium influx studies in single insertion lines indicated that AMT1;1 and AMT1;3 confer with 30 to 35% nearly the same capacity for ammonium uptake in nitrogen-deficient roots (Loqué et al., 2006), while AMT1;2 confers a lower capacity of 18 to 26% (Figure 2). Since the additive contribution of AMT1;1 and AMT1;3 to ammonium uptake in nitrogen-deficient roots accounts for 65 to 70% of the total uptake capacity (Loqué et al., 2006), AMT1;1, AMT1;2, and AMT1;3 together might explain ~90% of the overall ammonium uptake capacity in Arabidopsis roots. Transgenic lines in which the root-expressed AMT2;1 gene was targeted for silencing by RNAi did not reveal a contribution of this transporter to ammonium uptake by roots (Sohlenkamp et al., 2002). Therefore, one may expect the quadruple knockout (qko) of all four genes to have ~10% uptake capacity under nitrogen-deficient conditions. This expectation was closely matched by the observation of ammonium influx in qko (Figure 9A), showing that >90% of the high-affinity ammonium uptake in Arabidopsis roots is conferred by the individual capacities of AMT1;1, AMT1;2, and AMT1;3 working together in an additive manner. Interestingly, each of the three AMT capacities was enhanced when plants were shifted from nitrogen-sufficient to -deficient growth conditions (Figure 9A), most probably due to an increased protein synthesis, except

Figure 9. Ammonium Influx Analysis in Roots of the Quadruple (qko) and Triple Insertion Lines.

(A) Influx of 15N-labeled ammonium into roots of the wild type (Col-0), the quadruple insertion line qko, and triple insertion lines expressing AMT1;1 (qko+11), AMT1;2 (qko+12), AMT1;3 (qko+13), or AMT2;1 (qko+21). 15N-labeled ammonium was supplied at a concentration of 200 μM. Bars indicate means ± SD (n = 8 to 10 plants), and significant differences at P < 0.001 within each group are indicated by different letters.

(B) Concentration-dependent 15N-labeled ammonium influx into nitrogen-deficient roots (−N) of the quadruple and triple insertion lines. Bars indicate means ± SD (n = 8 to 10 plants). Six-week-old plants were precultured hydroponically under continuous supply of 2 mM ammonium nitrate (+N) or under nitrogen deficiency for 4 d (−N).
for AMT1;2 (Figures 1D, 2, and 6). Even though AMT1;2-mediated transport capacity increased under nitrogen deficiency (Figures 2A and 9A), AMT1;2 protein levels remained unchanged (Figures 2C and 2D), suggesting that posttranslational activation may have increased transport capacity (Neuha"user et al., 2007). Taken together, the three AMT genes differ in the inherent transport capacities they encode and in the magnitude of their modulation in response to the nitrogen nutritional status of the plant.

In vivo transport capacities of AMT1;1, AMT1;2, and AMT1;3 as determined in single insertion lines were not exactly the same as those determined using the triple insertion lines. The transport capacity of AMT1;2 (Figure 2) tended to be only slightly higher when comparing single insertion lines with the wild type than qko +12 with qko (Figure 9). By contrast, AMT1;1 and AMT1;3 contributed to 30 to 35% of the wild-type capacity in single insertion lines (Loquè et al., 2006), but this value increased to 55 to 60% when assayed in the corresponding triple insertion lines (Figure 9). Different Arabidopsis ecotypes were used as the

Figure 10. Nitrogen-Dependent Ammonium Influx and AMT Gene Expression in Roots of the Quadruple Insertion Line (qko).

(A) Concentration-dependent influx of 15N-labeled ammonium into nitrogen-sufficient (+N) or nitrogen-deficient roots (−N) of the quadruple insertion line qko. Bars indicate means ± SD (n = 5 to 6 plants).
(B) AMT1;4 and AMT1;5 mRNA abundance in roots of wild-type or triple insertion lines quantified by real-time RT-PCR relative to the constitutively expressed polyubiquitin control gene (UBQ2). Bars indicate means ± SD (n = 3). Six week-old plants were precultured hydroponically under continuous supply of 2 mM ammonium nitrate (+N) or under nitrogen deficiency for 4 d (−N).

Figure 11. Functional Analysis in Yeast and Localization in Roots of AMT1;5.

(A) The triple mep yeast mutant (31019b) was transformed with the vectors pYES2, pYES2-AMT1;3, pYES2-AMT1;5 (Ws), or pYES2-AMT1;5 (Col-0). Transformants were selected on YNB medium supplemented with 1 mM Arg. Five microliters of precultured yeast cell suspensions were spotted at onefold to fourfold dilutions on YNB medium supplemented with 2% galactose and either 0.2 or 2 mM NH4Cl or 1 mM Arg at pH 5.2. Cells were grown at 28°C for 6 d.
(B) Transgenic plants expressing an AMT1;5-promoter:GFP fusion construct were grown on agar plates supplemented with 2 mM ammonium nitrate for 2 weeks before being transferred to new media deprived of nitrogen and grown for 5 d. Whole-mount images from root tips and root hair zones were taken by confocal laser scanning microscopy. Bars = 200 μm.
source of mutant alleles in this study. Despite our effort to make the background of the triple insertion lines relatively homogeneous, it cannot be completely excluded that these lines may have differed for other genes influencing the traits being examined. However, segregation of other genes affecting ammonium uptake seems an unlikely explanation for the higher capacities conferred by AMT1;1 and AMT1;3 in the qko background relative to the wild type because ammonium uptake capacities of nitrogen-deficient wild-type Col-0, Col-gl, or Ws lines did not differ significantly (Loqué et al., 2006; Figure 9A; see Supplemental Figure 7 online). Moreover, a compensatory upregulation of mRNA or protein levels in response to the absence of other AMT proteins could also be ruled out to explain the higher capacities of AMT1;1 and AMT1;3 shown using the triple insertion lines (Figure 6). A compensatory posttranslational regulation might provide an alternative explanation for these differences. Recently, AMT1;1 was shown to be subjected to a C-terminal phosphorylation modification that inhibits ammonium transport activity (Loqué et al., 2007). Theoretically, a reduction in phosphorylation of AMT1;1 or AMT1;3 in the triple insertion lines expressing these respective proteins might have occurred in response to the absence of other expressed homologs to allow increased transport capacity.

The ammonium influx into nitrogen-sufficient and -deficient qko plants indicated the presence of at least another two ammonium uptake systems. A low-capacity transport system with a linear concentration dependency appeared to confer ammonium uptake in nitrogen-sufficient plants (Figure 10A). Its concentration-dependent kinetics was reminiscent of channel-mediated transport and might reflect the activity of NH₄⁺- and/or NH₃-conducting aquaporins in the root plasma membrane or of potassium channels, some of which also permeate ammonium (Uozumi et al., 1995; Moroni et al., 1998; Loqué et al., 2005). These or similar transporters could act as low-affinity ammonium transporters, whose contributions extend into the high-affinity range, albeit at a low capacity (Cerezo et al., 2001). In nitrogen-deficient qko plants, a second, saturable transport system appeared with a low Vₘₐₓ of ~4.5 μM and a very low capacity that corresponded only to ~5 to 10% of the wild-type transport capacity (Figures 9A and 10A). This transport system might reflect a plant adaptation to very low external ammonium availability, which cannot be efficiently used by AMT1;1 or AMT1;3. In agreement with the study by Engineer and Kranz (2007), quantitative RT-PCR analysis revealed that among the Arabidopsis AMT proteins, only AMT1;5 could potentially represent this second saturable transport system, since AMT1;5 transcripts accumulated under nitrogen deficiency, while AMT1;4 expression did not respond to nitrogen deficiency and was at least a thousand-fold lower under either nitrogen-deficient or -sufficient conditions (Figure 10B). Moreover, heterologous expression in yeast showed that AMT1;5 encodes a functional ammonium transporter (Figure 11A), which also suggested AMT1;5 may localize at the plasma membrane in Arabidopsis. In addition, promoter activity of AMT1;5 was observed in the rhizodermis and the root hairs (Figure 11B). These observations support a role for AMT1;5 in accessing external ammonium by uptake across the plasma membrane of rhizodermis cells (Figure 12). Nevertheless, these observations provide only correlative evidence that the residual nitrogen-dependent high-affinity ammonium uptake in the qko line depends on AMT1;5. Definitive proof would probably require RNAi-mediated silencing of AMT1;5 in the qko background or ethyl methanesulfonate mutagenesis in qko followed by a tilling approach to isolate amt1;5 mutant alleles, since T-DNA or transposon insertion mutants for this gene are not yet available. Nonetheless, the use of T-DNA or transposon insertion mutants for this gene in the generation of a multiple insertion line will be difficult due to the close genetic linkage between AMT1;5 and AMT1;3, and physiological studies will be hampered by the low transport capacity of AMT1;5.

Although AMT2;1 conferred high-affinity ammonium transport in yeast and localized to the plasma membrane (Sohlenkamp et al., 2000, 2002), AMT2;1 did not contribute to overall ammonium uptake in planta. Relative to the qko line, the qko-21 triple insertion line showed neither improved ammonium-dependent growth (Figure 8) nor higher ammonium influx (Figure 9). This observation is consistent with the previous failure to obtain a nitrogen-dependent growth phenotype by RNAi-mediated silencing of Arabidopsis AMT2;1 (Sohlenkamp et al., 2002). Moreover, AMT2;1 showed a high promoter activity in the root vasculature and a relatively weak activity in cortical cells near the root apex (Sohlenkamp et al., 2002). This particular expression pattern, together with the fact that AMT2;1 belongs to the AMT2 subfamily with potentially different biochemical transport properties to AMT1 transporters (Mayer et al., 2006), raises the possibility that AMT2;1 performs a role in a process other than ammonium acquisition from the rhizosphere. The qko and qko-21 lines

**Figure 12. Model Summarizing the Functions of AMT1-Type Transporters in High-Affinity Ammonium Uptake in Arabidopsis Roots.**
Schematic representation of the contribution to ammonium uptake and spatial expression in root tissues of AMT1;1, AMT1;3, AMT1;5 (all in red), and AMT1;2 (blue) under nitrogen deficiency. AMT-dependent ammonium influx is proportionally represented by the size of their arrows. Ammonium can enter the symplastic route (dashed red line) for radial transport toward the root stele via AMT1;1, AMT1;3, and AMT1;5, which are localized at the plasma membrane of rhizodermis cells, including root hairs. Ammonium can also bypass outer root cells via the apoplastic transport route (yellow line) and subsequently enter the root symplast by AMT2;1-mediated transport across the plasma membrane of endodermal (in the root hair zone) and cortical (in more basal root zones) cells. In the symplast, ammonium can either be assimilated into amino acids or loaded into the xylem by an as yet unidentified transport process. rhizo, rhizodermis; co, cortex; endo, endodermis; peric, pericycle; xyl, xylem.
generated in this study should provide a solid basis for future, more extended physiological analyses to uncover the role of AMT2;1.

The triple insertion lines also allowed estimation of substrate affinities of AMT transporters in planta (Figure 9B). AMT1;1 and AMT1;3 showed a very similar affinity for ammonium with $K_m$ values of 50.0 and 60.5 $\mu$M, respectively. Considering that ammonium concentrations in soil solutions rarely exceed 50 $\mu$M (Marschner, 1995), these $K_m$ values appear to suit their location in rhizodermis cells and root hairs (Figure 12). By contrast, AMT1;2 showed a significantly lower substrate affinity ($K_m = 233.9$ $\mu$M), suggesting adaptation to higher substrate concentrations. AMT1;2 is expressed in endodermal and cortical cells (Figure 4; Neuhäuser et al., 2007), where it resides in the plasma membrane (Figure 3). A location in inner root cells means that substrate for AMT1;2 must come from ammonium that has first been transported radially from the soil through the apoplastic in the outer cell layers (Figure 12). To obtain an estimate of the ammonium concentrations that AMT1;2 is facing, we measured ammonium concentrations in apoplastic washing fluid extracted from the roots of wild-type and amt1;2-1 plants (Figure 5). Interestingly, apoplastic ammonium concentrations in nitrogen-deficient or ammonium-resupplied roots were much higher than those present in the externally supplied solution. This is explained by the Casparian strip forming an apoplastic barrier, which favors a local accumulation of ammonium in the endodermal apoplast (Marschner, 1995). Moreover, continuous efflux of ammonium from cells may help contribute to the millimolar ammonium concentrations found in the apoplast of inner root tissues (Feng et al., 1994; Britto et al., 2001). Indeed, after extraction of ammonium by infiltration of leaf tissue with buffered solutions, apoplastic ammonium concentrations quickly recover and equilibrate in the lower millimolar concentration range (Husted and Schjoerring, 1995; Nielsen and Schjoerring, 1998; Mattsson and Schjoerring, 2002). This buffering capacity will most likely have increased ammonium concentrations in the microenvironment of AMT1;2 above the level supplied in our uptake studies. As a consequence, the determined $K_m$ of 234 $\mu$M (Figure 9B) represents an in vivo affinity constant, likely being higher than when assayed under conditions in which AMT1;2 directly faces the uptake solution. Indeed, when heterologously expressed in oocytes, AMT1;2 exhibited a $K_m$ value of $\sim 140$ $\mu$M (Neuhäuser et al., 2007), which is nevertheless more than 10 times higher as that for AMT1;1 determined under the same conditions (Mayer and Ludewig, 2006; Wood et al., 2006). Taken together, AMT1;2 obviously faces higher ammonium concentrations than the AMT1;1 and AMT1;3 rhizodermis-located proteins. The lower $K_m$ of AMT1;2 is therefore suited to this environment, enabling efficient substrate transport close to saturation of the transporter. We therefore conclude that the biochemical transport properties and specific location of AMT1;2 represent an adaptation to retrieval of ammonium that is released from the cortex and that enters the root tissue via the apoplastic transport route.

The findings of this study highlight two principles governing high-affinity ammonium uptake into Arabidopsis roots via multiple AMT1-type transport proteins. First, there is a spatial organization of AMT1 transporters, with those transporters possessing higher substrate affinities being located in outer root cells and those of lower affinity being located at the end of the apoplastic transport pathway. At the surface and root hairs of the rhizodermis, ammonium can be directly taken up from the soil solution by AMT1;1, AMT1;3, and AMT1;5 (Figure 12), allowing an immediate transfer into the root symplast for ammonium assimilation by the rhizodermis-localized cytosolic Gln synthetase (Ishiyama et al., 2004). Unassimilated ammonium might be stored in vacuoles (Miller et al., 2001) or further transported symplastically into the root stele, either for assimilation or loading into the xylem where it may accumulate in millimolar concentrations (see Supplemental Figure 3 online). Ammonium that enters the root via the apoplastic route may enter the root symplast through AMT1;2 at the endodermis (Figures 4 and 12). The endodermal expression pattern of AMT1;2 seems to be confined to the root hair zone, which constitutes $\sim 70\%$ of the total root surface (Marschner, 1995). Further up the root, AMT1;1, AMT1;2, and AMT1;3 expression extends to cortical cells (Loqué et al., 2006; Figure 4), which provide a large inner surface area for nutrient uptake. This spatial shift might be due to the limited life span of rhizodermis and root hair cells, whose physiological activities usually decrease within a few days (Fusseder, 1987; Neumann et al., 1999). An extension also of AMT1;2 expression to cortex cells in upper root zones might reflect a higher requirement for ammonium retrieval, considering that ammonia-releasing nitrogen catabolism probably increases in older root cells. The second principal highlighted by this study is that high-affinity ammonium transport in Arabidopsis roots is conferred by at least four transporters whose capacities likely decrease in the following order: AMT1;1 = AMT1;3 > AMT1;2 > AMT1;5. Although individual ammonium transport capacities, in particular those of AMT1;1 and AMT1;3, might depend on the presence of other transporters, ammonium transport capacities of all four AMT1 proteins increased under nitrogen deficiency (Figure 9; Loqué et al., 2006). To meet the plant’s nitrogen demand, it appears that its nutritional status determines the overall ammonium transport capacity, which in turn is broken down into individually modulated AMT1 capacities. Such a coordinated regulation could be brought about by a common regulator, for example, a transcription factor and/or a posttranslational modulator, which is subject to control by the nitrogen demand of the plant. However, nitrogen-sensing proteins or cis-acting elements that govern nitrogen demand–regulated expression of genes and proteins involved in nitrogen transport or assimilation still await to be uncovered.

METHODS

Isolation of Transposon Insertion Lines in AMT1;2 and Generation of Multiple Insertion Lines

The amt1;2-1 and amt1;2-2 insertion lines were isolated from the Arabidopsis thaliana transposon-tagged (dSpm) mutant collection (NASC). Insertions in tagged lines were identified by PCR using the transposon-specific Spm31 primer (5'-GGTCCTGTTAGACGCACTTTATCAATAG-3') and the AMT1;2 gene-specific primers FKQamtt12 (5'-CTTACTACTCTTCTGCGATGCGC-3') and R3KOamtt12 (5'-TTAAAGTGTTCTACACTATCGTC-3'). The amt1;2-1 line was isolated from the enhancer trap collection of Thomas Jack (Fartmouth College, NH) using as T-DNA–specific primer LBKQamtt1-1 (5'-TGCCTATATACTACGAGACGATC-3') and as AMT1;2 gene-specific primers F1KOamtt2-1 (5'-AGTGACCGCAGTCGAAGG-3') and R2KOamtt2-1 (5'-AGTGACCGCAGTCGAAGG-3').
The location of the transposons in the insertion lines was confirmed by sequencing. The
amt1;1-1 mutant (in Col-gl) and amt1;3-1 mutant (in Ws) were crossed to give the double insertion line (dko) amt1;1-1 amt1;3-1 (Loqué et al., 2006). A triple insertion line (tko) was obtained by crossing the dko with
amt2;2-1 (in Col-gl). The F1 plants were backcrossed to the dko and selfed. A homozygous tko line (amt1;1-1 amt1;3-1 amt2;2-1-1) isolated by PCR was used to generate a quadruple insertion line by crossing tko with
amt1;2-1 (in Col-0). After backcrossing to tko and selfing, a homozygous
amt1;1-1 amt1;3-1 amt1;2-1-1 amt1;2-1-1 line was isolated by PCR. Then, the
quadruple insertion line was backcrossed to wild-type Col-0 and selfed to obtain F2 and F3 populations. Within the F3 population, homozygous lines expressing only one of the four AMT genes were selected by PCR and assigned as qko+11 (qko+AMT1;1), qko+12 (qko+AMT1;2), qko+13 (qko+AMT1;3), and qko+21 (qko+AMT2;1). A homozygous
amt1;1-1 amt1;3-1 amt2;1-1 amt1;2-1 line (qko) that served as a reference line was also selected from this population. All lines were then selfed once (F4 generation) before using in phenotypic or physiological analyses.

Plant Culture
Arabidopsis seeds were germinated in the dark for 4 d and precultured on
rock wool moistened with tap water. After 1 wk, tap water was sub-
stituted for nutrient solution containing 1 mM KH2PO4, 1 mM MgSO4, 250
mM K2SO4, 250 uM CaCl2, 100 uM Na-Fe-EDTA, 50 uM KCl, 50 uM
H3BO3, 5 uM MnSO4, 1 mM ZnSO4, and 1 uM NaMoO4 (pH
adjusted to 6.0 by KOH). Unless indicated otherwise, 2 mM NH4NO3 was
supplied to provide nitrogen-sufficient conditions. The nutrient solution
was replaced once a week during the first 3 weeks, twice in the 4th week,
and 2 wk in the following weeks. Plants were grown hydroponically
under nonsterile conditions in a growth cabinet under the following re-
tinctions. Plants were grown under axenic conditions in a growth chamber
supplemented with different nitrogen sources at indicated concentra-
tions. Plants were grown without nitrogen source) solidified with Difco agar. The plants were precultured for
plated onto half-strength MS medium (containing 5 mM nitrate as sole
substrate (www.einheitserde.de) in nutrient solution under supply of
ammonium, or ammonium nitrate at different concentrations. In plate growth
tests of insertion lines, Arabidopsis seeds were surface sterilized and
seeds were surface sterilized and
in a growth cabinet under the following re-
tuations. Plants were grown under axenic conditions in a growth chamber
under the above-mentioned conditions except that the light intensity was
120 mmol photons m-2 s-1.

Collection of Apoplastic Fluid and Xylem Sap
Arabidopsis plants were grown hydroponically for 6 weeks. Apoplastic
fluid of the roots was extracted by centrifugation according to the method
of Yu et al. (1999) with some modifications: The harvested roots were
dried by blotting and fixed into a 15-mL Falcon tube. By centrifugation
at 500 g for 15 min at 4°C, the solution adhering to the root surface was
removed. In a subsequent centrifugation step at 1000 g for 15 min at 4°C,
root apoplastic fluid was collected. A negligible symplastic contamination
of the samples was determined. The apoplastic extracts and xylem sap
samples were stabilized with ice-cold 20 mM HCOOH in a 1:1 volume ratio
and stored at −20°C. Ammonium concentrations were analyzed by fluo-
rescence spectroscopy at neutral pH as described by Husted et al. (2000).

RNA Gel Blot Analysis
Total RNA was isolated by phenol-guanidine extraction followed by
chloride precipitation or was extracted using TRIzol reagent (In-
nitrogen). RNA (20 μg per lane) was resolved by electrophoresis in MOPS-
formaldehyde agarose gels, blotted onto Hybond N+ nylon membranes
(Amersham), and cross-linked to the membrane by incubation at 80°C for 2
h. The ORFs AMT1;2 and AMT2;1 and 3’-ends of AMT1;1, AMT1;2, and
AMT1;3 were used as probes for hybridization to total RNA. Hybridization
of random-primed 32P-radiolabeled probes was performed at 42°C in 50%
(v/v) formamide, 1% (w/v) sarkosyl, 5× SSC, and 100 μg mL−1 yeast tRNA.
Membranes were washed at 42°C twice in 2× SSC, 0.1% (w/v) SDS for
20 min, once in 0.2× SSC, 0.1% (w/v) SDS, and finally in 0.1× SSC, 0.1% (w/v) SDS for 20 min. Ethidium bromide staining of gels was used to monitor evenness of RNA loading.

Isolation of Microsomal Membrane and Plasma Membrane Fractions
Arabidopsis root and shoot tissues were frozen in liquid nitrogen and
ground in a mortar. The powder was homogenized in a buffer containing
250 mM Tris-HCl, pH 8.5, 290 mM sucrose, 25 mM EDTA, 5 mM
β-mercaptoethanol, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride
(PMSF). Homogenates were centrifuged at 10,000g for 15 min. Superna-
tants were filtered through nylon mesh (50 μM) and centrifuged at
100,000g for 30 min to pellet microsomal membrane fractions. The
drained pellet was resuspended in conservation buffer (5 mM bis-Tris-
propane, MES, pH 6.5, 250 mM sorbitol, 20% (w/v) glycerol, 1 mM DTT,
and 2 mM PMSF) and gently homogenized in a potter.

Plasma membranes were isolated by aqueous two-phase partitioning as described by Larsson et al. (1987). The drained pellet obtained from the above procedure was resuspended in microsomal buffer (5 mM KH2PO4, pH 7.8, 330 mM sucrose, and 3 mM KCl) and added to a 36-g phase partitioning system (final concentration: 6.4% dextran T-500, 6.4% poly-
ethylene glycol 3350, 5 mM KH2PO4, 3 mM KCl, and 330 mM sucrose). The two phases were mixed and centrifuged at 1500g for 5 min. Upper and lower phases were collected separately and repartitioned twice with fresh buffer. The purified phases were diluted with washing buffer and centrifuged at 100,000g for 60 min to pellet the membranes. The drained
pellets were resuspended in conservation buffer and gently homogenized
in a potter. All steps were performed at 4°C. Protein concentrations were
determined using a Bradford protein assay (Bio-Rad) with BSA as a standard.

Protein Gel Blot Analysis
Polyclonal antibodies were raised against peptides representing the C
terminus of AMT1;2 (n-PWGH GVEPT SRs-2) (Bioblot). The anti-
odies were affinity purified from serum using a nitrocellulose membrane
binding the corresponding peptide as described by Ludewig et al. (2003).
Proteins (5 to 10 μg per lane) were denatured in loading buffer (62.5 mM
Tris-HCl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 2.5% [v/v]
β-mercaptoethanol, 0.01% [w/v] bromphenol blue, and 1% PMSF) at
37°C for 30 min or 50°C for 5 min, separated on 10% SDS polyacrylamide
gels, and transferred to a polyvinylidene fluoride membrane (Immobilon-
P; Millipore) by electroblotting. Blots were developed using the ECL
Advance Western Blotting Detection Kit (Amersham) according to the
manufacturer’s protocol. Primary antibodies and the secondary antibody
(peroxidase-linked anti-rabbit IgG; Amersham) were diluted in blocking
solution at the following concentrations and combinations: anti-AMT1;1
1:400 with secondary antibody at 1:25,000, anti-AMT1;2 at 1:10,000,
anti-AMT1;3 at 1:5000, anti-AMT2;1 at 1:4000, anti-AHA2 at 1:10,000 (Delwitt et al., 1996), anti-VPPase (Takasu et al., 1997) at 1:20,000 (Schumacher et al., 1999) with secondary antibody at 1:10,000. MagiMark Western Standard (Invitrogen) was used as molecular weight
marker. Protein blots of DET3, a subunit of the vacuolar ATPase, were used as a control for equal loading.

Localization of AMT1;2 and AMT1;5 by GFP Fusion

For vector construction, DNA fragments of AMT genes were amplified from Arabidopsis (ecotype Col-0) genomic DNA by PCR using KOD plus DNA polymerase (Toyobo). All PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) and fully sequenced to confirm identity and lack of polymerase errors. An AMT1;2 promoter-ORF fragment covering the 2849-bp 5′-upstream region together with the 1542-bp ORF was amplified by PCR using the primers AMT1;2-2GF (5′-GAAGCTTTAGCT-TCTGTGGATATACTTACGAATAA-3′) and AMT1;2-GR (5′-TCCCGGGGAAACGTCAAGTGGTGGTGAGTGCAGA-3′). The underlined sequences indicate HindIII and SalI sites used for construction. The PCR product was cut out as a HindIII-Smal-ended fragment and ligated to a SalI-NotI fragment of EGFP (Clontech) to create a translational fusion at the C terminus of AMT1;2. This AMT1;2 promoter-ORF-GFP fusion cassette and an NptI-EcoRI-ended fragment of the nopaline synthase terminator (Tnos) from the pTH2 vector (Chiu et al., 1996) was cloned between the HindIII and EcoRI sites of pBP101 (Clontech) to obtain AMT1;2 PRO-ORF-GFP-Tnos fusion construct.

The AMT1;5 promoter fragment covering the 2115-bp 5′-upstream region in AMT1;5 was amplified by PCR using the primers AMT1;5-pro-F (5′-AAGCTTGTGCATATTAGTCGTCAAACTGTATTTA-3′) and AMT1;5-pro-R (5′-GGCGTCTCCGGCTAGATCT-3′). The amplified DNA fragment was cloned into pCR-BluntII-TOPO (Invitrogen) and sequenced. The specific primers AMT1;2-F (5′-CCCGGGGAAACGTCAAGTGGTGGTGAGTGCAGA-3′) and AMT1;2-R (5′-TCCCGGGGAAACGTCAAGTGGTGGTGAGTGCAGA-3′) were used for the transformation of the triple mep deletion yeast strain 31019b (Marini et al., 1997). Growth complementation assays were performed on solid YNB medium supplemented with 2% galactose and a nitrogen source (1 mM Arg and 0.2 or 2 mM ammonium chloride) and buffered at pH 5.2 by 50 mM MES-Tris.

Heterologous Expression of the Chimeric AMT1;2 Protein in Yeast

The ORF of AMT1;2 was amplified by PCR from reverse-transcribed Arabidopsis Col-0 cDNA using KOD-Δ polymerase (Toyobo) with the specific primers AMT1;2-F (5′-TCCCGGGGAAACGTCAAGTGGTGGTGAGTGCAGA-3′) and AMT1;2-R (5′-TCCCGGGGAAACGTCAAGTGGTGGTGAGTGCAGA-3′). The amplified fragment was cloned into pCR-BluntII-TOPO (Invitrogen) and DNA sequences were verified. The EcoRI fragment was subcloned into the yeast expression vector pYES2 (Invitrogen) resulting in the plasmid, which was used for the transformation of the triple mep deletion yeast strain 31019b (Marini et al., 1997). Growth complementation assays were performed on solid YNB medium supplemented with 2% galactose and a nitrogen source (1 mM Arg and 0.2 or 2 mM ammonium chloride) and buffered at pH 5.2 by 50 mM MES-Tris.

15N Uptake Analysis

Influx measurements of 15N-labeled NH₄⁺ in plant roots were conducted after rinsing the roots in 1 mM CaSO₄ solution for 1 min, followed by an incubation for 6 min in nutrient solution containing different concentrations of 15N-labeled NH₄⁺ (95 atom% 15N) as a sole nitrogen source, and finally washed in 1 mM CaSO₄ solution. Roots were harvested and stored at −70°C before freeze-drying. Each sample was ground, and 1.5 mg was used for 15N determination by isotope ratio mass spectrometry (Finngan). Values obtained for concentration-dependent ammonium influx up to 500 μM ammonium were directly fitted to the Michaelis-Menten equation.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are At4g13510 (AMT1;1), At1g64780 (AMT1;2), At3g24300 (AMT1;3), At4g28700 (AMT1;4), At3g24290 (AMT1;5), and At2g38290 (AMT2;1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The AMT1;2-2 Mutant Allele Is Nonfunctional When Heterologuously Expressed in Yeast.

Supplemental Figure 2. The Insertion Lines amt1;2-1 and amt1;2-2 Show Decreased Methylammonium Sensitivity.

Supplemental Figure 3. The Insertion Lines amt1;2-1 and amt1;2-2 Do Not Show Altered Ammonium Concentrations in the Xylem Sap.

Supplemental Figure 4. The amt2;1 T-DNA Insertion Line.
Supplemental Figure 5. Growth of the Quadruple Insertion Line (qko) on Millimolar Ammonium Concentrations.

Supplemental Figure 6. Time-Dependent Accumulation of AMT1;5 mRNA under Nitrogen Deficiency.

Supplemental Figure 7. Comparison of the Influx of $^{15}$N-Labeled Ammonium into Roots of Three Arabidopsis Ecotypes.

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The Organization of High-Affinity Ammonium Uptake in Arabidopsis Roots Depends on the Spatial Arrangement and Biochemical Properties of AMT1-Type Transporters

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