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A Critical Role of AMT2;1 in Root-To-Shoot Translocation of Ammonium in *Arabidopsis*

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ABSTRACT

Ammonium uptake in plant roots is mediated by AMT/MEP/Rh-type ammonium transporters. Out of five AMTs being expressed in *Arabidopsis* roots, four AMT1-type transporters contribute to ammonium uptake, whereas no physiological function has so far been assigned to the only homolog belonging to the MEP subfamily, AMT2;1. Based on the observation that under ammonium supply, the transcript levels of *AMT2;1* increased and its promoter activity shifted preferentially to the pericycle, we assessed the contribution of AMT2;1 to xylem loading. When exposed to ^{15}N-labeled ammonium, *amt2;1* mutant lines translocated less tracer to the shoots and contained less ammonium in the xylem sap. Moreover, in an *amt1;1 amt1;2 amt1;3 amt2;1* quadruple mutant (*qko*), co-expression of *AMT2;1* with either *AMT1;2* or *AMT1;3* significantly enhanced ^{15}N translocation to shoots, indicating a cooperative action between AMT2;1 and AMT1 transporters. Under N deficiency, *proAMT2;1-GFP* lines showed enhanced promoter activity predominantly in cortical root cells, which coincided with elevated ammonium influx conferred by AMT2;1 at millimolar substrate concentrations. Our results indicate that in addition to contributing moderately to root uptake in the low-affinity range, AMT2;1 functions mainly in root-to-shoot translocation of ammonium, depending on its cell-type-specific expression in response to the plant nutritional status and to local ammonium gradients.

Key words: nitrogen uptake, nitrogen translocation, ammonium assimilation, xylem loading, ammonia transport, ammonium influx

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INTRODUCTION

A critical aspect during plant growth and development is the plant's ability to efficiently meet the nutritional demand of aerial tissues via the long-distance delivery of nutrients taken up by the roots. In roots, the amount of nutrients destined for far-located tissues is determined by the activity of transporters that load the xylem vessels, while in shoots, transporters unloading the xylem can increase overall root-to-shoot translocation (Gaymard et al., 1998; Hamburger et al., 2002; Lin et al., 2008; Li et al., 2010; Chen et al., 2012). In the case of nitrogen (N), root-to-shoot allocation of different N forms is affected by a range of factors, such as the form and the amount of N available in the soil, the assimilation capacity of roots and shoots, and the growth conditions that affect the availability of carbon skeletons and reducing equivalents in roots (Smirnoff and Stewart, 1985). Whereas in most annual plants a significant proportion of nitrate taken up in roots is translocated to aerial parts, it has been previously assumed that ammonium, either taken up directly from the external solution or generated by nitrate reduction in roots, is almost exclusively assimilated in roots (van Beusichem et al., 1988; Kafkafi and Ganmore-Neumann, 1997; The term ammonium is used whenever the chemical form remains undefined, while NH_4^+ and NH_3 refer to the defined molecular species.) However, since ammonium assimilation in roots requires large amounts of carbon skeletons and reducing equivalents, it is conceivable that plants with limited root assimilatory capacity or conditions that reduce the allocation of carbon skeletons to roots may stimulate ammonium loading of the xylem to prevent the deleterious effects of its overaccumulation in roots. In this regard, it has turned out that early attempts to assess ammonium concentrations in plant samples produced often confounding results due to the lack of appropriate analytical methods that could guarantee sample stability as well

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as sensitivity and selectivity during ammonium detection (Schjoerring et al., 2002). The establishment of improved methods for ammonium detection in small volumes has revealed that significant amounts of ammonium are present in the xylem sap of various plant species (Finnemann and Schjoerring, 1999; Husted et al., 2000; Schjoerring et al., 2002). In xylem exudates of *Arabidopsis*, ammonium concentrations amounted to >4 mM (Yuan et al., 2007), while in ammonium-fed oilseed rape, these levels reached up to 8 mM, representing 11% of the total N found in the xylem sap (Finnemann and Schjoerring, 1999). The detection of ammonium concentrations in the millimolar range in root apoplasts (Yuan et al., 2007) further suggests that ammonium transporters may be required for xylem loading. However, the molecular mechanism governing root-to-shoot translocation of ammonium has remained unknown.

With regard to nitrate (NO_3^-), three members of the NPF (NRT1/PTR Family) family of nitrate/peptide transporters have been implicated in the control of root-to-shoot translocation of nitrate. Whereas NPF7.3/NRT1.5 mediates nitrate efflux into the xylem vessels (Lin et al., 2008), the nitrate influx transporters NPF7.2/NRT1.8 and, to some extent, NPF2.9/NRT1.9 retrieve nitrate from the xylem sap (Li et al., 2010; Wang and Tsay, 2011). In more mature parts of roots, where the dual-affinity nitrate transceptor NPF6.3/NRT1.1 is expressed in the central cylinder (Remans et al., 2006), evidence provided by the transport activity of this protein in a heterologous system and by *in planta* ^{15}N-nitrate translocation indicated that NPF6.3/NRT1.1 is also involved in root-to-shoot translocation of nitrate (Leran et al., 2013). Moreover, some of these transporters appear to cooperate with other transporters in order to maintain the cation-anion balance in the xylem sap. For instance, NPF7.3/NRT1.5 is not only involved in xylem loading of nitrate but also in potassium translocation (Drechsler et al., 2015).

In a wide range of organisms, transport of ammonium across membranes is mediated by proteins of the AMMONIUM TRANSPORTER/METHYLAMMONIUM PERMEASE/RHESUS PROTEIN (AMT/MEP/Rh) family (Ludewig et al., 2001; Loqué and von Wirén, 2004). In *Arabidopsis thaliana*, four homologs from the AMT family (*AMT1;1, AMT1;2, AMT1;3*, and *AMT1;5*) and one homolog from the MEP subfamily (*AMT2;1*) are expressed in roots, while *AMT1;4* is highly confined to pollen (Yuan et al., 2009). The root-expressed AMT1-type proteins AMT1;1, AMT1;2, AMT1;3, and AMT1;5 are the major transporters for high-affinity ammonium uptake into *Arabidopsis* roots (Loqué et al., 2006; Yuan et al., 2007). Two of these transporters, AMT1;1 and AMT1;3, show predominant localization in rhizodermal and cortical cells, including root hairs, and are responsible for approximately two-thirds of the high-affinity ammonium uptake capacity in roots (Loqué et al., 2006). The localization of AMT1;2 at the plasma membrane of endodermal and cortical cells, in turn, indicates that AMT1;2 may mediate the uptake of ammonium entering the root via the apoplastic transport route (Yuan et al., 2007).

Currently, the physiological function of AMT2;1 in plants still remains unclear. In contrast to the root-expressed AMT1-type transporters, no *in planta* evidence for a contribution to high-affinity ammonium influx has been detected for AMT2;1 (Yuan et al., 2007). However, according to growth complementation assays of a yeast mutant defective in ammonium uptake, AMT2;1 from *Arabidopsis thaliana* is a functional ammonium transporter (Sohlenkamp et al., 2000; Neuhäuser et al., 2009). Although its ammonium transport capacity (V_{max}) at pH 6.1 is at least ten times lower than that of AMT1;1, the apparent V_{max} of AMT2;1 seems to increase as the pH is raised (Sohlenkamp et al., 2002; Neuhäuser et al., 2009). Based on results obtained from yeast complementation assays, two-electrode voltage clamp studies, and homology modeling, it has been suggested that ammonium transport via AMT2;1 involves the recruitment of the ammonium ion (NH_4^+) at the vestibule of the external pore to allow for de-protonation and subsequent transport of the uncharged ammonia (NH_3) molecule through the pore (Sohlenkamp et al., 2000; Neuhäuser et al., 2009).

Previous studies also revealed that AMT2;1 localizes at the plasma membrane (Sohlenkamp et al., 2002; Neuhäuser et al., 2009). However, seemingly discrepant results have been reported regarding the tissue-specific localization of AMT2;1. In full-strength Murashige and Skoog (MS) medium, which contains ~40 mM nitrate and ~20 mM ammonium (Murashige and Skoog, 1962), GUS activity driven by 1.0 kb of the *AtAMT2;1* promoter has been detected mainly in the vascular tissue of roots, stems, leaves, and flowers (Sohlenkamp et al., 2002). Interestingly, when AMT2;1 localization was assessed in transgenic lines expressing a longer sequence of the *AMT2;1* promoter (i.e., 1.7 kb), *AMT2;1*-dependent GFP expression under low N supply (0–200 μM NH_4NO_3) was confined to rhizodermal cells, including root hairs, and was very weak in inner root tissues (Neuhäuser et al., 2009). The reason for this discrepancy in cell-type-specific localization and the consequence for the physiological function of AMT2;1 still remain elusive.

In this study, we further assessed the function of AMT2;1 by employing single insertion mutants defective in *AMT2;1* expression as well as double, triple, and quadruple *amt* knockout lines. These mutant lines were employed to determine ammonium uptake and translocation capacities. Together with tissue localization of *AMT2;1* expression in response to different N conditions, our results provide compelling evidence that AMT2;1 has a critical role in root-to-shoot translocation of ammonium, and to a minor extent, in ammonium uptake at elevated external substrate concentrations.

RESULTS

Regulation of *AMT2;1* Expression and Localization by Nitrogen

To assess how *AMT2;1* expression is regulated by N supply, transcript levels were determined in roots after exposure to different N forms. Relative to growth in nitrate, transcript levels of *AMT2;1* were more than two-fold higher when plants were grown in the absence of N for 5 days (Figure 1). In the presence of ammonium as the sole N source, *AMT2;1* mRNA levels increased only by about 50%, irrespective of whether 1 or 10 mM ammonium was supplied. By contrast, when the nitrate supply increased from 1 to 10 mM, *AMT2;1* transcript levels dropped further. These observations suggested that not only the plant N status but also the supply of different N forms exert a regulatory effect on the expression of this gene (Figure 1).

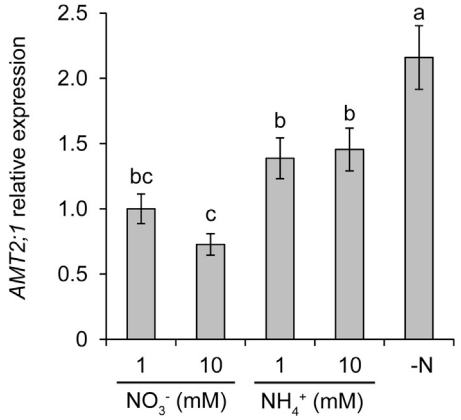

Figure 1. Nitrogen-Dependent *AMT2;1* Expression in *Arabidopsis* Roots.

Relative expression levels of *AMT2;1* were measured by quantitative RT–PCR, using *UBIQUITIN2* as internal control. Plants were cultivated on agar medium containing no nitrogen (−N) or the indicated concentrations of nitrate (NO_3^-) or ammonium (NH_4^+) for 5 days, after preculture on half-strength MS medium (containing 0.5 mM nitrate as sole N source) for 7 days. Values are means ± SE (n = 3 independent biological replicates). Different letters indicate significant differences among means according to Tukey's test at $P < 0.05$.

Earlier studies on the cell-type-specific localization of *AMT2;1* promoter activity have produced seemingly discrepant results. In one study, *AMT2;1* promoter activity was found to localize mainly in the innermost root tissue (Sohlenkamp et al., 2002), whereas AMT2;1-dependent GFP fluorescence was more pronounced in rhizodermal cells according to Neuhäuser et al. (2009). We speculated that the distinct localization patterns resulted from the use of different promoter fragments and/or different growth conditions, especially with respect to the form and amount of N supplied to plants. Using 1883 bp of the 5′-upstream sequence of *AMT2;1* for fusion with *GFP* allowed *AMT2;1* promoter activity to be traced in the mature zone of roots (Figure 2), while it was absent from root tips regardless of the N treatment (data not shown). Under N deficiency, *AMT2;1*-driven GFP expression was most pronounced in cortical cells, although it was also detected in the other cell types, including the epidermis (Figure 2A–2C). When nitrate was supplied to plants as the sole N source, *AMT2;1* promoter activity shifted slightly toward the endodermis, becoming almost undetectable in epidermal cells (Figure 2D–2F). The supply of only ammonium, on the other hand, caused *AMT2;1* expression to become more confined to endodermal and especially to pericycle cells (Figure 2G–2I). The treatment of plants with ammonium also resulted in the disappearance of *AMT2;1* promoter activity in epidermal cells. Altogether, these results indicate that *AMT2;1* promoter activity strongly depends on the form of N supply, with ammonium triggering localized expression of *AMT2;1* toward the pericycle.

Involvement of AMT2;1 in Ammonium Uptake in Roots

Earlier studies expressing AMT2;1 in yeast have proposed that this protein is impermeable to the toxic ammonium analog methylammonium (MeA) (Sohlenkamp et al., 2000, 2002). However, when the uptake of MeA was assessed at more alkaline external pH, a significant increase of ^{14}C-labeled MeA in AMT2;1-expressing yeast cells was recorded (Neuhäuser et al., 2009). Here, we grew the *amt1;1 amt1;2 amt1;3 amt2;1* quadruple knockout line (*qko*) on MeA together with the *amt1;1 amt1;2 amt1;3* triple knockout line (*qko+21*), in which *AMT2;1* is expressed in the absence of the three major high-affinity ammonium transporters (Yuan et al., 2007). Shoot biomass production was more strongly repressed by the presence of 50 mM MeA at pH 5.5 in *qko+21* plants relative to *qko* (Figure 3A and 3B). At higher MeA concentrations or at high pH, this difference was not observed (Figure 3A–3C).

Although AMT2;1 is able to mediate ammonium transport when expressed in yeast or *Xenopus laevis* oocytes (Sohlenkamp et al., 2000, 2002; Neuhäuser et al., 2009), this transporter does not contribute significantly to high-affinity ammonium uptake in roots (Yuan et al., 2007). In order to further investigate the role of AMT2;1 in roots, we assessed the contribution of this transporter to ammonium influx in roots of N-deficient plants in which *AMT2;1* expression was highest (Figure 1). At 0.2 and 0.5 mM external ammonium, short-term influx of ^{15}N-labeled NH_4^+ in *qko+21* was not significantly higher than that of *qko* (Figure 3D). However, when 1 mM ammonium was supplied, AMT2;1 conferred about 40% higher ammonium influx, while at 2 mM ammonium, this effect was reduced to 15%. Altogether, these results indicate that AMT2;1 slightly but significantly increases the root ammonium uptake capacity in the millimolar concentration range.

Involvement of AMT2;1 in Root-To-Shoot Translocation of Ammonium

The effect of ammonium on the transcriptional regulation and localization of *AMT2;1* suggested that this transporter may play a role in long-distance ammonium transport under ammonium supply. To test this hypothesis, we first compared ^{15}N accumulation in roots and shoots of plants co-expressing AMT2;1 together with either AMT1;3 or AMT1;2 in the *qko* background (Figure 4). In these experiments, ^{15}N-labeled NH_4^+ was supplied for 1 h to allow sufficient time for root-to-shoot translocation. At 200 μM external ammonium supply, AMT2;1 increased ^{15}N accumulation in roots by approx. 20% only in the presence of AMT1;3 but not AMT1;2 (Figure 4A). This went along with a 36% increase in ^{15}N accumulation in shoots of *qko+13+21* relative to *qko+13*, while the contribution of AMT2;1 was not significant in the *qko+12* background (Figure 4B). When plants were exposed to 4 mM external ammonium, ^{15}N accumulation in roots increased to much higher levels without showing any effect of AMT2;1 in either genetic background (Figure 4C). However, co-expression of AMT2;1 in *qko+13* or in *qko+12* insertion lines resulted in a 32% or 25% higher enrichment of ^{15}N in shoots, respectively (Figure 4D). These results suggested that at high supply, AMT2;1 facilitates ammonium translocation irrespective of whether it has been radially transported via the apoplastic or symplastic route.

To more directly assess the involvement of AMT2;1 in long-distance transport of ammonium, we collected xylem sap from *qko* and *qko+21* plants after their transfer to 10 mM ammonium or nitrate as the sole N source. Under these conditions, AMT2;1 should be more strongly expressed in inner root cells and at a higher level in the ammonium pre-treatment (Figures 1 and 2). In plants pre-cultured with nitrate, short-term ^{15}N-ammonium influx

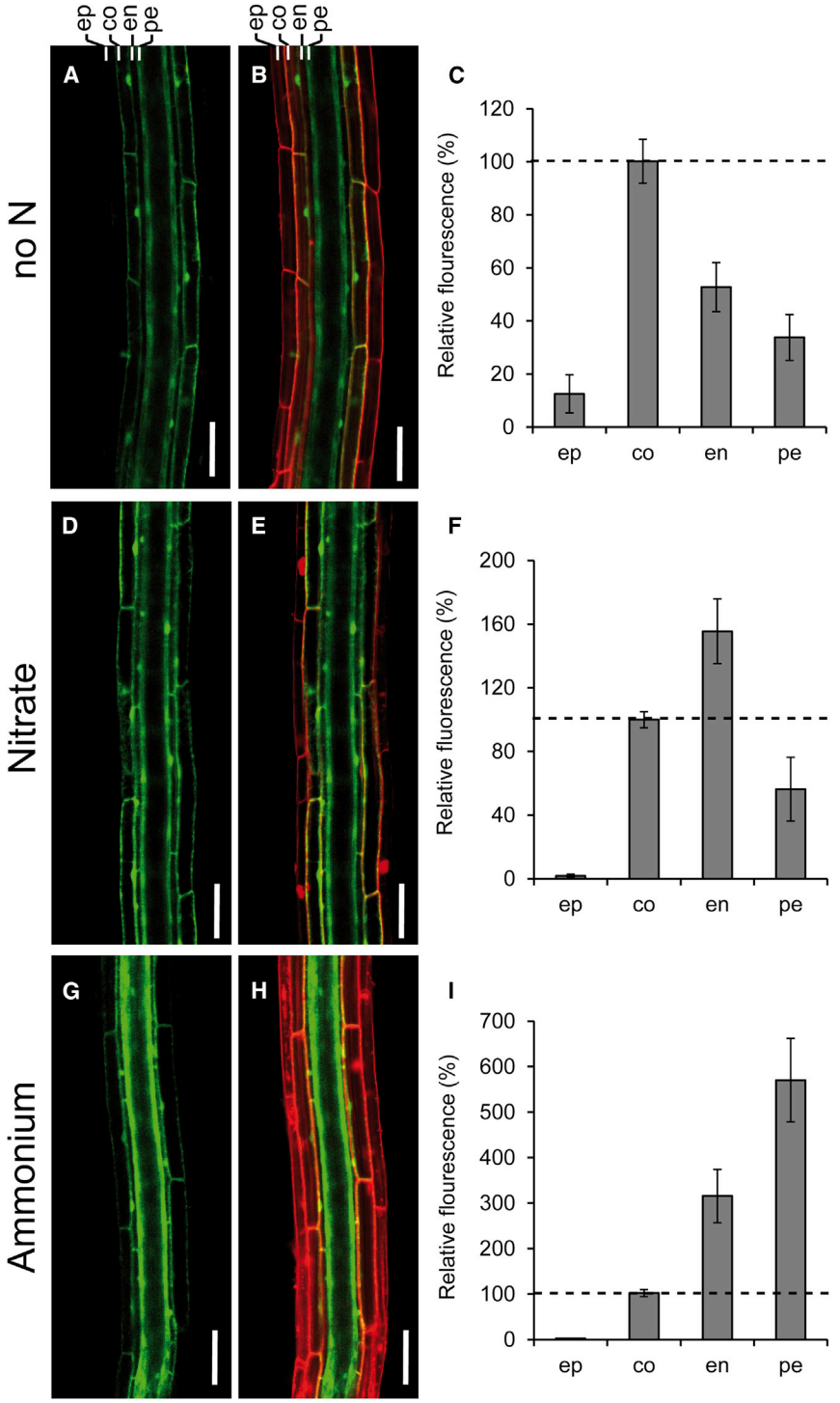

Figure 2. Localization of *AMT2;1* Promoter Activity Depends on the Form of Nitrogen Supply.

Transgenic plants expressing *proAMT2;1-GFP* were pre-cultured on half-strength MS medium with 2 mM nitrate as sole N source. After 10 days, plants were transferred to plant culture medium containing no N **(A–C)**, 10 mM nitrate **(D–F)**, or 10 mM ammonium **(G–I)**. GFP-derived fluorescence alone **(A, D, and G)** or in overlay with propidium iodide-dependent red fluorescence **(B, E, and H)**. Images were taken by confocal microscopy 3 days after transplanting. Scale bars, 50 μm. **(C, F, and I)** Quantitative readout of GFP-dependent fluorescence intensity in each individual cell layer was expressed relative to the levels detected in cortical cells (n = 10 roots per treatment, on which the fluorescence was measured in at least four different positions). Values are means ± SD. ep, epidermis; co, cortex; en, endodermis; pe, pericycle.

in roots was not significantly altered in *qko* plants by expression of AMT2;1 (Figure 5A). However, influx increased after short-term ammonium incubation, which was most likely due to the induction by ammonium of AMT1;5 and possibly further low-affinity transporters. Also in these ammonium pre-conditioned plants, there was no contribution of AMT2;1 to ammonium influx. As expected, the supply of ammonium to the nutrient solution led to a marked increase in ammonium concentrations in the xylem sap of both *qko* and *qko+21* plants (Figure 5B). Remarkably, the presence of AMT2;1 in the *qko+21* triple mutant resulted in an approx. 25% increase in ammonium levels in the xylem sap. At the same time, no significant difference in the xylem sap exudation rate was detected between *qko* and *qko+21* plants (Supplemental Figure 1), indicating that the transporter activity per se and not a secondary growth effect was responsible for elevated ammonium loading of the xylem (Figure 5B). As ammonium is largely converted to amino acids in roots (Tobin and Yamaya, 2001) and preferentially translocated in the xylem in the form of glutamine (Lam et al., 1995; Finnemann and Schjoerring, 1999; Sung et al., 2015), we also determined glutamine concentrations. These were strongly promoted by ammonium nutrition and approx. three-fold higher than those of ammonium but not affected by expression of AMT2;1 (Figure 5C). These results indicated that AMT2;1 indeed contributes to elevated ammonium translocation but only in the presence of ammonium in the medium. Although the increased ammonium influx and increased ammonium levels in the xylem sap of *qko+21* plants (Figures 3D and 5B) were not immediately accompanied by phenotypical changes, prolonged exposure to high ammonium suppressed the growth of these plants more severely than that of *qko* (Supplemental Figure 2).

To assess whether AMT2;1 can mediate ammonium efflux, we designed an assay using the ammonium uptake-defective yeast mutant triple-*mep*Δ (Supplemental Figure 3). Yeast cells were cultivated on arginine, which serves as an adequate N source to the triple-*mep*Δ mutant and results in the leakage of ammonium generated by its catabolism inside the cells

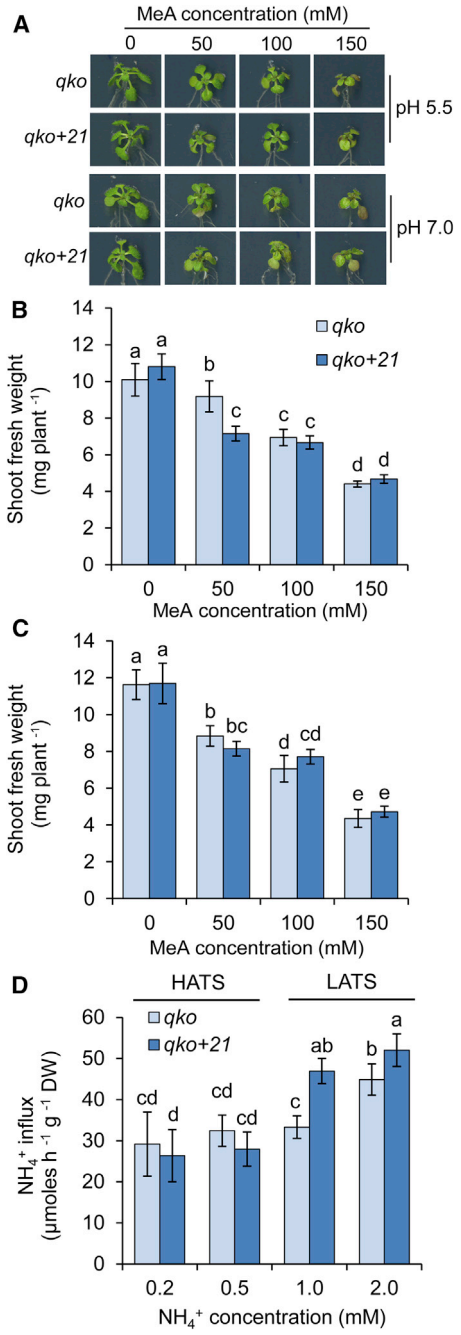

Figure 3. AMT2;1 Contributes to Ammonium Uptake in the Millimolar Concentration Range.

(A) Growth of *qko* and *qko+21* plants supplied with the indicated concentrations of methylammonium (MeA) at pH 5.5 or pH 7.0 for 8 days. The medium contained 1 mM nitrate. Plants were pre-cultured on half-strength MS medium containing 5 mM nitrate as sole nitrogen source for 7 days and exposed to nitrogen deficiency for 2 days before transferring to MeA treatments.

(B and C) Shoot fresh weights of plants grown for 8 days in the presence of the indicated concentrations of MeA at pH 5.5 **(B)** or pH 7.0 **(C)**, as described in **(A)**. Values are means ± SD (n = 4 independent biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

(D) Influx of ^{15}N-labeled ammonium (NH_4^+) into the roots of *qko* and *qko+21* plants. Plants were grown hydroponically for 5 weeks on nitrate and then grown in a nitrogen-free nutrient solution for 4 days. ^{15}N-labeled ammonium was supplied at increasing concentrations for a period of 6 min. Data are represented as means ± SD (n = 8–10 independent biological replicates). Different letters indicate significant differences according to Tukey's test ($P < 0.05$). HATS, high-affinity transport system; LATS, low-affinity transport system.

(Marini et al., 1997). As this mutant strain is not able to retrieve the ammonium lost by leakage, we monitored NH_4^+ concentrations in the external growth solution of triple-*mep*Δ expressing either AMT1;1 or AMT2;1. Whereas all transformants grew similarly in the arginine-containing liquid media (Supplemental Figure 3A), NH_4^+ concentrations increased gradually in the solution containing cells expressing the empty vector (Supplemental Figure 3B). In contrast, external NH_4^+ levels remained low in the medium containing triple-*mep*Δ expressing AtAMT1;1 or AtAMT2;1. Although not excluding a putative efflux activity of AMT2;1, these results further reinforced that AMT2;1 mediates ammonium import and functions in ammonium retrieval.

To verify the involvement of AMT2;1 in root-to-shoot translocation of ammonium by an alternative approach, we assessed ^{15}N partitioning in two independent *amt2;1* T-DNA insertion lines (Figure 6A and 6B). In these mutant lines, we anticipated that the large ammonium uptake capacity mediated by AMT1;1, AMT1;2, and AMT1;3 should increase the requirement for AMT2;1 in the long-distance transport of ammonium, compared with *qko*, in particular when root concentrations of this N form exceed the assimilation capacity in roots. Therefore, we transferred nitrate-grown plants to 10 mM ^{15}N-labeled ammonium for 2 h. In both *amt2;1-1* (Col-*gl* background) and *amt2;1-2* (Col-0 background), ^{15}N accumulation in roots was comparable and not significantly different from the corresponding wild-type plants (Figure 6C). However, the accumulation of ^{15}N in shoots significantly decreased in *amt2;1-1* as well as in *amt2;1-2* plants (Figure 6D). In order to verify the approach and estimate the proportion of ammonium that contributed to ^{15}N translocation to shoots, we also assessed ^{15}N partitioning in a mutant defective in the expression of *GLN1;2*, which encodes a root-expressed, ammonium-inducible cytosolic glutamine synthetase (Ishiyama et al., 2004). In the *gln1;2-1* mutant, more ^{15}N accumulated in roots, and approx. 50% less ^{15}N was translocated to shoots than in wild-type plants (Figure 6C and 6D). Considering that in roots some glutamine may still have been synthesized via GLN1;1 and GLN1;3, this experiment suggested that only up to 50% of the translocated ^{15}N remained in the form of ammonium and that AMT2;1 conferred 20%–30% of this ammonium translocation capacity to the shoots.

To further investigate the role of AMT2;1 in root ammonium uptake in the presence of all AMT1-type transporters, we assessed short-term ^{15}N-labeled ammonium influx in N-starved wild-type and *amt2;1-1* plants. Although ammonium influx rates in *amt2;1-1* plants were indistinguishable from wild-type over a wide range of ammonium concentrations, they were significantly lower, i.e., by ~23%, when 10 mM ammonium was supplied (Figure 7A). Notably, *amt2;1-1* was not affected in short-term ammonium influx when plants were already pre-conditioned to high ammonium. As our experiments indicated a substantial contribution of AMT2;1 to ammonium translocation only in ammonium-supplied plants, we then compared NH_4^+ concentrations in the xylem sap of wild-type and *amt2;1-1* mutant plants

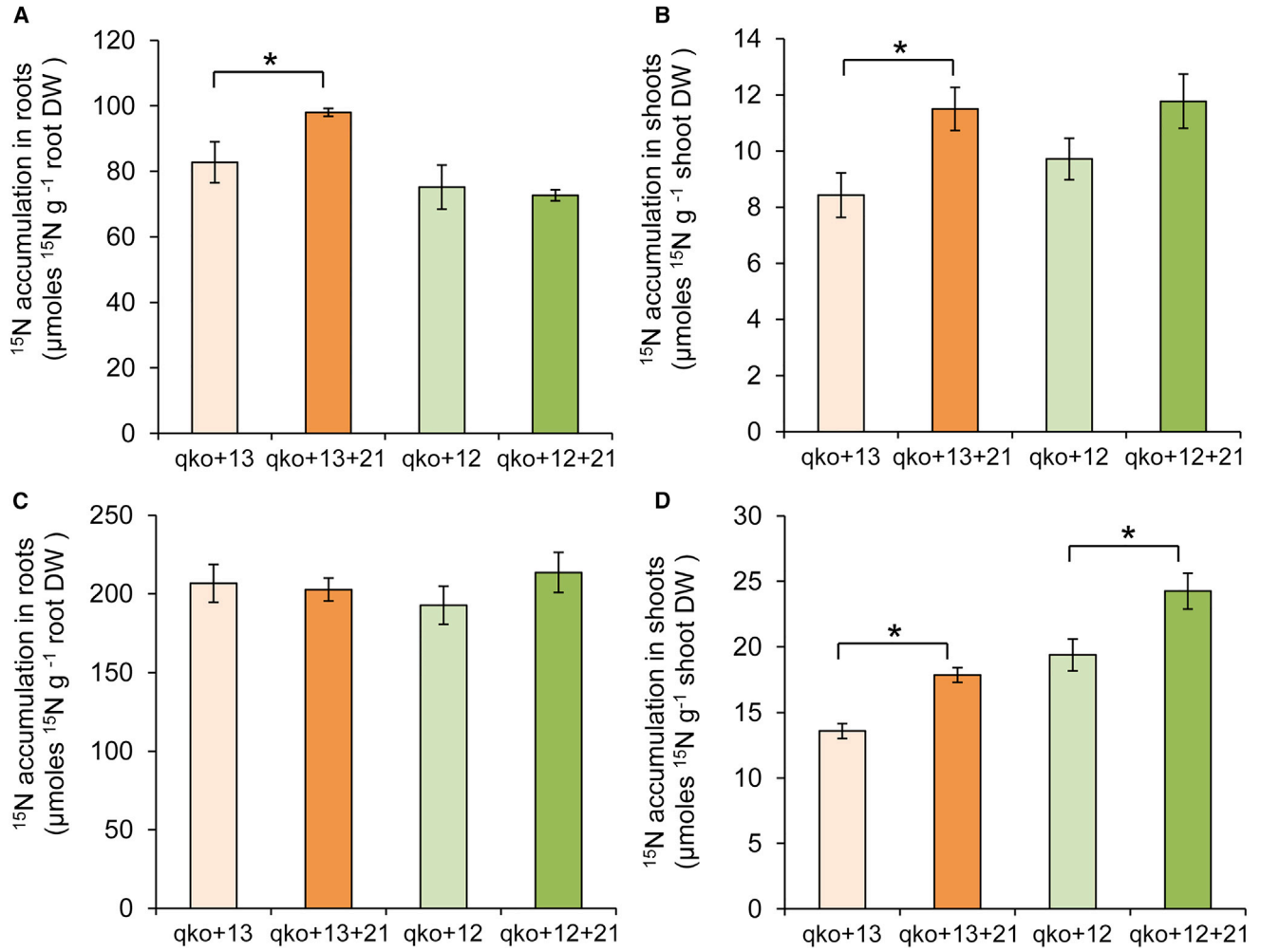

Figure 4. AMT2;1 Contributes to Nitrogen Accumulation in Roots and Shoots.

^{15}N accumulation in roots **(A and C)** and shoots **(B and D)** of *qko* plants expressing AMT1;3 (*qko+13*), AMT1;2 (*qko+12*), or either of them together with AMT2;1 (*qko+13+21* or *qko+12+21*, respectively). Plants were grown hydroponically in a complete nutrient solution containing 2 mM nitrate as sole N form followed by 3 days of nitrogen starvation before transfer to 200 µM **(A and B)** or 4 mM **(C and D)** ^{15}N-labeled ammonium (NH_4^+) for 1 h. Values are means ± SD (n = 4 independent biological replicates). Significant differences at $P < 0.05$ as determined by Student's t-test are indicated by an asterisk.

exposed to 10 mM ammonium for 2 days. In N-deficient plants, NH_4^+ levels in the xylem sap were still in the millimolar range and only tended to be lower in *amt2;1-1* (Figure 7B). However, in ammonium-preconditioned plants, when NH_4^+ concentrations in the xylem sap were four-fold higher, significantly lower concentrations were detected in the xylem sap of *amt2;1-1* plants. This independent observation underscored a significant contribution of AMT2;1 to root-to-shoot translocation of ammonium, and to a smaller extent, to root ammonium uptake.

DISCUSSION

Plants with access to external ammonium as a sole N source have been shown to translocate considerable amounts of ammonium to shoots, although the majority of this N form is usually converted into amino acids already in roots (Finnemann and Schjoerring, 1999; Schjoerring et al., 2002). Despite extensive investigations on the physiological roles of AMT-type transporters in ammonium nutrition, it has remained open whether any of these ammonium transporters might play a role in xylem loading. We show here that AMT2;1 makes a substantial contribution to root-to-shoot translocation of ammonium in particular when plants are exposed to elevated ammonium supplies. Furthermore, in N-deficient roots AMT2;1 can increase ammonium influx at elevated external substrate concentrations. Thus, AMT2;1, which belongs to the MEP-type subfamily of bidirectional ammonium transporters (Soupene et al., 2002), shows a novel physiological feature of AMT-type transporters, as it contributes to ammonium uptake or translocation depending on its cell-type-specific expression in response to the plant nutritional status and local ammonium gradients.

AMT2;1 Mediates Root-To-Shoot Translocation of Ammonium

Based on the observation that preculture with ammonium as a sole N source enhanced *AMT2;1* transcript levels (Figure 1) and confined them mainly to pericycle cells (Figure 2G–2I), the hypothesis was raised that AMT2;1 may be involved in long-distance ammonium translocation under ammonium nutrition.

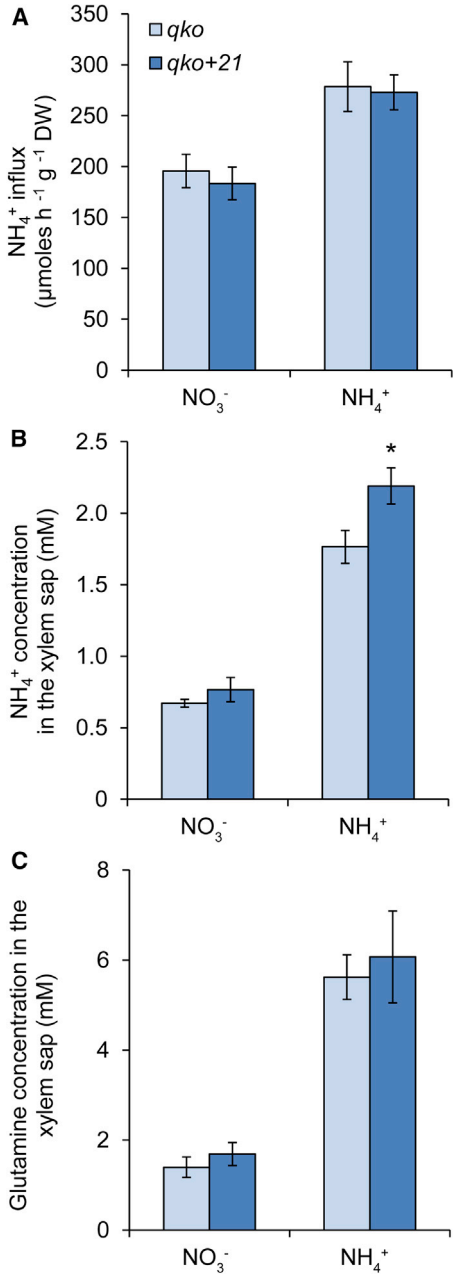

Figure 5. Contribution of AMT2;1 to Xylem Loading.
Short-term ammonium (NH_4^+) influx **(A)** and the concentrations of NH_4^+ **(B)** or glutamine **(C)** in the xylem sap of *qko* and *qko+21* plants. Plants were grown hydroponically in complete nutrient solution containing 3 mM nitrate (NO_3^-) as the sole nitrogen source. After 6 weeks, plants were transferred to 10 mM NO_3^- or 10 mM NH_4^+ for 2 days. Values are means ± SD (n = 10 independent biological replicates for NH_4^+ influx or three independent biological replicates consisting of five plants for NH_4^+ or glutamine concentrations in the xylem sap). *$P < 0.05$, Student's t-test compared with *qko*.

Using the most direct approach to assess ammonium accumulation in the xylem showed indeed that (1) in *amt2;1-1* insertion lines xylem sap concentrations of ammonium were lower than in wild-type plants (Figure 7B), and (2) in an independent genetic approach, the xylem sap of *qko+21* plants contained significantly more ammonium than that of *qko* plants (Figure 5B). This was not the result of different xylem exudation rates (Supplemental Figure 1) or of different ammonium uptake rates (Figures 5A and 7A). As expected, ammonium concentrations in the xylem sap of *qko* and *qko+21* plants were much lower than those in wild-type and *amt2;1-1* plants, not only because of a lower overall uptake capacity for ammonium due to the lacking expression of other AMTs but also because plants were incubated for a shorter period in 10 mM external ammonium. Nevertheless, in both experimental settings, AMT2;1 increased xylem sap concentrations of ammonium by approx. 20%, indicating a considerable robustness of its transport function. This function of AMT2;1 in ammonium translocation strictly depended on the preconditioning of plants to external ammonium, as neither N-deficient plants, which showed highest overall transcript levels of *AMT2;1* (Figure 1), nor nitrate-grown plants, which showed *AMT2;1* promoter activity also in inner root cells (Figure 2D–2F), allowed detection of a significant contribution of AMT2;1 in terminating the radial transport of ammonium toward the xylem (Figures 5 and 7). Previous studies have shown that AMT2;1 is a plasma membrane protein that can mediate high-affinity ammonium transporter when expressed in yeast (Sohlenkamp et al., 2000, 2002). However, different from AMT1-type transporters, which mediate electrogenic NH_4^+ fluxes (Ludewig et al., 2003; Mayer et al., 2006), AMT2;1 mediates electroneutral transport of uncharged NH_3 although AMT2;1 still possesses a high-affinity recruitment site for NH_4^+ (Neuhäuser et al., 2009). This transport mechanism may allow effective substrate binding also at acidic pH, i.e., when NH_3 concentrations are very low, which is in agreement with yeast complementation studies and the proposed import function from the apoplast (Sohlenkamp et al., 2002). However, uncoupling NH_3 from H^+ cotransport likely decreases transport efficiency into an alkaline compartment such as the cytosol and may be responsible for the lower transport velocity reported for AMT2;1 relative to AMT1;1 (Sohlenkamp et al., 2002). On the other hand, non-electrogenic transport of NH_3 likely favors substrate release into an acidic compartment, where co-transported H^+ would impair the transport process. Thus, at least in principle, AMT2;1 could transport its substrate more efficiently from the cytosol into the apoplast than AMT1-type transporters do. However, so far the only evidence that AMT2;1 may exhibit ammonium export activity is the increased tolerance to methylammonium conferred by this protein when expressed in wild-type yeast (Neuhäuser et al., 2009). Our attempt to demonstrate ammonium efflux in yeast rather indicated a role of AMT2;1 in ammonium retrieval (Supplemental Figure 3). Unfortunately, the electroneutral transport of NH_4^+ by AMT2;1 (Neuhäuser et al., 2009) largely limits the possibility to more directly demonstrate a putative efflux function of this transporter by electrophysiological studies. Since there is no experimental evidence disproving the possibility that AMT2;1 mediates ammonium efflux, it still remains open whether or not AMT2;1 may act as a bidirectional ammonium transporter.

Several channels and transporters known to play a major function in root-to-shoot translocation of nutrients are expressed in the plasma membrane of pericycle cells. Examples are the stelar outward-rectifying potassium channel SKOR (Gaymard et al., 1998), the boron exporter BOR1 (Takano et al., 2002), and the nitrate transporter NPF7.3/NRT1.5 (Lin et al., 2008). Thus, in

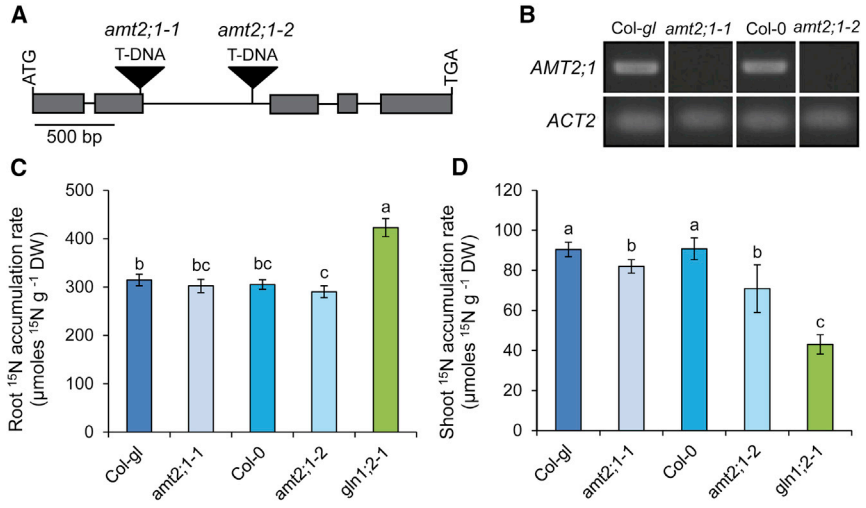

Figure 6. Lower ^{15}N Translocation to Shoots in *amt2;1* Insertion Mutants.

(A) Schematic representation of the exon-intron structure of *AMT2;1* including the T-DNA integration sites in the lines *amt2;1-1* and *amt2;1-2*. Gray boxes represent exons and black lines represent introns.

(B) RT–PCR analysis of *AMT2;1* transcripts in *amt2;1-1*, *amt2;1-2* and in the corresponding wild-types Col-*gl* and Col-0, respectively. Expression of *ACTIN2* (*ACT2*) served as a loading control.

(C and D) ^{15}N concentrations in roots **(C)** and shoots **(D)** of Col-*gl*, *amt2;1-1*, Col-0, *amt2;1-2*, and *gln1;2-1* grown hydroponically with 10 mM ammonium as sole N source for 3 days, after preculture in nutrient solution containing 2 mM KNO_3. Six-week-old plants were transferred to nutrient solution containing 10 mM ^{15}N-labeled ammonium for 2 h before harvest. Values are means ± SD (n = 7–8 independent biological replicates). Different letters indicate significant differences among means according to Tukey's test at $P < 0.05$.

order to reconcile the strong upregulation of *AMT2;1* in the pericycle of ammonium-treated roots (Figure 2G–2I) and the increased AMT2;1-dependent ammonium levels in the xylem sap (Figures 5B and 7B), we propose that AMT2;1 contributes to root-to-shoot ammonium translocation by facilitating the radial transport of this N form toward the vasculature. According to this hypothesis, the ammonium-dependent repositioning of *AMT2;1* expression in the innermost cell layers could help concentrate ammonium specifically in the pericycle cells that are directly adjacent to xylem vessels.

AMT2;1-mediated ammonium accumulation in the xylem sap made a significant contribution to long-distance ammonium translocation from roots to shoots. In two independent *amt2;1* knockout lines, ^{15}N accumulation in shoots was significantly reduced already after 2 h of exposure to ^{15}N-labeled ammonium (Figure 6D). As these plants were pre-cultured with ammonium, we further verified whether AMT2;1-dependent ammonium translocation is confined exclusively to plants exposed to high ammonium supplies and may rather represent a strategy used by plants to cope with an excessive ammonium accumulation in root tissues (Kronzucker et al., 1998). Therefore, plants were pre-cultured under N deficiency before exposure to ^{15}N-labeled ammonium in the high-affinity range. In this case, co-expression of AMT2;1 with AMT1;3 but not with AMT1;2 significantly increased ^{15}N accumulation in roots and shoots (Figure 4A and 4B). Elevated root ^{15}N levels, however, were indicative of a contribution of AMT2;1 to ammonium influx into rather than out of root cells. In contrast, at 4 mM external ^{15}N-labeled ammonium, co-expression of AMT2;1 with any of the two AMT1-type transporters could not further increase ^{15}N levels in roots but could significantly increase ^{15}N levels in shoots (Figure 4C and 4D). This observation clearly indicated a predominant function of AMT2;1 in root-to-shoot translocation of ammonium, which obviously gains in importance at elevated ammonium supplies. Thus, a part of the previously reported dynamic interactions between root influx, long-distance translocation of ammonium, and futile ammonium cycling (Kronzucker et al., 1998; Britto et al., 2001; Loqué and von Wirén, 2004; Coskun et al., 2013) likely goes back to the N-status-dependent and cell-type-specific expression of AMT2-type ammonium transporters.

The Predominant Physiological Function of AMT2;1 Is Determined by Its Nitrogen-Status-Dependent Cell-Type-Specific Expression

Among all root-expressed AMT-type transporters, only AMT2;1 has not yet been implicated with ammonium uptake (Sohlenkamp et al., 2002; Yuan et al., 2007). Here, we re-assessed ammonium uptake by AMT2;1 in the wild-type and *qko* background by supplying increasing concentrations of ^{15}N-labeled ammonium to N-deficient plants, which induces expression of *AMT2;1* predominantly in outer roots cells (Figure 2A–2C). AMT2;1 made a small but significant contribution to net ammonium influx only at millimolar substrate concentrations (Figures 3D and 7A). In addition, we also showed that AMT2;1 can efficiently retrieve ammonium when expressed in the triple-*mep*Δ yeast mutant (Supplemental Figure 3B). These observations support functional expression studies in yeast and oocytes, showing that AMT2;1 is able to mediate cellular ammonium import (Sohlenkamp et al., 2002; Neuhäuser et al., 2009). In wild-type plants, the net contribution of AMT2;1 to ammonium influx was negligible, because the capacity of AMT1 transporters outcompetes AMT2;1 in the micromolar concentration range (Yuan et al., 2007, 2013). In the millimolar range, the small contribution of AMT2;1 to ammonium influx is most likely due the existence of other yet poorly defined low-affinity transporters, such as AMF-type ammonium transporters (Chiasson et al., 2014) or potassium channels (Szczerba et al., 2008; ten Hoopen et al., 2010).

The present study shows that the amount and form of N supply not only regulates *AMT2;1* transcript levels but also modifies the cell-type-specific localization of *AMT2;1* promoter activity (Figures 1 and 2). Although *AMT2;1* expression increased under

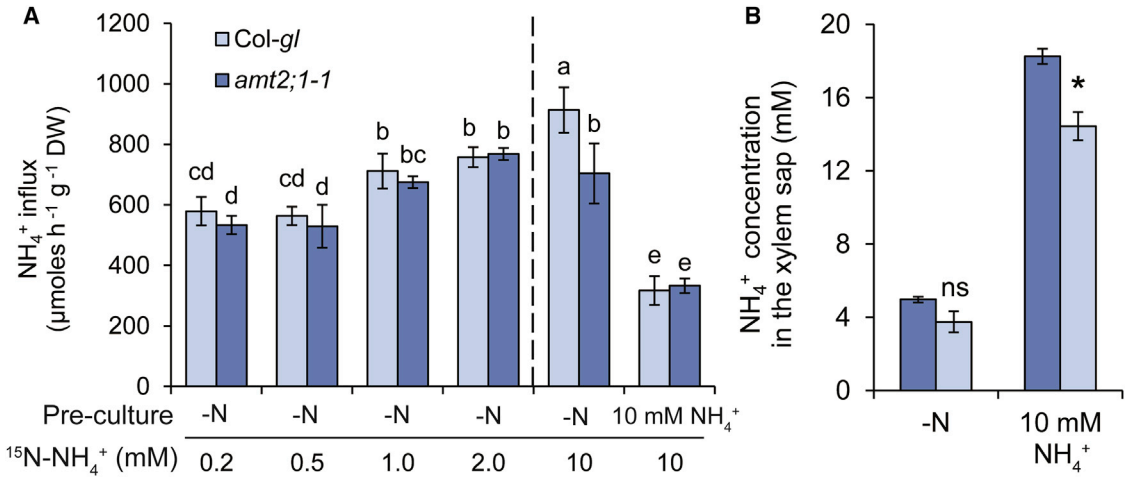

Figure 7. Ammonium Uptake and Loading of the Xylem Are Altered by AMT2;1 According to the Plant N Status.

Short-term ammonium (NH_4^+) influx **(A)** and NH_4^+ concentrations in the xylem sap **(B)** of Col-*gl* and *amt2;1-1* plants, which were cultured hydroponically in nutrient solution containing 2 mM KNO_3^- for 5 weeks before transfer to nutrient solution lacking nitrogen (−N) or containing 10 mM NH_4^+ as the sole N source. After 2 days on treatments, short-term NH_4^+ influx was assessed, and xylem exudates were collected for NH_4^+ analysis. For the influx experiment, ^{15}N-labeled NH_4^+ was supplied at the indicated concentrations. Values are means ± SD (n = 5 and 4 independent biological replicates for NH_4^+ influx and xylem sap analysis, respectively).

In **(A)**, different letters indicate significant differences according to Tukey's test ($P < 0.05$), whereas in **(B)** significant differences to Col-*gl* were determined by Student's t-test (*$P < 0.05$; ns, not significant).

low N compared with nitrate or ammonium (Figure 1), the most conspicuous effect was the ammonium-dependent stimulation of *AMT2;1* promoter activity in pericycle cells (Figure 2). Noteworthy, the dependence of *AMT2;1* localization on the amount and form of N supplied to plants also shed light on seemingly conflicting results reported in previous studies (Sohlenkamp et al., 2002; Neuhäuser et al., 2009). According to our results, differences in localization reported before were mostly related to the contrasting nutrient composition used in these studies, especially regarding N supply. While Sohlenkamp et al. (2002) cultivated plants used for GUS assays in full-strength MS medium, which contains ~20 mM ammonium, the study of Neuhäuser et al. (2009) supplemented *proAMT2;1-GFP* plants with less than 0.2 mM N, a condition that rapidly provokes N deficiency in *Arabidopsis* (Gruber et al., 2013). Notably, we found evidence that the shift in cell-type-specific localization of *AMT2;1* is associated with different physiological functions. When N starvation enhances expression in the outermost cells (Figure 2A–2C), AMT2;1 contributes to ammonium uptake, as long as high levels of this N form are supplied to plants (Figures 3D and 7A). In plants pre-conditioned to high ammonium, the increased expression of *AMT2;1* in endodermal and pericycle cells (Figure 2G–2I) is associated with changes in ammonium levels in the xylem sap (Figures 5B and 7B) but not in ammonium uptake (Figures 5A and 7A).

Interplay between Root Ammonium Assimilation and Translocation

Upon high external supply, excessive uptake of ammonium can result in ammonium toxicity, if this N form is not quickly assimilated or stored in vacuoles (Li et al., 2014). The genes that encode the cytosolic isoform of glutamine synthetase (i.e., GS1), which is the major GS isoform in roots, are differentially responsive to ammonium availability (Ishiyama et al., 2004). Among them, *GLN1;2* is upregulated in *Arabidopsis* roots a few hours after exposing plants to elevated ammonium supply, and expression is mainly confined to pericycle cells along the root axis (Ishiyama et al., 2004). Most GS1 activity detected in ammonium-treated roots is related to GLN1;2, as this was the only GLN1 isoenzyme markedly induced by ammonium (Ishiyama et al., 2004; Lothier et al., 2011). Thus, a large proportion of the ammonium taken up or produced by nitrate reduction is directly assimilated in roots as long as carbon skeletons and NADH (for NADH-GOGAT) are not limiting. The predominant expression of *GLN1;2* in the vasculature at high external ammonium supply suggests that the conversion of ammonium to glutamine takes place mainly in the root vasculature, where this amino acid can be immediately transferred to xylem vessels. In line with this assumption, we observed that glutamine levels strongly increased in xylem sap upon ammonium nutrition (Figure 5C). In addition, supply of 10 mM ^{15}N-labeled ammonium to *gln1;2* resulted in a 52% reduction in shoot ^{15}N compared with wild-type plants, whereas ^{15}N concentration in roots raised to significantly higher levels in *gln1;2* (Figure 6C–6D). These results indicated that only part of the overall ammonium taken up at high external supply can be destined to aerial parts when root ammonium assimilation is impaired.

Glutamine is the major N form translocated in the xylem of ammonium-fed oilseed rape (Finnemann and Schjoerring, 1999) and the major amino acid found in the xylem sap of *Arabidopsis thaliana* (Lam et al., 1995). When the ammonium concentration in roots was increased by supplying high levels of ammonium, ~2.0 mM of NH_4^+ were detected in the xylem sap of plants lacking the major high-affinity ammonium transporters (Figure 5B) and up to 18 mM in wild-type plants (Figure 7B). In oilseed rape grown on ammonium, as much as 11% of the total N translocated in xylem sap was in the form of ammonium

(Finnemann and Schjoerring, 1999). In the same study, it was also observed that GS activity in roots was repressed in response to high N availability, while the translocation of ammonium to shoots was enhanced. The repression of GS could be associated with carbon limitation and might be important to protect the root against excessive drainage of photoassimilates. On the other hand, enhanced translocation of ammonium could ensure a steady supply of N to the shoots also under such growth conditions (Finnemann and Schjoerring, 1999). Our results and those reported by Ishiyama et al. (2004) suggest that the coordination between ammonium-induced assimilation and translocation is at least in part mediated by GLN1;2 and AMT2;1 and occurs predominantly in the pericycle of roots.

METHODS

Plant Materials and Growth Conditions

The *amt2;1-1* insertion line, which is in a Col-*gl* background, was isolated from the enhancer trap collection of Thomas Jack (Campisi et al., 1999) as described previously (Yuan et al., 2007). The homozygous lines *amt2;1-2* (SALK_119678C) and *gln1;2-1* (SALK_145235C), which are in a Col-0 background, were acquired from the SALK collection. Disruption of *AMT2;1* expression in the *amt2;1* insertion lines was confirmed by qualitative RT–PCR using the expression of *ACT2* as loading control. For this analysis, the following primers were used: AMT2;1-RT-For: 5′-CGG GAA AGA TAG AAT AAC AAA ATG G-3′; AMT2;1-RT-Rev: 5′-ATT GCT CCG ATG ACA GAA GG-3′; ACT2-RT-For: 5′-GAC CTT GCT GGA CGT GAC CTT AC-3′; ACT2-RT-Rev: 5′-GTA GTC AAC AGC AAC AAA GGA GAG C-3′.

Generation and selection of *qko*, *qko+12* (*qko+AMT1;2*), *qko+13* (*qko+AMT1;3*) and *qko+21* (*qko+AMT2;1*) were described previously (Yuan et al., 2007). The double recomplemented lines *qko+12+21* (*qko+AMT1;2+AMT2;1*) and *qko+13+21* (*qko+AMT1;3+AMT2;1*) were obtained by backcrossing *qko+21* to Col-0 followed by segregation analysis in the F2 population.

In experiments carried out in agar plates, *Arabidopsis* seeds were surface sterilized and sown onto modified half-strength MS medium containing 5 mM nitrate as sole N source and solidified with Difco agar. After 7 days of preculture, seedlings were transferred to vertical plates containing half-strength MS medium supplemented with different N forms at indicated concentrations. Plants were grown under axenic conditions in a growth cabinet under the following regime: 10/14 h light/dark; light intensity 120 μmol m^{-2} s^{-1}; temperature 22°C/18°C. For hydroponic culture, *Arabidopsis* seeds were pre-cultured on rock wool moistened with tap water. After 1 week, tap water was replaced by nutrient solution containing 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 μM K_2SO_4, 250 μM $CaCl_2$, 100 μM Na-Fe-EDTA, 50 μM KCl, 50 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, and 1 μM $NaMoO_4$ (pH adjusted to 6.0 by KOH). Unless indicated otherwise, 2 mM KNO_3 was supplied to provide N-sufficient conditions. During the first 3 weeks, the nutrient solution was replaced once a week, twice a week in the fourth week, and every 2 days in the following weeks. Plants were grown hydroponically in a growth chamber under the above-mentioned conditions except that the light intensity was 280 μmol photons m^{-2} s^{-1}.

Localization of *AMT2;1* Promoter Activity

For the *proAMT2;1-GFP* construct, the primers *2;1-F-Sal*I (5′-CGT CGA CAT TAT ATT TAA GAA TGA GAC AAA TTC TA-3′) and *2;1-R-BamH*I (5′-GGG ATC CTT TGT TAT TCT ATC TTT CCC GGA GTT GA-3′) were used to amplify the 1883-bp 5′-upstream genomic sequence of *AMT2;1* before ligation with EGFP and nopaline synthase terminator sequences using the *Sal*I and *BamH*I sites of pBI101 (Clontech, Palo Alto, CA, USA). *Arabidopsis* plants were transformed using the GV3101 (pMP90) strain of *Agrobacterium tumefaciens* according to the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on agar media with half-strength MS supplemented with 0.5% (w/v) sucrose and 50 mg l^{-1} kanamycin sulfate. At least six independent T2 lines were assessed, and the results of one representative line are shown.

An LSM 510 Meta (Carl Zeiss MicroImaging GmbH) laser scanning confocal microscope was used for the analysis of *proAMT2;1-GFP* plants. Roots were stained with propidium iodide (10 μg ml^{-1}) for 10 min. GFP-dependent fluorescence was assessed by excitation at 488 nm with an argon laser and 505- to 530-nm band-pass filter. Propidium iodide-derived fluorescence was imaged under 488 nm excitation and by filtering the emitted light at 458–514 nm. The Zeiss LSM 510 software version 3.0 was used for image recording and fluorescence quantification. All confocal sections across samples were recorded with the same microscope settings.

Real-Time Quantitative PCR

Total RNA was extracted using the QIAzol Lysis reagent (QIAGEN) following the manufacturer's instructions. Prior to cDNA synthesis, samples were treated with DNase (Thermo Fisher Scientific). Reverse transcription was performed using SuperScript II (Thermo Fisher Scientific) reverse transcriptase and $Oligo(dT)_{12\text{-}18}$. Real-time PCR was performed using a Mastercycler ep realplex (Eppendorf) and QuantiTect SYBR Green qPCR mix (QIAGEN). The following gene-specific primer pairs were used: AMT2;1_for, 5′-TAT GCT CTT TGG GGA GAT GG-3′; AMT2;1_rev, 5′-TGA CAC CTC TAG CAC CAT GAA C-3′; UBQ2_for, 5′-CCA AGA TCC AGG ACA AAG AAG GA-3′; UBQ2_rev, 5′-TGG AGA GCA TAA CAC TTG C-3′. Primer specificity was confirmed by analysis of the melting curves and agarose gel electrophoresis of the PCR products. Relative expression levels were calculated according to Pfaffl (2001).

^{15}N Uptake and Accumulation

To assess the contribution of AMT2;1 to short-term ammonium uptake, roots of N-deficient plants were rinsed in 1 mM $CaSO_4$ solution for 1 min and then transferred to nutrient solution containing different concentrations of ^{15}N-labeled NH_4^+ (95 atom % ^{15}N) as the sole N source. After 6 min incubation in uptake solution, roots were washed with 1 mM $CaSO_4$ to remove apoplastic ^{15}N and stored at −80°C before freeze drying. Root and shoot ^{15}N accumulation was assessed by incubating N-starved plants in a nutrition solution containing different concentrations of ^{15}N-labeled NH_4^+ for 1 h. Roots were rinsed in 1 mM $CaSO_4$ for 1 min before and after exposure to ^{15}N-labeling solution. Shoots and roots were harvested separately. ^{15}N concentration was determined by isotope ratio mass spectrometry (Horizon, NU Instruments).

Collection of Xylem Sap and Ammonium Measurements

Xylem sap was collected by excision of the shoots below the rosette with a sharp razor blade. Exudating sap was sampled over a period of 60 min in a mounted silicon tube with an internal diameter of 1.0 or 1.5 mm and a wall thickness of 1.0 or 0.75 mm, respectively. Subsequently, xylem exudates of five plants grown in one pot were pooled in one microcentrifuge tube, giving one replicate. The tube contained 400 μl of ice-cold 20 mM HCOOH (xylem sap:HCOOH volume ratio of about 1:1) in order to stabilize the sample and thus prevent the degradation of amino acids and other labile N metabolites to ammonium during extraction and analysis, as described by Husted et al. (2000). Finally, the volume of the stabilized xylem exudate samples was determined and the samples stored at −20°C until analysis.

Ammonium concentrations in stabilized xylem sap samples were determined with an HPLC-system by derivatization with *o*-phthalaldehyde (OPA) and detection with fluorescence spectroscopy at neutral pH as described by Husted et al. (2000). The HPLC pump was used to continuously pump the carrier stream through the system at a flow rate of 0.8 ml min^{-1}. The carrier consisted of 3 mM OPA, 10 mM β-mercaptoethanol as the reducing agent, and 100 mM phosphate

buffer adjusted to pH 6.8. The samples were then injected into the carrier stream, which entered the reaction coil in the column oven, where they were heated to 80°C. At this temperature, ammonium reacts with OPA to form an alkylthioisoindole fluorochrome. This fluorochrome was detected at an excitation wavelength of 410 nm and an emission wavelength of 470 nm using a fluorescence spectrophotometer (F2000 Hitachi, Tokyo, Japan).

Statistical Analysis

All statistical analysis was performed using SigmaPlot 11.0. Comparisons of sample means were performed either by Student's *t*-test ($P < 0.05$) or one-way analysis of variance ($P < 0.05$) followed by Tukey's post-hoc multiple comparisons tests, as indicated in the legends of each figure.

ACCESSION NUMBERS

The Arabidopsis Genome Initiative identifiers for the genes described in this article are as follows: *AMT2;1* (AT2G38290), *AMT1;2* (AT1G64780), *AMT1;3* (AT3G24300), *GLN1;2* (AT1G66200), *UBQ2* (AT2G36170) and *ACT2* (AT3G18780).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.F.H.G, L.Y., and N.v.W.; Investigation, R.F.H.G., A.M.L., F.D., and L.Y.; Resources, D.R.; Writing – Original Draft, R.F.H.G and N.v.W.; Writing – Review and Editing, R.F.H.G and N.v.W.

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