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 sub-family, 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 15N-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 15N 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


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 NH4+ and NH3 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...
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 (NO₃⁻), 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 receptor 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 ¹⁵N-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 (NH₄⁺) at the vestibule of the external pore to allow for de-protonation and subsequent transport of the uncharged ammonia (NH₃) 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 NH₄NO₃) 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).
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 15N-labeled NH₄⁺ 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 15N 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, 15N-labeled NH₄⁺ was supplied for 1 h to allow sufficient time for root-to-shoot translocation. At 200 μM external ammonium supply, AMT2;1 increased 15N 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 15N 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, 15N 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 15N 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 longitudinal 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 15N-ammonium influx...
The 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-mepD (Supplemental Figure 3). Yeast cells were cultured on arginine, which serves as an adequate N source to the triple-mepD mutant and results in the leakage of ammonium generated by its catabolism inside the cells.
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$_D$ 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$_D$ 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-0 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 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 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.
exposed to 10 mM ammonium for 2 days. In N-deficient plants, 
\( \text{NH}_4^+ \) levels in the xylem sap were still in the millimolar range
and only tended to be lower in \( \text{amt2;1-1} \) (Figure 7B). However,
in ammonium-preconditioned plants, when \( \text{NH}_4^+ \) concentrations
in the xylem sap were four-fold higher, significantly lower concen-
trations were detected in the xylem sap of \( \text{amt2;1-1} \) 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.
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 ammonium influx (*Ludewig et al., 2003; Mayer et al., 2006*), *AMT2;1* mediates electroneutral transport of uncharged ammonium, although *AMT2;1* still possesses a high-affinity recruitment site for ammonium (*Neuhäuser et al., 2009*). This transport mechanism may allow effective substrate binding also at acidic pH, i.e., when NH₃ 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 NH₃ 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 NH₃ 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 NH₄⁺ 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...
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, 15N accumulation in shoots was significantly reduced already after 2 h of exposure to 15N-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 15N-labeled ammonium (Figure 7A). In addition, we also showed that AMT2;1 can efficiently retrieve ammonium when expressed in the triple-mep1 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; Neuhausser 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...
ammonium 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 15N-labeled ammonium to gln1;2 resulted in a 52% reduction in shoot 15N compared with wild-type plants, whereas 15N 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 NH4+ 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.
Molecular Plant

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

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

To assess the contribution of AMT2;1 to short-term ammonium uptake, roots of N-deficient plants were rinsed in 1 mM CaSO₄ solution for 1 min and then transferred to nutrient solution containing different concentrations of ¹⁵N-labeled NH₄⁺ (95 atom % ¹⁵N) as the sole N source. After 6 min incubation in uptake solution, roots were washed with 1 mM CaSO₄ to remove apoplastic ¹⁵N and stored at −80°C before freeze drying. Root and shoot ¹⁵N accumulation was assessed by incubating N-starved plants in a nutrition solution containing different concentrations of ¹⁵N-labeled NH₄⁺ for 1 h. Roots were rinsed in 1 mM CaSO₄ for 1 min before and after exposure to ¹⁵N-labeling solution. Shoots and roots were harvested separately. ¹⁵N 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, given 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 (pH 7.0).
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 alkyloxyisooindole fluorescence. 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.

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