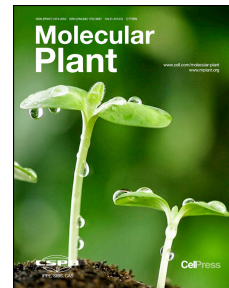


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A critical role of AMT2;1 in root-to-shoot translocation of ammonium in *Arabidopsis*

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1 **A critical role of AMT2;1 in root-to-shoot translocation of**
2 **ammonium in *Arabidopsis***

3

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15

16 **Short title:** Root-to-shoot translocation of ammonium by AMT2;1

17

18 **Short Summary:**

19 The physiological function of AMT2;1, the sole MEP-type ammonium transporter in
20 *Arabidopsis thaliana*, has remained elusive. In this study we demonstrate that
21 AMT2;1 is involved in root-to-shoot translocation of ammonium and, to a minor
22 extent, ammonium uptake depending on the regulation of its cell type-specific
23 expression by the plant nutritional status and local ammonium gradients.

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26

27 **ABSTRACT**

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

46

47 **Key words:** nitrogen uptake, nitrogen translocation, ammonium assimilation, xylem
48 loading, ammonia transport, ammonium influx, glutamine synthetase

49

50 **INTRODUCTION**

51 A critical aspect during plant growth and development is the plant's ability to
52 efficiently meet the nutritional demand of aerial tissues via the long-distance delivery
53 of nutrients taken up by the roots. In roots the amount of nutrients that is destined for
54 far-located tissues is determined by the activity of transporters that load the xylem
55 vessels, while in shoots transporters unloading the xylem can increase overall root-
56 to-shoot translocation (Chen et al., 2012; Gaymard et al., 1998; Hamburger et al.,
57 2002; Li et al., 2010; Lin et al., 2008). In the case of nitrogen (N), root-to-shoot
58 allocation of different N forms is affected by a range of factors, such as the form and
59 the amount of N available in the soil, the assimilation capacity of roots and shoots
60 and the growth conditions that affect the availability of carbon skeletons and reducing
61 equivalents in roots (Smirnoff and Stewart, 1985). Whereas in most annual plants a
62 significant proportion of nitrate taken up in roots is translocated to aerial parts, it has
63 been previously assumed that ammonium¹, either taken up directly from the external
64 solution or generated by nitrate reduction in roots, is almost exclusively assimilated in
65 roots (Kafkafi and Ganmore-Neumann, 1997; van Beusichem et al., 1988). However,
66 since ammonium assimilation in roots requires large amounts of carbon skeletons
67 and reducing equivalents, it is conceivable that plants with limited root assimilatory
68 capacity or conditions that reduce the allocation of carbon skeletons to roots may
69 stimulate ammonium loading of the xylem to prevent the deleterious effects of its
70 over-accumulation in roots. In this regard, it has turned out that early attempts to
71 assess ammonium concentrations in plant samples produced often confounding
72 results due to the lack of appropriate analytical methods that could guarantee sample
73 stability as well as sensitivity and selectivity during ammonium detection (Schjoerring
74 et al., 2002). The establishment of improved methods for ammonium detection in
75 small volumes has revealed that significant amounts of ammonium are present in the
76 xylem sap of various plant species (Finnemann and Schjoerring, 1999; Husted et al.,
77 2000; Schjoerring et al., 2002). In xylem exudates of *Arabidopsis*, ammonium
78 concentrations mounted up to > 4 mM (Yuan et al., 2007), while in ammonium-fed
79 oilseed rape these levels reached up to 8 mM, representing 11% of the total N found
80 in the xylem sap (Finnemann and Schjoerring, 1999). The detection of ammonium
81 concentrations in the millimolar range in root apoplasts (Yuan et al., 2007), further

¹ The term ammonium is used whenever the chemical form remains undefined, while NH_4^+ and NH_3 refer to the defined molecular species.

82 suggests that ammonium transporters may be required for xylem loading. However,
83 the molecular mechanism involved in root-to-shoot translocation of ammonium has
84 remained unknown.

85 With regard to nitrate (NO_3^-), so far three members of the NPF (NRT1/PTR Family)
86 family of nitrate/peptide transporters have been implicated in the control of root-to-
87 shoot translocation of nitrate. Whereas NPF7.3/NRT1.5 mediates nitrate efflux into
88 the xylem vessels (Lin et al., 2008), the nitrate influx transporters NPF7.2/NRT1.8
89 and, to some extent, NPF2.9/NRT1.9 retrieve nitrate from the xylem sap (Li et al.,
90 2010; Wang and Tsay, 2011). In more mature parts of roots, where the dual-affinity
91 nitrate transporter NPF6.3/NRT1.1 is expressed in the central cylinder (Remans et
92 al., 2006), evidence provided by the transport activity of this protein in a heterologous
93 system and by *in planta* ^{15}N -nitrate translocation indicated that NPF6.3/NRT1.1 is
94 also involved in root-to-shoot translocation of nitrate (Leran et al., 2013). Moreover,
95 some of these transporters appear to cooperate with other transporters in order to
96 maintain the cation-anion balance in the xylem sap. For instance, NPF7.3/NRT1.5 is
97 not only involved in xylem loading of nitrate but also in potassium translocation
98 (Drechsler et al., 2015).

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

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

128 Previous studies have further revealed that the AMT2;1 protein localizes at the
129 plasma membrane (Neuhäuser et al., 2009; Sohlenkamp et al., 2002). However,
130 seemingly discrepant results have been reported regarding the tissue-specific
131 localization of AMT2;1. In full-strength Murashige and Skoog medium, which contains
132 ~40 mM nitrate and ~20 mM ammonium (Murashige and Skoog, 1962), GUS activity
133 driven by 1.0 kb of the *AtAMT2;1* promoter has been detected mainly in the vascular
134 tissue of roots, stems, leaves and flowers (Sohlenkamp et al., 2002). Interestingly,
135 when AMT2;1 localization was assessed in transgenic lines expressing a longer
136 sequence of the *AMT2;1* promoter (i.e. 1.7 kb), *AMT2;1*-dependent GFP expression
137 under low N supply (0 to 200 μ M NH_4NO_3) was confined to rhizodermal cells,
138 including root hairs, and was very weak in inner root tissues (Neuhäuser et al., 2009).
139 The reason for this discrepancy in cell type-specific localization and the consequence
140 for the physiological function of AMT2;1 still remain elusive.

141 In the present work, we re-assessed the function of AMT2;1 by employing single
142 insertion mutants defective in *AMT2;1* expression as well as double, triple and
143 quadruple *amt* knockout lines. These lines were employed to determine ammonium
144 uptake and translocation capacities. Together with tissue localization of *AMT2;1*
145 expression in response to different N conditions our results provide compelling

146 evidence that *AMT2;1* is involved in root-to-shoot translocation of ammonium, and to
147 a minor extent, in ammonium uptake at elevated external substrate concentrations.

148

149 RESULTS

150 Regulation of *AMT2;1* expression and localization by nitrogen

151 To assess how *AMT2;1* expression is regulated by N supply, transcript levels were
152 determined in roots after exposure to different N forms. Relative to growth in nitrate,
153 transcript levels of *AMT2;1* were more than two-fold higher when plants were grown
154 in the absence of N for 5 days (Figure 1). In the presence of ammonium as the sole N
155 source, *AMT2;1* mRNA levels increased only by about 50% irrespective of whether 1
156 or 10 mM ammonium were supplied. By contrast, when nitrate supply increased from
157 1 to 10 mM, *AMT2;1* transcript levels further dropped. These observations suggested
158 that not only the plant N status but also the supply of different N forms exert a
159 regulatory effect on the expression of this gene (Figure 1).

160 Earlier studies on the cell-type specific localization of *AMT2;1* promoter activity have
161 produced seemingly discrepant results, as in one study *AMT2;1* promoter activity was
162 found to localize mainly in the innermost root tissue (Sohlenkamp et al., 2002),
163 whereas *AMT2;1*-dependent GFP fluorescence was more pronounced in rhizodermal
164 cells according to Neuhäuser et al. (2009). We speculated that the distinct
165 localization patterns resulted from the use of different promoter fragments and/or
166 different growth conditions, especially with respect to the form and amount of N
167 supplied to plants. Using 1883 bp of the 5'-upstream sequence of *AMT2;1* for fusion
168 with *GFP* allowed tracing *AMT2;1* promoter activity in the mature zone of roots
169 (Figure 2) while it was absent from root tips regardless of the N treatment (data not
170 shown). Under N deficiency, *AMT2;1*-driven GFP expression was most pronounced
171 in cortical cells, although being detectable also in the other cell types, including the
172 epidermis (Figure 2A-2C). When nitrate was supplied to plants as the sole N source,
173 *AMT2;1* promoter activity shifted slightly towards the endodermis, becoming almost
174 undetectable in epidermal cells (Figure 2D-2F). The supply of only ammonium, on the
175 other hand, caused *AMT2;1* expression to become more confined to endodermal and
176 especially to pericycle cells (Figure 2G-2I). The treatment of plants with ammonium
177 also resulted in the disappearance of *AMT2;1* promoter activity in epidermal cells.

178 Altogether, these results indicate that promoter activity of *AMT2;1* strongly depends
179 on the form of N supply, with ammonium triggering localized expression of *AMT2;1*
180 towards the pericycle.

181

182 **Involvement of *AMT2;1* in ammonium uptake in roots**

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

195 Although *AMT2;1* is able to mediate ammonium transport when expressed in yeast or
196 *Xenopus laevis* oocytes (Neuhäuser et al., 2009; Sohlenkamp et al., 2000;
197 Sohlenkamp et al., 2002), this transporter does not contribute significantly to high-
198 affinity ammonium uptake in roots (Yuan et al., 2007). In order to further investigate
199 the role of *AMT2;1* in roots, we assessed the contribution of this transporter to
200 ammonium influx in roots of N-deficient plants in which *AMT2;1* expression was
201 highest (Figure 1). At 0.2 and 0.5 mM external ammonium, short-term influx of ¹⁵N-
202 labeled NH₄⁺ in *qko+21* was not significantly higher than that of *qko* (Figure 3D).
203 However, when 1 mM ammonium was supplied, *AMT2;1* conferred about 40% higher
204 ammonium influx, while at 2 mM ammonium this effect was reduced to 15%.
205 Altogether, these results indicated that *AMT2;1* slightly but significantly increases the
206 root ammonium uptake capacity in the millimolar concentration range.

207

208 **Involvement of *AMT2;1* in root-to-shoot translocation of ammonium**

209 The effect of ammonium on the transcriptional regulation and localization of *AMT2;1*
210 suggested that this transporter may play a role in long-distance ammonium transport
211 under ammonium supply. To test this hypothesis, we first compared ¹⁵N

212 accumulation in roots and shoots of plants co-expressing AMT2;1 together with either
213 AMT1;3 or AMT1;2 in the *qko* background (Figure 4). In these experiments, ¹⁵N-
214 labeled NH₄⁺ was supplied for one hour to allow sufficient time for root-to-shoot
215 translocation. At 200 μM external ammonium supply, AMT2;1 increased ¹⁵N
216 accumulation in roots by approx. 20% only in presence of AMT1;3 but not of AMT1;2
217 (Figure 4A). This went along with a 36% increase in ¹⁵N accumulation in shoots of
218 *qko+13+21* relative to *qko+13*, while the contribution of AMT2;1 was not significant in
219 *qko+12* background (Figure 4B). When plants were exposed to 4 mM external
220 ammonium, ¹⁵N accumulation in roots raised to much higher levels without showing
221 any effect of AMT2;1 in either genetic background (Figure 4C). However, co-
222 expression of AMT2;1 in *qko+13* or in *qko+12* triple insertion lines resulted in a 32%
223 or 25% higher enrichment of ¹⁵N in shoots, respectively (Figure 4D). These results
224 suggested that at high supply AMT2;1 facilitates ammonium translocation irrespective
225 of whether it has been radially transported via the apoplastic or symplastic route.

226 To more directly assess the involvement of AMT2;1 in long-distance transport of
227 ammonium, we collected xylem sap from *qko* and *qko+21* plants after their transfer to
228 10 mM ammonium or nitrate as the sole N source. Under these conditions, AMT2;1
229 should be more strongly expressed in inner root cells and at a higher level in the
230 ammonium pre-treatment (Figures 1 and 2). In plants pre-cultured with nitrate, short-
231 term ¹⁵N-ammonium influx in roots was not significantly altered in *qko* plants by
232 expression of AMT2;1 (Figure 5A). However, influx increased after short-term
233 ammonium incubation, which was most likely due to the induction by ammonium of
234 AMT1;5 and possibly further low-affinity transporters. Also in these ammonium pre-
235 conditioned plants, there was no contribution of AMT2;1 to ammonium influx. As
236 expected, the supply of ammonium to the nutrient solution led to a marked increase
237 in ammonium concentrations in the xylem sap of both *qko* and *qko+21* plants (Figure
238 5B). Remarkably, the presence of AMT2;1 in the *qko+21* triple mutant resulted in an
239 approx. 25% increase in ammonium levels in the xylem sap. At the same time, no
240 significant difference in the xylem sap exudation rate was detected between *qko* and
241 *qko+21* plants (Supplemental Figure 1), indicating that the transporter activity *per se*
242 and not a secondary growth effect was responsible for elevated ammonium loading
243 of the xylem (Figure 5B). As ammonium is largely converted to amino acids in roots
244 (Tobin and Yamaya, 2001) and preferentially translocated in the xylem in the form of
245 glutamine (Finnemann and Schjoerring, 1999; Lam et al., 1995; Sung et al., 2015),

246 we also determined glutamine concentrations. These were strongly promoted by
247 ammonium nutrition and approx. 3-fold higher than those of ammonium but not
248 affected by expression of AMT2;1 (Figure 5C). These results indicated that AMT2;1
249 indeed contributes to elevated ammonium translocation but only in the presence of
250 ammonium in the medium. Although the increased ammonium influx and increased
251 ammonium levels in the xylem sap of *qko+21* plants (Figures 3D and 5B) were not
252 immediately accompanied by phenotypical changes, prolonged exposure to high
253 ammonium suppressed the growth of these plants more severely than that of *qko*
254 (Supplemental Figure 2).

255 In order to assess whether AMT2;1 can mediate ammonium efflux, we designed an
256 assay using the ammonium uptake-defective yeast mutant *triple-mepΔ*
257 (Supplemental Figure 3). Yeast cells were cultivated on arginine, which serves as an
258 adequate N source to the *triple-mepΔ* mutant and results in the leakage of
259 ammonium generated by its catabolism inside the cells (Marini et al., 1997). As this
260 mutant strain is not able to retrieve the ammonium lost by leakage, we monitored
261 NH_4^+ concentrations in the external growth solution of *triple-mepΔ* expressing either
262 AMT1;1 or AMT2;1. Whereas all transformants grew similarly in the arginine-
263 containing liquid media (Supplemental Figure 3A), NH_4^+ concentrations increased
264 gradually in the solution containing cells expressing the empty vector (Supplemental
265 Figure 3B). In contrast, external NH_4^+ levels remained low in the medium containing
266 *triple-mepΔ* expressing AtAMT1;1 or AtAMT2;1. Although not excluding a putative
267 efflux activity of AMT2;1, these results further reinforced that AMT2;1 mediates
268 ammonium import and functions in ammonium retrieval.

269 To verify the involvement of AMT2;1 in root-to-shoot translocation of ammonium in an
270 alternative approach, we assessed ^{15}N partitioning in two independent *amt2;1* T-DNA
271 insertion lines (Figure 6A and 6B). In these lines we anticipated that the large
272 ammonium uptake capacity mediated by AMT1;1, AMT1;2 and AMT1;3 should
273 increase the requirement for AMT2;1 in the long-distance transport of ammonium, as
274 compared to *qko*, in particular when root concentrations of this N form exceed the
275 assimilation capacity in roots. Therefore, we transferred nitrate-grown plants to 10
276 mM ^{15}N -labeled ammonium for 2 h. In both lines, *amt2;1-1* (Col-*gl* background) and
277 *amt2;1-2* (Col-0 background), ^{15}N accumulation in roots was comparable and not
278 significantly different from the corresponding wild-type plants (Figure 6C). However,

279 the accumulation of ^{15}N in shoots significantly decreased in *amt2;1-1* as well as in
280 *amt2;1-2* plants (Figure 6D). In order to verify the approach and estimate the
281 proportion of ammonium that contributed to ^{15}N translocation to shoots, we also
282 assessed ^{15}N partitioning in a mutant defective in the expression of *GLN1;2*, which
283 encodes a root-expressed, ammonium-inducible cytosolic glutamine synthetase
284 (Ishiyama et al., 2004). In the *gln1;2-1* mutant, more ^{15}N accumulated in roots and
285 approx. 50% less ^{15}N was translocated to shoots than in wild-type plants (Figure 6C
286 and 6D). Considering that in roots some glutamine may still have been synthesized
287 via *GLN1;1* and *GLN1;3*, this experiment suggested that only up to 50% of the
288 translocated ^{15}N remained in the form of ammonium and that *AMT2;1* conferred 20-
289 30% of this ammonium translocation capacity to the shoots.

290 To further investigate a role of *AMT2;1* in root ammonium uptake in the presence of
291 all *AMT1*-type transporters, we assessed short-term ^{15}N -labeled ammonium influx in
292 N-starved wild-type and *amt2;1-1* plants. Although ammonium influx rates in *amt2;1-1*
293 plants were indistinguishable from wild type over a wide range of ammonium
294 concentrations, they were significantly lower, i.e. by ~23%, when 10 mM ammonium
295 was supplied (Figure 7A). Notably, *amt2;1-1* was not affected in short-term
296 ammonium influx when plants were already pre-conditioned to high ammonium. As
297 our experiments indicated a substantial contribution of *AMT2;1* to ammonium
298 translocation only in ammonium-supplied plants, we then compared NH_4^+
299 concentrations in the xylem sap of wild-type and *amt2;1-1* mutant plants exposed to
300 10 mM ammonium for 2 days. In N-deficient plants, NH_4^+ levels in the xylem sap
301 were still in the millimolar range and only tended to be lower in *amt2;1-1* (Figure 7B).
302 However, in ammonium-preconditioned plants, when NH_4^+ concentrations in the
303 xylem sap were fourfold higher, significantly lower concentrations were detected in
304 the xylem sap of *amt2;1-1* plants. This independent observation underscored a
305 significant contribution of *AMT2;1* to root-to-shoot translocation of ammonium, and to
306 a smaller extent, to root ammonium uptake.

307

308 DISCUSSION

309 Plants with access to external ammonium as a sole N source have been shown to
310 translocate considerable amounts of ammonium to shoots, although the majority of

311 this N form is usually converted into amino acids already in roots (Finnemann and
312 Schjoerring, 1999; Schjoerring et al., 2002). Despite extensive investigations on the
313 physiological roles of AMT-type transporters in ammonium nutrition, it has remained
314 open whether any of these ammonium transporters might play a role in xylem
315 loading. We show here that *AMT2;1* makes a substantial contribution to root-to-shoot
316 translocation of ammonium in particular when plants are exposed to elevated
317 ammonium supplies. Furthermore, in N-deficient roots *AMT2;1* can increase
318 ammonium influx at elevated external substrate concentrations. Thus *AMT2;1*, which
319 belongs to the MEP-type subfamily of bidirectional ammonium transporters (Soupene
320 et al., 2002), shows a novel physiological feature of AMT-type transporters, as it
321 contributes to ammonium uptake or translocation depending on its cell type-specific
322 expression in response to the plant nutritional status and local ammonium gradients.

323

324 ***AMT2;1* mediates root-to-shoot translocation of ammonium**

325 Based on the observation that preculture with ammonium as a sole N source
326 enhanced *AMT2;1* transcript levels (Figure 1) and confined them mainly to pericycle
327 cells (Figure 2G-2I), the hypothesis was raised that *AMT2;1* may be involved in long-
328 distance ammonium translocation under ammonium nutrition. Using the most direct
329 approach to assess ammonium accumulation in the xylem showed indeed that i) in
330 *amt2;1-1* insertion lines xylem sap concentrations of ammonium were lower than in
331 wild-type plants (Figure 7B), and ii) in an independent genetic approach that the
332 xylem sap of *qko+21* plants contained significantly more ammonium than that of *qko*
333 plants (Figure 5B). This was not the result of different xylem exudation rates
334 (Supplemental Figure 1) or of different ammonium uptake rates (Figure 5A and 7A).
335 As expected, ammonium concentrations in the xylem sap of *qko* and *qko+21* plants
336 were much lower than those in wild-type and *amt2;1-1* plants, not only because of a
337 lower overall uptake capacity for ammonium due to the lacking expression of other
338 AMTs, but also because plants were incubated for a shorter period in 10 mM external
339 ammonium. Nevertheless, in both experimental settings *AMT2;1* increased xylem sap
340 concentrations of ammonium by approx. 20%, indicating a considerable robustness
341 of its transport function. This function of *AMT2;1* in ammonium translocation strictly
342 depended on the preconditioning of plants to external ammonium, as neither N-
343 deficient plants, which showed highest overall transcript levels of *AMT2;1* (Figure 1),
344 nor nitrate-grown plants, which showed *AMT2;1* promoter activity also in inner root

345 cells (Figure 2D-2F), allowed detecting a significant contribution of *AMT2;1* in
346 terminating the radial transport of ammonium towards the xylem (Figures 5 and 7).
347 Previous studies have shown that *AMT2;1* is a plasma membrane protein that can
348 mediate high-affinity ammonium transporter when expressed in yeast (Sohlenkamp
349 et al., 2000; Sohlenkamp et al., 2002). However, different from *AMT1*-type
350 transporters, which mediate electrogenic NH_4^+ fluxes (Ludewig et al., 2003; Mayer et
351 al., 2006), *AMT2;1* mediates electroneutral transport of uncharged NH_3 although
352 *AMT2;1* still possesses a high-affinity recruitment site for NH_4^+ (Neuhäuser et al.,
353 2009). This transport mechanism may allow effective substrate binding also at acidic
354 pH, i.e. when NH_3 concentrations are very low, which is in agreement with yeast
355 complementation studies and the proposed import function from the apoplast
356 (Sohlenkamp et al., 2002). However, uncoupling NH_3 from H^+ cotransport likely
357 decreases transport efficiency into an alkaline compartment such as the cytosol and
358 may be responsible for the lower transport velocity reported for *AMT2;1* relative to
359 *AMT1;1* (Sohlenkamp et al., 2002). On the other hand, non-electrogenic transport of
360 NH_3 likely favours substrate release into an acidic compartment, where co-
361 transported H^+ would impair the transport process. Thus, at least in principle, *AMT2;1*
362 could transport its substrate more efficiently from the cytosol into the apoplast than
363 *AMT1*-type transporters do. However, so far the only evidence that *AMT2;1* may
364 exhibit ammonium export activity is the increased tolerance to methylammonium
365 conferred by this protein when expressed in wild-type yeast (Neuhäuser et al., 2009).
366 Our attempt to demonstrate ammonium efflux in yeast rather indicated a role of
367 *AMT2;1* in ammonium retrieval (Supplemental Figure 3). Unfortunately, the
368 electroneutral transport of NH_4^+ by *AMT2;1* (Neuhäuser et al., 2009) largely limits the
369 possibility to more directly demonstrate a putative efflux function of this transporter by
370 electrophysiological studies. Since there is no experimental evidence disproving the
371 possibility that *AMT2;1* mediates ammonium efflux, it still remains open whether or
372 not *AMT2;1* may act as a bidirectional ammonium transporter.

373 Several channels and transporters known to play a major function in root-to-shoot
374 translocation of nutrients are expressed in the plasma membrane of pericycle cells.
375 Examples are the stelar outward-rectifying potassium channel *SKOR* (Gaymard et
376 al., 1998), the boron exporter *BOR1* (Takano et al., 2002) and the nitrate transporter
377 *NPF7.3/NRT1.5* (Lin et al., 2008). Thus, in order to reconcile the strong upregulation
378 of *AMT2;1* in the pericycle of ammonium-treated roots (Figure 2G-2I) and the

379 increased AMT2;1-dependent ammonium levels in the xylem sap (Figures 5B and
380 7B), we propose that AMT2;1 contributes to root-to-shoot ammonium translocation by
381 facilitating the radial transport of this N form towards the vasculature. According to
382 this hypothesis, the ammonium-dependent repositioning of *AMT2;1* expression in the
383 innermost cell layers could help concentrating ammonium specifically in the pericycle
384 cells that are directly adjacent to xylem vessels.

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

408

409 **The predominant physiological function of AMT2;1 is determined by its** 410 **nitrogen status-dependent cell type-specific expression**

411 Among all root-expressed AMT-type transporters, only AMT2;1 has not yet been
412 implicated with ammonium uptake (Sohlenkamp et al., 2002; Yuan et al., 2007).

413 Here, we reassessed ammonium uptake by *AMT2;1* in the wild-type and *qko*
414 background by supplying increasing concentrations of ¹⁵N-labeled ammonium to N-
415 deficient plants, which induces expression of *AMT2;1* predominantly in outer roots
416 cells (Figure 2A-2C). Only at millimolar substrate concentrations, *AMT2;1* made a
417 small but significant contribution to net ammonium influx (Figure 3D and 7A). In
418 addition, we also show that *AMT2;1* can efficiently retrieve ammonium when
419 expressed in the triple-*mepΔ* yeast mutant (Supplemental Figure 3B). These
420 observations support functional expression studies in yeast and oocytes showing that
421 *AMT2;1* is able to mediate cellular ammonium import (Sohlenkamp et al., 2002;
422 Neuhäuser et al., 2009). In wild-type plants, the net contribution of *AMT2;1* to
423 ammonium influx was negligible, because the capacity of *AMT1* transporters
424 outcompetes *AMT2;1* in the micromolar concentration range (Yuan et al., 2007; Yuan
425 et al., 2013). In the millimolar range, the small contribution of *AMT2;1* to ammonium
426 influx is most likely due the existence of other yet poorly defined low-affinity
427 transporters, such as AMF-type ammonium transporters (Chiasson et al., 2014) or
428 potassium channels (Szczzerba et al., 2008; ten Hoopen et al., 2010).

429 The present study shows that the amount and form of N supply not only regulates
430 *AMT2;1* transcript levels, but also modifies the cell type-specific localization of
431 *AMT2;1* promoter activity (Figures 1 and 2). Although *AMT2;1* expression increased
432 under low N as compared to nitrate or ammonium (Figure 1), the most conspicuous
433 effect was the ammonium-dependent stimulation of *AMT2;1* promoter activity in
434 pericycle cells (Figure 2). Noteworthy, the dependence of *AMT2;1* localization on the
435 amount and form of N supplied to plants also shed light on seemingly conflicting
436 results reported in previous studies (Neuhäuser et al., 2009; Sohlenkamp et al.,
437 2002). According to our results, differences in localization reported before were
438 mostly related to the contrasting nutrient composition used in these studies,
439 especially regarding N supply. While Sohlenkamp et al. (2002) cultivated plants used
440 for GUS assays in full-strength MS medium, which contains ~20 mM ammonium, the
441 study of Neuhäuser et al. (2009) supplemented *proAMT2;1-GFP* plants with less than
442 0.2 mM N, a condition that rapidly provokes N deficiency in Arabidopsis (Gruber et
443 al., 2013). Notably, we raised evidence that the shift in cell type-specific localization
444 of *AMT2;1* is associated with different physiological functions. When N starvation
445 enhances expression in the outermost cells (Figure 2A-2C), *AMT2;1* contributes to
446 ammonium uptake, as long as high levels of this N form are supplied to plants

447 (Figures 3D and 7A). In plants pre-conditioned to high ammonium, the increased
448 expression of *AMT2;1* in endodermal and pericycle cells (Figure 2G-2I) is associated
449 to changes of ammonium levels in the xylem sap (Figures 5B and 7B) but not of
450 ammonium uptake (Figures 5A and 7A).

451

452 **The interplay between root ammonium assimilation and translocation**

453 Upon high external supply, excessive uptake of ammonium can result in ammonium
454 toxicity, if this N form is not quickly assimilated or stored in vacuoles (Li et al., 2014).
455 The genes that encode the cytosolic isoform of glutamine synthetase (i.e. GS1),
456 which is the major GS isoform in roots, are differentially responsive to ammonium
457 availability (Ishiyama et al., 2004). Among them, *GLN1;2* is up-regulated in
458 Arabidopsis roots few hours after exposing plants to elevated ammonium supply and
459 expression was mainly confined to pericycle cells along the root axis (Ishiyama et al.,
460 2004). Most GS1 activity detected in ammonium-treated roots is related to *GLN1;2*,
461 as this was the only *GLN1* isoenzyme markedly induced by ammonium (Ishiyama et
462 al., 2004; Lothier et al., 2011). Thus, a large proportion of the ammonium taken up or
463 produced by nitrate reduction is directly assimilated in roots as long as carbon
464 skeletons and NADH (for NADH-GOGAT) are not limiting. The predominant
465 expression of *GLN1;2* in the vasculature at high external ammonium supply suggests
466 that the conversion of ammonium to glutamine takes place mainly in the root
467 vasculature, where this amino acid can be immediately transferred to xylem vessels.
468 In line with this assumption, we observed that glutamine levels strongly increased in
469 xylem sap upon ammonium nutrition (Figure 5C). In addition, supply of 10 mM ¹⁵N-
470 labeled ammonium to *gln1;2* resulted in a 52% reduction in shoot ¹⁵N compared to
471 wild-type plants, whereas ¹⁵N concentration in roots raised to significantly higher
472 levels in *gln1;2* (Figure 6C-6D). These results indicated that only part of the overall
473 ammonium taken up at high external supply can be destined to aerial parts when root
474 ammonium assimilation is impaired.

475 Glutamine is the major N form translocated in the xylem of ammonium-fed oilseed
476 rape (Finnemann and Schjoerring, 1999) and the major amino acid found in the
477 xylem sap of *Arabidopsis thaliana* (Lam et al., 1995). When the ammonium
478 concentration in roots was increased by supplying high levels of ammonium, ~2.0

479 mM of NH_4^+ were detected in the xylem sap of plants lacking the major high-affinity
480 ammonium transporters (Figure 5B) and up to 18 mM in wild-type plants (Figure 7B).
481 In oilseed rape grown on ammonium as much as 11% of the total N translocated in
482 xylem sap was in the form of ammonium (Finnemann and Schjoerring, 1999). In the
483 same study it was also observed that GS activity in roots was repressed in response
484 to high N availability, while the translocation of ammonium to shoots was enhanced.
485 The repression of GS could be associated with carbon limitation and might be
486 important to protect the root against an excessive drainage of photoassimilates. On
487 the other hand, an enhanced translocation of ammonium could ensure a steady
488 supply of N to the shoots also under such growth conditions (Finnemann and
489 Schjoerring, 1999). Our results and those reported by Ishiyama et al. (2004) suggest
490 that the coordination between ammonium-induced assimilation and translocation is at
491 least in part mediated by *GLN1;2* and *AMT2;1* and occurs predominantly in the
492 pericycle of roots.

493

494 MATERIALS AND METHODS

495 Plant materials and growth conditions

496 The *amt2;1-1* insertion line, which is in Col-*gl* background, was isolated from the
497 enhancer trap collection of Thomas Jack (Campisi et al., 1999) as described
498 previously (Yuan et al., 2007). The homozygous lines *amt2;1-2* (SALK_119678C)
499 and *gln1;2-1* (SALK_145235C), which are in Col-0 background, were acquired from
500 the SALK collection. Disruption of *AMT2;1* expression in the *amt2;1* insertion lines
501 was confirmed by qualitative RT-PCR using the expression of *ACT2* as loading
502 control. For this analysis, the following primers were used: *AMT2;1*-RT-For: 5'-
503 CGGGAAAGATAGAATAACAAAATGG-3'; *AMT2;1*-RT-Rev: 5'-ATTGCTCCGATG
504 ACAGAAGG-3'; *ACT2*-RT-For: 5'-GACCTTGCTGGACGTGACCTTAC-3'; *ACT2*-RT-
505 Rev: 5'-GTAGTCAACAGCAACAAAGGAGAGC-3'.

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

511 In experiments carried out in agar plates, Arabidopsis seeds were surface sterilized
512 and sown onto modified half-strength Murashige and Skoog (MS) medium containing
513 5 mM nitrate as sole N source and solidified with Difco agar. After 7 days of
514 preculture, seedlings were transferred to vertical plates containing half-strength MS
515 medium supplemented with different N forms at indicated concentrations. Plants were
516 grown under axenic conditions in a growth cabinet under the following regime: 10/14
517 h light/dark; light intensity $120 \mu\text{mol m}^{-2} \text{s}^{-1}$; temperature $22^{\circ}\text{C}/18^{\circ}\text{C}$. For hydroponic
518 culture, Arabidopsis seeds were precultured on rock wool moistened with tap water.
519 After 1 week, tap water was replaced by nutrient solution containing 1 mM KH_2PO_4 , 1
520 mM MgSO_4 , 250 μM K_2SO_4 , 250 μM CaCl_2 , 100 μM Na-Fe-EDTA, 50 μM KCl, 50 μM
521 H_3BO_3 , 5 μM MnSO_4 , 1 μM ZnSO_4 , 1 μM CuSO_4 , and 1 μM NaMoO_4 (pH adjusted to
522 6.0 by KOH). Unless indicated otherwise, 2 mM KNO_3 was supplied to provide N-
523 sufficient conditions. During the first 3 weeks, the nutrient solution was replaced once
524 a week, in the 4th week twice a week and in the following weeks every 2 days. Plants
525 were grown hydroponically in a growth chamber under the above-mentioned
526 conditions except that the light intensity was $280 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

527

528 **Localization of *AMT2;1* promoter activity**

529 For the *proAMT2;1-GFP* construct, the primers *2;1-F-SalI* (5'-
530 CGTCGACATTATATTTAAGAATGAGACAAATTCTA-3') and *2;1-R-BamHI* (5'-
531 GGGATCCTTTGTTATTCTATCTTTCCCGGAGTTGA-3') were used to amplify the
532 1883-bp 5'-upstream genomic sequence of *AMT2;1* before ligation with EGFP and
533 nopaline synthase terminator sequences using the *SalI* and *BamHI* sites of pBI101
534 (Clontech, Palo Alto, CA, USA). Arabidopsis plants were transformed using the
535 GV3101 (pMP90) strain of *Agrobacterium tumefaciens* according to the floral dip
536 method (Clough and Bent, 1998). Transgenic plants were selected on agar media
537 with half-strength MS supplemented with 0.5% (w/v) sucrose and 50 mg L^{-1}
538 kanamycin sulfate. At least six independent T2 lines were assessed and the results
539 of one representative line are shown.

540 A LSM 510 Meta (Carl Zeiss MicroImaging GmbH) laser scanning confocal
541 microscope was used for the analysis of *proAMT2;1-GFP* plants. Roots were stained
542 with propidium iodide ($10 \mu\text{g mL}^{-1}$) for 10 minutes. GFP-dependent fluorescence was
543 assessed by excitation at 488 nm with an argon laser and 505- to 530-nm band-pass

544 filter. Propidium iodide-derived fluorescence was imaged under 488 nm excitation
545 and by filtering the emitted light at 458 to 514 nm. The Zeiss LSM 510 software
546 version 3.0 was used for image recording and fluorescence quantification. All
547 confocal sections across samples were recorded with the same microscope settings.

548

549 **Real-time quantitative PCR**

550 Total RNA was extracted using the QIAzol™ Lysis reagent (Qiagen) following the
551 manufacturer's instructions. Prior to cDNA synthesis, samples were treated with
552 DNase (Thermo Fisher Scientific). Reverse transcription was performed using
553 SuperScript™ II (Thermo Fisher Scientific) reverse transcriptase and Oligo(dT)₁₂₋₁₈.
554 Real-time PCR was performed using a Mastercycler ep realplex (Eppendorf) and
555 QuantiTect SYBR Green qPCR mix (Qiagen). The following gene-specific primer
556 pairs were used: AMT2;1_for, 5'-TATGCTCTTTGGGGAGATGG-3'; AMT2;1_rev, 5'-
557 TGACACCTCTAGCACCATGAAC-3' ; UBQ2_for, 5'-
558 CCAAGATCCAGGACAAAGAAGGA-3'; UBQ2_rev, 5'-TGGAGAGCATAACACTTGC-
559 3'). Primer specificity was confirmed by analysis of the melting curves and agarose
560 gel electrophoresis of the PCR products. Relative expression levels were calculated
561 according to Pfaffl (2001).

562

563 **¹⁵N uptake and accumulation**

564 To assess the contribution of AMT2;1 to short-term ammonium uptake, roots of N-
565 deficient plants were rinsed in 1 mM CaSO₄ solution for 1 min and then transferred to
566 nutrient solution containing different concentrations of ¹⁵N-labeled NH₄⁺ (95 atom%
567 ¹⁵N) as the sole N source. After 6 min incubation in uptake solution, roots were
568 washed with 1 mM CaSO₄ to remove apoplastic ¹⁵N and stored at -80°C before
569 freeze drying. Root and shoot ¹⁵N accumulation was assessed by incubating N-
570 starved plants in a nutrition solution containing different concentration of ¹⁵N-labeled
571 NH₄⁺ for one hour. Roots were rinsed in 1 mM CaSO₄ for 1 min before and after
572 exposure to ¹⁵N-labelling solution. Shoots and roots were harvested separately. ¹⁵N
573 concentration was determined by isotope ratio mass spectrometry (Horizon, NU
574 Instruments).

575

576

577

578 **Collection of xylem sap and ammonium measurements**

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

589 Ammonium concentrations in stabilized xylem sap samples were determined with a
590 HPLC-system by derivatization with *o*-phthalaldehyde (OPA) and detection with
591 fluorescence spectroscopy at neutral pH as described by Husted et al. (2000). The
592 HPLC pump was used to continuously pump the carrier stream through the system at
593 a flow rate of 0.8 ml min^{-1} . The carrier consisted of 3 mM OPA, 10 mM
594 β -mercaptoethanol as the reducing agent and 100 mM phosphate buffer adjusted to
595 pH 6.8. The samples were then injected into the carrier stream, which entered the
596 reaction coil in the column oven, where they were heated to 80°C . At this
597 temperature, ammonium reacts with OPA to form an alkylthioisindole fluorochrome.
598 This fluorochrome was detected at an excitation wavelength of 410 nm and an
599 emission wavelength of 470 nm using a fluorescence spectrophotometer (F2000
600 Hitachi, Tokyo, Japan).

601

602 **Statistical analysis**

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

607

608

609 ACCESSION NUMBERS

610 The Arabidopsis Genome Initiative identifiers for the genes described in this article
611 are as follows: *AMT2;1* (At2g38290), *AMT1;2* (At1g64780), *AMT1;3* (At3g24300),
612 *GLN1;2* (At1g66200), *UBQ2* (At2g36170) and *ACT2* (At3g18780).

613

614 SUPPLEMENTAL INFORMATION

615 **Supplemental Figure 1.** Volume of xylem sap collected from *qko* and *qko+21* plants.

616 **Supplemental Figure 2.** Phenotypical analysis of *qko* and *qko+21* plants after
617 prolonged exposure to high ammonium.

618 **Supplemental Figure 3.** *AMT2;1* mediates ammonium retrieval in yeast.

619

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624

625 AUTHOR CONTRIBUTIONS

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627 and L.Y.; Resources, D.R.; Writing – Original Draft, R.F.H.G and N.v.W.; Writing –
628 Review and Editing, R.F.H.G and N.v.W.

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634

635 **FIGURE LEGENDS**636 **Figure 1. Nitrogen-dependent *AMT2;1* expression in Arabidopsis roots.**

637 Relative expression levels of *AMT2;1* were measured by quantitative RT-PCR, using
 638 *UBIQUITIN2* as internal control. Plants were cultivated on agar medium containing no
 639 nitrogen (-N) or the indicated concentrations of nitrate (NO_3^-) or ammonium (NH_4^+) for
 640 5 days, after precultured on half-strength Murashige and Skoog (MS) medium
 641 (containing 0.5 mM nitrate as sole N source) for 7 days. Values are means \pm SE ($n =$
 642 3 independent biological replicates). Different letters indicate significant differences
 643 among means according to Tukey's test at $P < 0.05$.

644

645 **Figure 2. Localization of *AMT2;1* promoter activity depends on the form of
646 nitrogen supply.**

647 Transgenic plants expressing *proAMT2;1-GFP* were precultured on half-strength MS
 648 medium with 2 mM nitrate as sole N source. After 10 days plants were transferred to
 649 plant culture medium containing no N (**A, B and C**), 10 mM nitrate (**D, E and F**) or 10
 650 mM ammonium (**G, H and I**). GFP-derived fluorescence alone (**A, D, G**) or in overlay
 651 with propidium iodide-dependent red fluorescence (**B, E, H**). Images were taken by
 652 confocal microscopy 3 days after transplanting. Scale bars = 50 μm . (**C, F, I**)
 653 Quantitative read-out of GFP-dependent fluorescence intensity in each individual cell
 654 layer was expressed relative to the levels detected in cortical cells ($n = 10$ roots per
 655 treatment, on which the fluorescence was measured in at least 4 different positions).
 656 Values are means \pm SD. ep, epidermis; co, cortex; en, endodermis; pe, pericycle.

657

658 **Figure 3. *AMT2;1* contributes to ammonium uptake in the millimolar
659 concentration range.**

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

665 **(B and C)** Shoot fresh weights of plants grown for 8 days in the presence of the
 666 indicated concentrations of MeA at pH 5.5 (**B**) or pH 7.0 (**C**), as described in **(A)**.

667 Values are means \pm SD ($n = 4$ independent biological replicates). Different letters
668 indicate significant differences according to Tukey's test ($P < 0.05$).

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

676

677 **Figure 4. AMT2;1 contributes to nitrogen accumulation in roots and shoots.**

678 ^{15}N accumulation in roots **(A, C)** and shoots **(B, D)** of *qko* plants expressing AMT1;3
679 (*qko+13*), AMT1;2 (*qko+12*) or either of them together with AMT2;1 (*qko+13+21* or
680 *qko+12+21*, respectively). Plants were grown hydroponically in a complete nutrient
681 solution containing 2 mM nitrate as sole N form followed by 3 days of nitrogen
682 starvation before transfer to 200 μM **(A and B)** or 4 mM **(C and D)** ^{15}N -labeled
683 ammonium (NH_4^+) for 1 hour. Values are means \pm SD ($n = 4$ independent biological
684 replicates). Significant differences at $P < 0.05$ as determined by Student's *t*-test are
685 indicated by an asterisk.

686

687 **Figure 5. Contribution of AMT2;1 to xylem loading.**

688 Short-term ammonium (NH_4^+) influx **(A)** and the concentrations of NH_4^+ **(B)** or
689 glutamine **(C)** in the xylem sap of *qko* and *qko+21* plants. Plants were grown
690 hydroponically in complete nutrient solution containing 3 mM nitrate (NO_3^-) as the
691 sole nitrogen source. After 6 weeks, plants were transferred to 10 mM NO_3^- or 10 mM
692 NH_4^+ for 2 days. Values are means \pm SD ($n = 10$ independent biological replicates for
693 NH_4^+ influx or 3 independent biological replicates consisting of 5 plants for NH_4^+ or
694 glutamine concentrations in the xylem sap). * $P < 0.05$, Student's *t*-test compared
695 with *qko*.

696

697 **Figure 6. Lower ^{15}N translocation to shoots in *amt2;1* insertion mutants.**

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

701 in *amt2;1-1*, *amt2;1-2* and in the corresponding wild types Col-*gl* and Col-0,
702 respectively. Expression of *ACTIN2* (*ACT2*) served as a loading control.

703 **(C and D)** ¹⁵N concentrations in roots **(C)** and shoots **(D)** of Col-*gl*, *amt2;1-1*, Col-0,
704 *amt2;1-2* and *gln1;2-1* grown hydroponically with 10 mM ammonium as sole N source
705 for 3 days, after preculture in nutrient solution containing 2 mM KNO₃. Six-week-old
706 plants were transferred to nutrient solution containing 10 mM ¹⁵N-labeled ammonium
707 for 2 h, before harvest. Values are means ± SD (*n* = 7-8 independent biological
708 replicates). Different letters indicate significant differences among means according
709 to Tukey's test at *P* < 0.05.

710

711 **Figure 7. Ammonium uptake and loading of the xylem are altered by AMT2;1**
712 **according to the plant N status.**

713 Short-term ammonium (NH₄⁺) influx **(A)** and NH₄⁺ concentrations in the xylem sap **(B)**
714 of Col-*gl* and *amt2;1-1* plants, which were cultured hydroponically in nutrient solution
715 containing 2 mM KNO₃⁻ for 5 weeks before transfer to nutrient solution lacking
716 nitrogen (-N) or containing 10 mM NH₄⁺ as the sole N source. After 2 days on
717 treatments, short-term NH₄⁺ influx was assessed and xylem exudates were collected
718 for NH₄⁺ analysis. For the influx experiment, ¹⁵N-labeled NH₄⁺ was supplied at the
719 indicated concentrations. Values are means ± SD (*n* = 5 and 4 independent biological
720 replicates for NH₄⁺ influx and xylem sap analysis, respectively). In **(A)**, different
721 letters indicate significant differences according to Tukey's test (*P* < 0.05), whereas in
722 **(B)** significant differences to Col-*gl* were determined by Student's *t*-test (*, *P* < 0.05;
723 ns, not significant).

724

725

726 REFERENCES

- 727 **Britto, D.T., Siddiqi, M.Y., Glass, A.D., and Kronzucker, H.J.** (2001). Futile
728 transmembrane NH₄⁺ cycling: a cellular hypothesis to explain ammonium toxicity in plants.
729 Proc. Natl. Acad. Sci. USA **98**:4255-4258.
- 730 **Campisi, L., Yang, Y., Yi, Y., Heilig, E., Herman, B., Cassista, A.J., Allen, D.W., Xiang,**
731 **H., and Jack T.** (1999). Generation of enhancer trap lines in Arabidopsis and
732 characterization of expression patterns in the inflorescence. Plant J. **17**:699-707.
- 733 **Chen, C.Z., Lv, X.F., Li, J.Y., Yi, H.Y., and Gong, J.M.** (2012). Arabidopsis NRT1.5 is
734 another essential component in the regulation of nitrate reallocation and stress tolerance.
735 Plant Physiol. **159**:1582-1590.

- 736 **Chiasson, D.M., Loughlin, P.C., Mazurkiewicz, D., Mohammadidehcheshmeh, M.,**
737 **Fedorova, E.E., Okamoto, M., McLean, E., Glass, A.D., Smith, S.E., Bisseling, T., et**
738 **al.** (2014). Soybean SAT1 (Symbiotic Ammonium Transporter 1) encodes a bHLH
739 transcription factor involved in nodule growth and NH_4^+ transport. *Proc. Natl. Acad. Sci.*
740 *USA* **111**:4814-4819.
- 741 **Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for *Agrobacterium*-
742 mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**:735-743.
- 743 **Coskun, D., Britto, D.T., Li, M., Becker, A., and Kronzucker, H.J.** (2013). Rapid ammonia
744 gas transport accounts for futile transmembrane cycling under $\text{NH}_3/\text{NH}_4^+$ toxicity in plant
745 roots. *Plant Physiol.* **163**:1859-1867.
- 746 **Drechsler, N., Zheng, Y., Bohner, A., Nobmann, B., von Wirén, N., Kunze, R., and**
747 **Rausch, C.** (2015). Nitrate-dependent control of shoot K homeostasis by the Nitrate
748 Transporter1/Peptide Transporter family member NPF7.3/NRT1.5 and the Stelar K^+
749 Outward Rectifier SKOR in *Arabidopsis*. *Plant Physiol.* **169**:2832-2847.
- 750 **Finnemann, J., and Schjoerring, J.K.** (1999). Translocation of NH_4^+ in oilseed rape plants
751 in relation to glutamine synthetase isogene expression and activity. *Physiol. Plantarum*
752 **105**:469-477.
- 753 Gaynard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-
754 Ferriere, N., Thibaud, J.B., and Sentenac, H. (1998). Identification and disruption of a
755 plant shaker-like outward channel involved in K^+ release into the xylem sap. *Cell* **94**:647-
756 655.
- 757 **Gruber, B.D., Giehl, R.F.H., Friedel, S., and von Wirén, N.** (2013). Plasticity of the
758 *Arabidopsis* root system under nutrient deficiencies. *Plant Physiol.* **163**:161-179.
- 759 **Hamburger, D., Rezzonico, E., Petetot, J.M.C., Somerville, C., and Poirier, Y.** (2002).
760 Identification and characterization of the *Arabidopsis* PHO1 gene involved in phosphate
761 loading to the xylem. *Plant Cell* **14**:889-902.
- 762 **Husted, S., Hebborn, C.A., Mattsson, M., and Schjoerring, J.K.** (2000). A critical
763 experimental evaluation of methods for determination of NH_4^+ in plant tissue, xylem sap
764 and apoplastic fluid. *Physiol. Plantarum* **109**:167-179.
- 765 **Ishiyama, K., Inoue, E., Watanabe-Takahashi, A., Obara, M., Yamaya, T., and**
766 **Takahashi, H.** (2004). Kinetic properties and ammonium-dependent regulation of
767 cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. *J. Biol. Chem.* **279**:16598-
768 16605.
- 769 **Kafkafi, U., and Ganmore-Neumann, R.** (1997). Ammonium in plant tissue: Real or artifact?
770 *J. Plant Nutr.* **20**:107-118.
- 771 **Kronzucker, H.J., Schjoerring, J.K., Erner, Y., Kirk, G.J.D., Siddiqi, M.Y., and Glass,**
772 **A.D.M.** (1998). Dynamic interactions between root NH_4^+ influx and long-distance N
773 translocation in rice: Insights into feedback processes. *Plant Cell Physiol.* **39**:1287-1293.
- 774 **Lam, H.M., Coschigano, K., Schultz, C., Melo-Oliveira, R., Tjaden, G., Oliveira, I., Ngai,**
775 **N., Hsieh, M.H., and Coruzzi, G.** (1995). Use of *Arabidopsis* mutants and genes to study
776 amide amino acid biosynthesis. *Plant Cell* **7**:887-898.

- 777 **Leran, S., Munos, S., Brachet, C., Tillard, P., Gojon, A., and Lacombe, B.** (2013).
778 *Arabidopsis* NRT1.1 is a bidirectional transporter involved in root-to-shoot nitrate
779 translocation. *Mol. Plant* **6**:1984-1987.
- 780 **Li, B., Li, G., Kronzucker, H.J., Baluska, F., and Shi, W.** (2014). Ammonium stress in
781 *Arabidopsis*: signaling, genetic loci, and physiological targets. *Trends Plant Sci.* **19**:107-
782 114.
- 783 **Li, J.Y., Fu, Y.L., Pike, S.M., Bao, J., Tian, W., Zhang, Y., Chen, C.Z., Zhang, Y., Li, H.M.,**
784 **Huang, J., et al.** (2010). The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate
785 removal from the xylem sap and mediates cadmium tolerance. *Plant Cell* **22**:1633-1646.
- 786 **Lin, S.H., Kuo, H.F., Canivenc, G., Lin, C.S., Lepetit, M., Hsu, P.K., Tillard, P., Lin, H.L.,**
787 **Wang, Y.Y., Tsai, C.B., et al.** (2008). Mutation of the *Arabidopsis* NRT1.5 nitrate
788 transporter causes defective root-to-shoot nitrate transport. *Plant Cell* **20**:2514-2528.
- 789 **Loqué, D., and von Wirén, N.** (2004). Regulatory levels for the transport of ammonium in
790 plant roots. *J. Exp. Bot.* **55**:1293-1305.
- 791 **Loqué, D., Yuan, L., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K.,**
792 **Takahashi, H., and von Wirén, N.** (2006). Additive contribution of AMT1;1 and AMT1;3 to
793 high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient
794 *Arabidopsis* roots. *Plant J.* **48**:522-534.
- 795 **Lothier, J., Gaufichon, L., Sormani, R., Lemaitre, T., Azzopardi, M., Morin, H., Chardon,**
796 **F., Reisdorf-Cren, M., Avice, J.C., and Masclaux-Daubresse, C.** (2011). The cytosolic
797 glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium
798 homeostasis in *Arabidopsis* rosettes when nitrate supply is not limiting. *J. Exp. Bot.*
799 **62**:1375-1390.
- 800 **Ludewig, U., von Wirén, N., Rentsch, D., and Frommer, W.B.** (2001). Rhesus factors and
801 ammonium: a function in efflux? *Genome Biol.* **2**:1-5.
- 802 **Ludewig, U., Wilken, S., Wu, B.H., Jost, W., Obrdlik, P., El Bakkoury, M., Marini, A.M.,**
803 **Andre, B., Hamacher, T., Boles, E., et al.** (2003). Homo- and hetero-oligomerization of
804 ammonium transporter-1 NH₄⁺ uniporters. *J. Biol. Chem.* **278**:45603-45610.
- 805 **Marini, A.M., Soussi-Boudekou, S., Vissers, S., and Andre, B.** (1997). A family of
806 ammonium transporters in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **17**:4282-4293.
- 807 **Mayer, M., Dynowski, M., and Ludewig, U.** (2006). Ammonium ion transport by the AMT/Rh
808 homologue LeAMT1;1. *Biochem J.* **396**:431-437.
- 809 **Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bio assays
810 with tobacco tissue cultures. *Physiol. Plantarum* **15**:473-&.
- 811 **Neuhäuser, B., Dynowski, M., and Ludewig, U.** (2009). Channel-like NH₃ flux by
812 ammonium transporter AtAMT2. *FEBS Lett.* **583**:2833-2838.
- 813 **Pfaffl, M.W.** (2001). A new mathematical model for relative quantification in real-time RT-
814 PCR. *Nucleic Acids Res.* **29**:e45.
- 815 **Remans, T., Nacry, P., Pervent, M., Filleur, S., Diatloff, E., Mounier, E., Tillard, P., Forde,**
816 **B.G., and Gojon, A.** (2006). The *Arabidopsis* NRT1.1 transporter participates in the
817 signaling pathway triggering root colonization of nitrate-rich patches. *Proc. Natl. Acad. Sci.*
818 *USA* **103**:19206-19211.

- 819 **Schjoerring, J.K., Husted, S., Mack, G., and Mattsson, M.** (2002). The regulation of
820 ammonium translocation in plants. *J. Exp. Bot.* **53**:883-890.
- 821 **Smirnov, N., and Stewart, G.R.** (1985). Nitrate assimilation and translocation by higher
822 plants: Comparative physiology and ecological consequences. *Physiol. Plantarum* **64**:133-
823 140.
- 824 **Sohlenkamp, C., Shelden, M., Howitt, S., and Udvardi, M.** (2000). Characterization of
825 *Arabidopsis* AtAMT2, a novel ammonium transporter in plants. *FEBS Lett.* **467**:273-278.
- 826 **Sohlenkamp, C., Wood, C.C., Roeb, G.W., and Udvardi, M.K.** (2002). Characterization of
827 *Arabidopsis* AtAMT2, a high-affinity ammonium transporter of the plasma membrane.
828 *Plant Physiol.* **130**:1788-1796.
- 829 **Soupe, E., Lee, H., and Kustu, S.** (2002). Ammonium/methylammonium transport (Amt)
830 proteins facilitate diffusion of NH₃ bidirectionally. *Proc. Natl. Acad. Sci. USA* **99**:3926-
831 3931.
- 832 **Sung, J., Sonn, Y., Lee, Y., Kang, S., Ha, S., Krishnan, H.B., and Oh, T.K.** (2015).
833 Compositional changes of selected amino acids, organic acids, and soluble sugars in the
834 xylem sap of N, P, or K-deficient tomato plants. *J. Plant Nutr. Soil Sci.* **178**:792-797.
- 835 **Szczerba, M.W., Britto, D.T., Balkos, K.D., and Kronzucker, H.J.** (2008). Alleviation of
836 rapid, futile ammonium cycling at the plasma membrane by potassium reveals K⁺-
837 sensitive and -insensitive components of NH₄⁺ transport. *J. Exp. Bot.* **59**:303-313.
- 838 **Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., Hayashi, H.,
839 Yoneyama, T., and Fujiwara, T.** (2002). *Arabidopsis* boron transporter for xylem loading.
840 *Nature* **420**:337-340.
- 841 **ten Hoopen, F., Cuin, T.A., Pedas, P., Hegelund, J.N., Shabala, S., Schjoerring, J.K.,
842 and Jahn, T.P.** (2010). Competition between uptake of ammonium and potassium in
843 barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences. *J.*
844 *Exp. Bot.* **61**:2303-2315.
- 845 **Tobin, A.K., and Yamaya, T.** (2001). Cellular compartmentation of ammonium assimilation
846 in rice and barley. *J. Exp. Bot.* **52**:591-604.
- 847 **van Beusichem, M.L., Kirkby, E.A., and Baas, R.** (1988). Influence of nitrate and
848 ammonium nutrition on the uptake, assimilation, and distribution of nutrients in *Ricinus*
849 *communis*. *Plant Physiol.* **86**:914-921.
- 850 **von Wirén, N., and Merrick, M.** (2004). Regulation and function of ammonium carriers in
851 bacteria, fungi, and plants. In: *Molecular Mechanisms Controlling Transmembrane*
852 *Transport*--Boles, E., and Krämer, R., eds. Heidelberg, Germany: Springer Berlin Press.
853 95-120.
- 854 **Wang, Y.Y., and Tsay, Y.F.** (2011). *Arabidopsis* nitrate transporter NRT1.9 is important in
855 phloem nitrate transport. *Plant Cell* **23**:1945-1957.
- 856 **Yuan, L., Gu, R., Xuan, Y., Smith-Valle, E., Loqué, D., Frommer, W.B., and von Wirén, N.**
857 (2013). Allosteric regulation of transport activity by heterotrimerization of *Arabidopsis*
858 ammonium transporter complexes in vivo. *Plant Cell* **25**:974-984.
- 859 **Yuan, L., Loqué, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., Takahashi, H., and
860 von Wirén, N.** (2007). The organization of high-affinity ammonium uptake in *Arabidopsis*

861 roots depends on the spatial arrangement and biochemical properties of AMT1-type
862 transporters. *Plant Cell* **19**:2636-2652.

863 **Yuan, L., Graff, L., Loqué, D., Kojima, S., Tsuchiya, Y.N., Takahashi, H., and von Wirén,**
864 **N.** (2009). AtAMT1;4, a pollen-specific high-affinity ammonium transporter of the plasma
865 membrane in *Arabidopsis*. *Plant Cell Physiol.* **50**:13-25.

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