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Regulation of NH$_4^+$ transport by essential cross-talk between AMT monomers through the carboxyl-tails

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Abstract
The ammonium transport across plant plasma membranes is facilitated by AMT/Rh-type ammonium transporters, which have homologs also in most organisms. In the root of the plant *Arabidopsis thaliana*, AMTs have been identified which function directly in the high affinity NH$_4^+$ acquisition from soil. Here we show that AtAMT1;2 has a distinct role, as it is located in the plasma membrane of the root endodermis. AtAMT1;2 functions as a comparatively low affinity NH$_4^+$ transporter. Mutations at the highly conserved carboxyl-terminus of AMTs, including one that mimics phosphorylation at a putative phosphorylation site, impair NH$_4^+$ transport activity. Co-expressing these mutants along with wild type AtAMT1;2 substantially reduced the activity of the wild type transporter. A molecular model of AtAMT1;2 provides a plausible explanation for the dominant inhibition, as the carboxyl-terminus of one monomer directly contacts the neighboring subunit. It is suggested that part of the cytoplasmic carboxyl-terminus of a single monomer can gate the AMT trimer. This regulatory mechanism for rapid and efficient inactivation of NH$_4^+$ transporters may apply to several AMT members to prevent excess influx of cytotoxic ammonium.

Introduction
Ammonium (NH$_4^+/\text{NH}_3$) transporters of the AMT/Rh family are identified throughout all domains of life, including archae, bacteria, fungi, plants and mammals (Ludewig et al., 2001; von Wirén and Merrick, 2004). The ammonium transporters (AMTs) from different species appear to have contrasting transport mechanisms, depending on their physiological role (Ludewig, 2006). While the function of AMTs from plants is the net import and accumulation of NH$_4^+$ (Mayer et al., 2006), the Rh glycoproteins, in contrast, appear to conduct NH$_3$ or facilitate NH$_4^+/\text{H}^+$ exchange for export and disposal (Westhoff et al., 2002; Zidi-Yahiaoui et al., 2005; Mayer et al., 2006). Similarly, procaryotic AMT homologs appear to be NH$_3$ channels (Khademi et al., 2004; Zheng et al., 2004; Andrade et al., 2005; Javelle et al., 2005). The genome of many species contains several homologous *AMT/Rh* genes and six *AMTs* are found in the model plant *Arabidopsis thaliana* (Loque and von Wirén, 2004).

The transcripts of at least three AMTs from *Arabidopsis* are regulated by nitrogen availability in roots (Gazzarrini et al., 1999). In several plant species studied, AMT
transcripts are up-regulated by nitrogen limitation. In roots AtAMT1;1 is localized in the plasma membrane of the rhizodermis, cortex and pericycle (Mayer and Ludewig, 2006) and is responsible for about 30% of the ammonium acquisition in Arabidopsis roots (Kaiser et al., 2002). AtAMT1;1 is partially co-localized with AtAMT1;3, which is also expressed in the rhizodermis and cortex and participates in another 30% of NH₄⁺ uptake of roots (Loque et al., 2006). The residual 40% of ammonium influx in roots appears thus to be carried by AtAMT1;2, which is studied here, and AtAMT2;1 which has been mainly localized to the vasculature, but promoter activity was also identified in the cortex and root tip (Sohlenkamp et al., 2002).

Whether plant AMT transporter activity is fine-tuned by post-transcriptional mechanisms is unknown, but the homologous bacterial AmtB is negatively regulated by reversible binding of the signal transduction P-II protein GlnK to AmtB (Coutts et al., 2002). The deletion of part of the cytoplasmic tail impairs that interaction and reduces the transport activity by ~70% (Coutts et al., 2002). A similar regulation of plant AtAMTs is unlikely since the single P-II protein from Arabidopsis is located in chloroplasts (Smith et al., 2002). However, the cytosolic carboxyl- (C) terminus has been shown to be important for transport function in AMTs from Lycopersicon esculentum (tomato): the transport by AMTs was inhibited by a specific mutation in that region (Ludewig et al., 2003). An exchange of a conserved glycine by aspartate (G458D in LeAMT1;1 and G465D in LeAMT1;2) abolished transport, although the mutation did not affect AMT localization at the plasma membrane (Ludewig et al., 2003).

The importance of this glycine had been initially identified in the homologous Mep transporters from the yeast Saccharomyces cerevisiae. In the mep1-1 mutation, the corresponding glycine was exchanged to aspartate (G413D) causing inactivation of Mep1p (Marini et al., 1997; Marini et al., 2000). The mutant had reduced, albeit residual transport activity. Most notably, the mutant transporter was able to impair the transport of co-expressed Mep2p or Mep3p, indicating cross-talk between different Mep transporters (Marini et al., 2000). Similar observations were reported for ammonium transporters in another fungal species, Aspergillus nidulans (Monahan et al., 2002). Likewise, the equivalent glycine mutations in NH₄⁺ transporters from tomato inactivated co-expressed transporters in a dominant way. Cross-inhibition by mutant subunits was taken as evidence for homo- and possibly hetero-oligomerization by plant AMTs (Ludewig et al., 2003). Although these data had
implicated the importance of the cytosolic C-terminus in the transport activity of AMTs, the physiological relevance of these observations remained nebulous. Identification of the high-resolution structures of the prokaryotic AmtB from *E. coli* did not clarify how the carboxy-terminus is involved in the transport of NH$_4^+$ (Khademi et al., 2004; Zheng et al., 2004). Although the cytosolic C-terminus has not structurally been resolved, it appeared that this tail was not a central constituent of the pore, questioning its importance for transport activity. The analysis of homologous transporters in the fungal species *Candida albicans* revealed that the complete deletion of the cytoplasmic C-terminus impaired AMT function, but shorter truncations covering the region around the conserved glycine were functional in ammonium transport. Interestingly, the cytoplasmic C-terminus was essential for ammonium-related signalling (Biswas and Morschhauser, 2005).

In this study we propose a physiological molecular mechanism of how the carboxyl-terminus regulates the activity of AMT transporters. We concentrated on AtAMT1;2, which is the only root-expressed AMT in Arabidopsis that has not been celluarly localized and conflicting data on the methylammonium transport properties by AtAMT1;2 had been published (Gazzarrini et al., 1999; Shelden et al., 2001). Here it is shown that AtAMT1;2 is a comparatively low-affinity NH$_4^+$ transporter which is preferentially localized in the root endodermis. Specific mutations in the conserved C-terminus of AMTs, including an exchange in a putative phosphorylation site, impair transport. Most interestingly, a molecular model shows that part of the carboxy-tail of one monomer attaches to its neighbor; this provides a reasonable explanation for the observed cross-inhibition of functional, co-expressed AMT monomers. It is concluded that this provides a physiological mechanism to effectively prevent excess ammonium influx and toxicity.

**Results**

**Preferential localization of AtAMT1;2 in the plasma membrane of endodermal root cells**

The transcripts of several NH$_4^+$ transporters have been identified in roots, including those of AtAMT1;1 and AtAMT1;2 (Gazzarrini et al., 1999). In apical zones of the roots, the GFP-fusion protein with AtAMT1;1 under the control of the endogenous promoter was preferentially identified in epidermal and cortical cell layers (Fig.1 A,B,C), as had been previously shown (Loque et al., 2006; Mayer and Ludewig,
In contrast, the GFP-tagged AtAMT1;2 was preferentially found in the plasma membrane of the root endodermis, when expressed from the endogenous promoter (Fig. 1 D,E,F). A minor fraction of AtAMT1;2-GFP fluorescence was also observed in cortical cells. Trans-cellular apoplastic transport across the endodermal cell layer is blocked by the casparian strip, thus AtAMT1;2 transfers NH$_4^+$ from the apoplast of outer cell layers into the endodermal cytoplasm for further release into the stele.

**Transport characteristics of AtAMT1;2 in oocytes**

NH$_4^+$ induced large inward currents in AtAMT1;2-expressing oocytes (Fig. 2A). Similar to the results with other AMT transporters, ionic currents elicited by addition of ammonium were exclusively inward, even at positive voltages. The inward current elicited by 1 mM ammonium was larger than the current induced by the equivalent amount of MeA (Fig. 2A). At -100 mV, the concentration needed to achieve half maximal currents was ~140 µM for NH$_4^+$ and ~1.9 mM for MeA$^+$ (Fig. 2B). The NH$_4^+$ concentration needed to saturate AtAMT1;2 was much higher than for AtAMT1;1, which has a more than 10-fold higher affinity (Mayer and Ludewig, 2006; Wood et al., 2006). It is possible that the different Km values somewhat reflect the apoplastic ammonium concentrations at the rhizodermis and endodermis.

The concentration needed to achieve half maximal currents (“the K$_m$”) was analyzed at each voltage separately and was found to differ. Less ammonium was required to elicit half maximal currents at more negative voltages. This indicates that both, higher ammonium and more negative voltage lead to saturation (Fig. 2C). A similar finding has been made with LeAMT1;1 (Ludewig et al., 2002). Assuming a single binding site for NH$_4^+$ the transport and saturation are characterized by the entry of NH$_4^+$ into the pore and its exit, either back into the external medium or to the cytoplasmic side (this corresponds to transport). The entry (and the exit) rate of NH$_4^+$, and thus the K$_m$, depend on the membrane voltage, as long as the binding site is located within the membrane electric field. The slope of the voltage dependence of the K$_m$ was fitted and the fractional electrical distances $\delta_{NH4^+} = 0.56$ and $\delta_{MeA^+} = 0.26$ were obtained. These values can be interpreted in the way that the binding sites for NH$_4^+$ and MeA$^+$ are located 56% and 26%, respectively, inside the membrane electric field, measured from the outside. Interestingly, the steep voltage dependence indicates that NH$_4^+$ enters deeply into the pore and that it has to cross more than half of the membrane electric field to reach this site.
AtAMT-transport activity is inactivated by mutations in the carboxyl-terminus

The sequences of AtAMT1;1 and AtAMT1;2 share high similarity that even extends into the cytoplasmic carboxyl-terminus (Fig.3A). Specific mutations of a highly conserved glycine in this region have been shown to inactivate AMT/MEP transporters from fungi and tomato (Marini et al., 2000; Ludewig et al., 2003; Smith et al., 2003). However, the role of the cytoplasmic tail is unclear and how the mutation inhibits the transport function remains obscure.

The sequences of AtAMT1;1 and AtAMT1;2 are identical over a stretch of 17 amino acids in the carboxyl-terminus (Fig.3A). Within that sequence, the conserved and functionally indispensable glycine is identified (Fig.3A). In addition, a partially conserved threonine is recognized, which was phosphorylated in a large-scale screen for plasma membrane phospho-proteins (Nuhse et al., 2004). The phosphorylation of this threonine may suggest that post-translational modifications are involved in the regulation of AMT function. It is worth mentioning that the identified phospho-peptide (ISSEDEMAGMDMpTR) was isolated from Arabidopsis suspension cells that were grown in ammonium-rich media (2 mM) (Nuhse et al., 2004). When grown in nitrogen rich media, the high affinity uptake systems are efficiently shut off in roots (Loque et al., 2006).

The threonine is conserved in a number of AMTs from different plant species and even in some bacterial and archaeal AMTs (Suppl. Fig.1). In several other AMTs, including the prokaryotic AMT-1 from Archaeoglobus fulgidus, this threonine is replaced by serine. Other amino acids are found at the equivalent position in most fungal homologs while the carboxy-tail of mammalian Rh glycoproteins fails to show significant conservation with AMTs.

Whether modifications in the relevant threonine (T460 in AtAMT1;1) affect NH₄⁺ transport was tested by introducing mutations at this position. The threonine was replaced by aspartate which is negatively charged at physiological pH and may mimic phosphorylation. The exchange in AtAMT1;1 inactivated NH₄⁺ transport, as determined by growth assays of triple−mepΔ yeast expressing the T460D mutant construct (Fig.3B). By contrast, the mutation of T460 to the uncharged alanine, which is the corresponding residue in AtAMT1;5, yielded a functional transporter (Fig.3B).

Equivalent mutations in AtAMT1;2 or a GFP-tagged version of AtAMT1;2 had similar effects (Fig.4A). The T472D mutant was inactive, while the T472A mutant was
functional (Fig.4A). In addition, the mutant in which the adjacent glycine (G468) was exchanged to aspartate was also non-functional (Fig.4A). The equivalent mutation inactivates fungal homologs (Marini et al., 2000; Monahan et al., 2002) and two AMTs from tomato (Ludewig et al., 2003). By contrast, mutants having a partially conserved serine at position 461 (Suppl. Fig. 1) exchanged to alanine or aspartate were both functional (Fig.4A).

The mutations at positions 461, 468 and 472 did not affect the localization pattern of the GFP-tagged AtAMT1;2 proteins expressed in yeast, suggesting that targeting of the membrane proteins was unaffected (Fig.4B). This resembles the properties of the dominant glycine to aspartate mutant in yeast (Marini et al., 2000) and tomato (Ludewig et al., 2003).

The transport rates of the AtAMT1;2 wild type and the mutants T472D, T472A and G468D were also quantified using $^{14}$C-methylammonium ($^{14}$C-MeA) transport assays in yeast. The T472D and G468D mutants had no residual transport activity when expressed in yeast (Suppl. Fig.3A). In contrast, robust $^{14}$C-methylammonium uptake was observed for the AtAMT1;2 wild type and only slightly reduced uptake for the T472A mutant (Suppl. Fig.3B). Taken together, a specific mutation that mimics phosphorylation at the putative phosphorylation site in the carboxyl-terminal tail of AMTs abolishes NH$_4^+$ transport.

**A molecular model of AtAMT1;2 predicts carboxyl-terminal interactions**

The crystal structures of prokaryotic AMT homologs were used to generate an AtAMT1;2 homology model. The highest resolution structures of *E.coli* AmtB (1U7G) and *A.fulgidus* AMT-1 (2B2F) were taken as templates (Khademi et al., 2004; Andrade et al., 2005). The structures aligned well within the core transmembrane region (Fig.5). Major deviations in the AtAMT1;2 homology model were restricted to the external and internal loops that connect the transmembrane helices. Interestingly, a surprisingly precise alignment and structural model was obtained from the highly conserved cytosolic C-terminus, which forms a structure that includes two short helices (CH1 & CH2) (Fig.5A). The C-terminus was fully ordered and resolved in the AfAMT-1 structure; it aligns with the cytoplasmic face of the same subunit, but also forms hydrogen bonds with the adjacent monomer in the trimer. The same fold is observed in the AtAMT1;2 model; the carboxyl-terminus of the neighboring chain B (blue) is positioned on top of the cytoplasmic surface of chain A (red) (Fig.5).
The structure of AfAMT-1 and the model suggest that subunit interactions by the carboxyl-terminus are of functional importance. A close inspection of the position of the putative phosphorylation site threonine 472 identified tight packing with its neighbors, including two residues from the M7-M8 linker of the adjacent subunit (residues G323 and H324). The side chain was accessible from the cytoplasm, which opens the possibility for its modification (Suppl. Fig.2). By contrast, the serine 461 was positioned further away from the adjacent subunit at the beginning of CH1. Interestingly, glycine 468, which when mutated to aspartate inactivates AMTs from many species, is located in the hinge between helices CH1 and CH2 and is adjacent to T472 (Suppl. Fig.2). It is obvious that a side chain larger than in glycine cannot be accommodated at that position. Any exchange to another amino acid must disrupt the helix-loop-helix structure and the interaction with adjacent residues. Similarly, in AfAMT-1, the oxygen of the corresponding backbone glycine forms two hydrogen bonds with the serine that corresponds to the threonine in AtAMTs. The first hydrogen bond is formed from the glycine (G379:O) to the backbone amino group of serine (S383:N) and the second with the hydroxy group of the serine side chain (S383:OH). Whether modifications in a single subunit also affect proper functioning of co-assembled monomers was tested using co-expression of mutant and wild type in Xenopus oocytes.

**NH₄⁺ transport by AtAMT1;2 mutants in oocytes**

The large magnitude of the currents elicited by ammonium in AtAMT1;2-expressing oocytes allowed a reliable and quantitative comparison with currents by mutant transporters (Fig.6A). Consistent with the non-functionality of the mutants T472D and G468D in yeast, injection of equal amounts of cRNA did not lead to detectable NH₄⁺ currents. The currents from oocytes expressing these mutants were indistinguishable from water injected and non-injected controls (Fig.6A). In contrast, NH₄⁺ currents were detected in T472A mutant-expressing oocytes, but these were of almost 10-fold lower magnitude compared to the AtAMT1;2 wild type.

**Cross-inhibition by co-expressed non-functional monomers**

Further analysis was done by co-expression of equal amounts of mutant and wild type cRNA. Doubling the amount of injected cRNA roughly doubled the NH₄⁺ current by AtAMT1;2 (Fig.6B). However, co-expression of equal amounts of cRNA from wild type and mutant G468D drastically reduced the NH₄⁺ current to below that of
AtAMT1;2 expressed alone. This is consistent with data from tomato AMTs, where the corresponding mutant also inhibited transport by wild type monomers (Ludewig et al., 2003). NH₄⁺ transport by AtAMT1;2 is reduced to 15% by the co-expressed mutant T472D (Fig.6B). Assuming equal processing and stability of the wild type and mutant proteins, a binomial distribution of wild type/mutant subunits within the trimeric complexes is expected. If a single mutant monomer is sufficient to inactivate the entire trimer, only the trimers consisting completely of wild type monomers will be active and only 12.5% of residual current will remain.

The mutant T472A which elicited less current than the wild type also partially inhibited the NH₄⁺ current by the co-expressed AtAMT1;2. The current was, however, larger than in co-injections of wild type and non-functional mutants. Despite the reduced NH₄⁺ transport by the T472A mutant, the residual activity was sufficient to restore yeast growth to the wild type level (Fig.4A). Taken together, the data suggest that the correct carboxy-terminal fold is required for AtAMT activity and that disruption of the carboxyl-tail of a single monomer will inactivate the entire AMT trimer.

**Weak temperature dependence of AtAMT1;2**

If gating and conformational coupling between co-assembled monomers occurs, larger conformational rearrangements might be involved. We tested that hypothesis by measuring the temperature-dependence of the NH₄⁺ currents. A weak temperature-dependence was observed for AtAMT1;2 currents ($Q_{10} = 1.5$), which is consistent with a diffusion controlled transport process and minimal conformational rearrangements during transport (Fig.7A).

In contrast, a steeper, but still weak temperature dependence ($Q_{10} = 1.9$) was measured in the partially active T472A mutant, suggesting that some minor conformational rearrangements between active and inactive states may occur. The apparent activation enthalpy was 50 kJ/mol (compared to 33 kJ/mol in AtAMT1;2) (Fig.7A).

**Discussion**

Nutrients, such as ammonium, are mostly absorbed at the epidermis (rhizodermis) and move symplastically through the cortex to the stele. However, nutrients may enter the symplasm later in cortical and endodermal cells, but the casparian strip provides a major barrier for further apoplastic movement. While AtAMT1;1 and AtAMT1;3 are involved in the high-affinity loading of NH₄⁺ at the rhizodermis (Loque
et al., 2006; Mayer and Ludewig, 2006; Wood et al., 2006), AtAMT1;2 has a lower affinity to NH$_4^+$ and is preferentially localized in the root endodermis. This tissue shares functional aspects with the polarized epithelia in animal tissues. It is worth mentioning that the fluorescence from GFP-tagged AtAMT1:2 was stronger at the cortical side. This observation, however, needs confirmation by independent methods. Further transport in the stele involves AtAMT1;1 (Loque et al., 2006; Mayer and Ludewig, 2006) and AtAMT2;1 (Sohlenkamp et al., 2002).

Although the AtAMT1;2 sequence contains a putative plastid transit peptide (Shelden et al., 2001), the protein is localized to the plasma membrane. The transport properties of AtAMT1;2 for NH$_4^+$ were analyzed in oocytes. Half maximal currents were measured at ~140 µM, while the currents by MeA$^+$ were half-maximal in the mM range. This contrasts to previous studies where a K$_m$=40 µM for MeA$^+$ had been determined in yeast (Gazzarrini et al., 1999). In another study, yeast-expressing AtAMT1;2 had biphasic uptake kinetics for $^{14}$C-MeA$^+$ with K$_{ms}$ of 36 µM and 3 mM (Shelden et al., 2001). We detected very little current by MeA$^+$ in the µM range but observed significant currents at higher methylammonium concentrations. Monophasic transport characteristics were measured for NH$_4^+$.

The previous identification of a phosphorylated threonine in the carboxyl-terminus of AMTs opened the possibility that the transporter function is regulated by phosphorylation (Nuhse et al., 2004). The mutational exchange of the relevant threonine in AtAMT1;1 and AtAMT1;2 to aspartate adds a negative charge at that position; both mutants were non-functional. By contrast, the exchange to alanine restored their function. The mutations at the threonine 472 in AtAMT1;2 did not only impair the transport within the mutated monomer, but additionally inhibited co-expressed wild type monomers. A similar dominant cross-inhibition of NH$_4^+$ transport by a glycine to aspartate exchange in the C-terminus had been reported for the tomato orthologs (Ludewig et al., 2003). This inhibition was confirmed here for AtAMT1;2. Importantly, GFP-tagged wild type and mutant proteins did not differ in their subcellular localization properties, in accordance with earlier data (Ludewig et al., 2003).

Conformational coupling between adjacent subunits can be rationalized based on the structure of AfAMT-1 and the homology model of AtAMT1;2, but such static models have clear limitations. Crystal structures of a plant AMT and protein dynamics may be
required to prove whether conformational changes occur within the cytoplasmic parts of AMTs. The cytosolic TM5-TM6 and TM9-TM10 linkers were partially disordered and varied within the different crystal structures of EcAmtB, which may indicate some flexibility of these peptides (Zheng et al., 2004). Similarly, the structure of the carboxyl-terminus of EcAmtB (1U7G) was disordered which, however, may simply have resulted from the histidine-tag that was attached to AmtB for purification. EcAmtB interacts with the regulating GlnK protein, which negatively regulates transport. Interestingly, a deletion in the C-terminus reduces the activity of AmtB to roughly 30% and impairs the binding of GlnK to AmtB (Coutts et al., 2002). Very recently, the structure of AmtB in complex with GlnK was resolved (Conroy et al., 2007; Gruswitz et al., 2007). This structure revealed that the carboxy-tail of EcAmtB adopts the identical fold as in AfAMT-1 and in the AtAMT1;2 model structure. The effects of mutations in the C-terminal region on EcAmtB function were also recently investigated (Severi et al., 2007). Remarkably similar conclusions regarding potential interactions between AmtB monomers and the possible role of the C-terminal region have been drawn. Two classes of mutants were identified, either with residual (~25%) activity, or essentially inactive. These two mutant classes were interpreted to reflect two distinct states of the carboxy-terminus and hence EcAmtB (Severi et al., 2007).

A weak temperature dependence corresponding to a $Q_{10}$ of 1.5 was observed for the wild type currents. A $Q_{10}$ value of ~1.3-1.6 is characteristic for diffusion limited processes but enzymatic reactions and large conformational changes in proteins are frequently associated with a higher $Q_{10}$ of 2-3 (Liu et al., 1996). The observed $Q_{10}$ value is compatible with minor conformational changes during transport and a channel-like $\text{NH}_4^+$ transport mechanism. However, minor conformational changes that would be required in a $\text{H}^+$ coupled $\text{NH}_3$ co-transporter mechanism (= net $\text{NH}_4^+$ transport) cannot be excluded. An even lower temperature dependence of transport has been reported for EcAmtB (Javelle et al., 2005) and the structurally related Rh glycoproteins (Zidi-Yahiaoui et al., 2005).

When compared with the wild type AtAMT1;2, the transport activity of the T472A mutant was stronger reduced in oocytes than in yeast. It is possible that other cytosolic factors differently regulate wild type AtAMT1;2 activity in these systems; e.g. one might speculate that AtAMT1;2 is partially phosphorylated in yeast, but not in oocytes. Furthermore, the reduced transport activity of T472A correlated with a larger
activation enthalpy which corresponds to a $Q_{10}$ of 1.9. It is likely that the overall mechanism of NH$_4^+$ conduction is not altered. However, it was evident from the structure of the carboxyl-terminus (Fig.5) that any exchange in T472 must alter the original fold. We suggest that the active, conducting state is less favored in the mutant T472A and that the equilibrium between the conformations is shifted to the inactive state. This gives a plausible explanation for the reduced current in that mutant, especially at the lower temperatures at which the oocyte experiments were performed. In contrast, protein modifications at the conserved stretch of the C-terminus appear to manipulate the ratio between active and inactive states in the wild type (Fig.7B). If phosphorylation has the same effect as the aspartate mutation, a specific kinase will then be required for inactivation to occur. It is likely that the appropriate kinase for a plant protein is not endogenously expressed in oocytes. This explains the weak temperature dependence of the wild type in oocytes, as it is constitutively active.

Regulation by cytosolic peptides is not unprecedented in membrane transport. For example, the molecular mechanism of inactivation in K$^+$ ion channels involves reversible blockage of the conduction pathway by the peptide tail (Hoshi et al., 1990). Similarly, plant aquaporins are gated by phosphorylation at flexible cytosolic loops. Aquaporins are tetramers with each monomer forming a solute channel and transition between the open and closed conformations leaves the overall pore architecture intact (Tornroth-Horsefield et al., 2006). Interestingly, interactions between aquaporin monomers affect their overall activity (Fetter et al., 2004).

A schematic model of AMT gating is presented in Fig.7B. The post-translational modification of a single monomer and disruption of the conserved part of its carboxy-tail fold appear to be sufficient to inactivate its physical neighbors and the entire trimer. This is a much more efficient shut-off than activation mechanism. Its physiological role is likely to minimize excess influx of cytotoxic ammonium; this allows plant roots to rapidly cope with variable levels of nitrogen supply. AtAMT1;1, AtAMT1;2 and AtAMT1;3 are in direct contact with the external root apoplast. These AMTs are highly conserved in the relevant region, including the threonine. The conservation of the putative phosphorylation site in AMTs from many plant species suggests that the proposed regulation is potentially a feature of many plant AMTs.
Materials and methods

Plasmid Constructs - The promoter and coding regions of AtAMT1;2 (At1g64780; 3011 bp) were amplified from genomic Col-0 DNA by PCR using Phusion polymerase (New England Biolabs, Ipswich, MA). The sequence was subcloned into the plant transformation binary vector pTKan+GFP. The following primers were used: (5’->3’) GGGGATCCTAAACTTCTCTTATCCAAAAAACCCTGTACC and CCTCTAGAAACAGTCAAGGTCGGTGTAGGAGTCGAGCT. The reverse primer was designed to eliminate the STOP codon and to generate a translational fusion with GFP. For oocyte and yeast expression, the open reading frame of AtAMT1;2 was amplified from the genomic fragment and subcloned into the oocyte expression vector pOO2 and the yeast expression vector pDR199. The fragment with eliminated STOP codon was also amplified and inserted in frame in front of the GFP(S65T) sequence in the yeast expression vector. Mutations were introduced by the Quickchange method from Stratagene. All PCR fragments were verified by full-length sequencing.

Plant growth and analysis - Arabidopsis thaliana plants (ecotype Col-0) were grown in soil and transformed using the GV3101 agrobacterium strain by spraying. Transgenic plant selection and segregation for kanamycin resistance analysis was performed on agar plates with 50 µg/ml kanamycin. Homozygous 10-day old plants carrying a single T-DNA insertion were analyzed by confocal microscopy (Leica DMRE microscope equipped with a confocal head TCS SP; Leica, Wetzlar, Germany). Before imaging, the cell wall was counterstained for 3 min with a 1:50 dilution from a stock of 1 mg/ml propidium iodide.

Expression in yeast – The plasmids containing the respective open reading frames were heat shock-transfected in the ura-ammonium transporter defective yeast strain (31019b; triple-mepΔ) (Marini et al., 1997). The nitrogen deficient growth medium was YNB w/o amino acids and ammonium sulfate (Difco), supplemented with 3% glucose and 3 mM NH4Cl as sole nitrogen source. No buffer was added. Growth of yeast was not affected by expression of the different constructs under non-selective conditions. 14C-MeA uptakes were performed at an optical density of 5 using 100 µM MeA as described (Gazzarrini et al., 1999).

Electrophysiological measurements, preparation and injection of oocytes – These methods have been described in more detail elsewhere (Mayer et al., 2006). Briefly, oocytes were taken from adult females, dissected by collagenase treatment (2 µg/ml,
1,5 h) and injected with 50 nl of cRNA (10-50 ng/ml). Oocytes were kept in ND96 for 3 days at 16 °C and then placed in a small recording chamber. The recording solution was (in mM): 110 CholineCl, 2 CaCl$_2$, 2 MgCl$_2$, 5 N-morpholinoethane sulfonate (MES), pH adjusted to 5.5 with Tris(hydroxymethyl) aminomethane (TRIS). Variable ammonium concentrations were added as NH$_4$Cl salt. Currents without added ammonium were subtracted at each voltage. The concentration dependence of currents were fitted using the following equation: $I=I_{\text{max}}/(1+K_m/c)$, where $I_{\text{max}}$ is the maximal current at saturating concentration, $K_m$ is the substrate concentration permitting half-maximal currents, and $c$ is the experimentally used concentration. The voltage dependence $\delta$ of the $K_m$ was calculated using the following equation: $K_m(\delta)=K_m(0mV)^*\exp(\delta*e^*V/k*T)$, where $\delta$ is fractional electrical distance, $e$ is elementary charge, $V$ is membrane potential, $k$ is Boltzmann's constant and $T$ is absolute temperature. The temperature was measured with a small thermoelement (EBRO TFN520 coupled to TPN110-30), which was directly positioned in the recording chamber. The NH$_4^+$ currents were extracted by subtracting background currents at each temperature. The (apparent) activation enthalpies ($\Delta H$) were extracted from the data using the Arrhenius equation: $I(T)=(A/T)\exp(-\Delta H/[RT])$ where $I$ is the current (which corresponds to a transport rate) at the temperature $T$, $R$ is the gas constant, and $A$ is a constant factor. The $Q_{10}$ was calculated from $\Delta H$ according to $Q_{10} = I(T+10 K)/I(T)$ for $T = 293^\circ K$. In all figures means ± SD are given, except for Fig.2D, which shows means ± SEM.

**Homology modeling** - The primary sequence of AtAMT1;2 was aligned with Archaeoglobus fulgidus AfAMT-1 (22% identity) and EcAmtB (25% identity) using ClustalW. Structural fitting was done using MODELLER 7v7 (available at [http://salilab.org/modeller/](http://salilab.org/modeller/)) and the display of the structures involved VMD, similar to as described (Mayer et al., 2006).

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Figure legends

**FIGURE 1:** Differential expression of AtAMT1;1 and AtAMT1;2 in roots. (A) Fluorescence from plant roots expressing AtAMT1;1-GFP from the endogenous promoter. (B) Same root with propidium iodide cell wall stain (Ep: epidermis; Co: cortex) and overlay (C). Fluorescence from homozygous plant roots expressing pAtAMT1;2::AtAMT1;2-GFP. (E) Propidium iodide stain (End: endodermis) and overlay (F). Scaling bars: 20 µm.

**FIGURE 2:** Functional characteristics of AtAMT1;2 in *Xenopus* oocytes. (A) Current-voltage plot of currents by AtAMT1;2 induced by ammonium (1 mM, open circles) and methylammonium (1 mM, closed circles). (B) Saturation of AtAMT1;2 by NH$_4^+$ (open circles) and MeA$^+$ (closed circles). Voltage-dependence of the saturation constants for NH$_4^+$ (C) and MeA$^+$ transport (D).

**FIGURE 3:** The cytosolic carboxyl-terminus of AMT transporters affects NH$_4^+$ transport. (A) Alignment of amino acid sequences of several plant NH$_4^+$ transporters with *AfAMT-1*. The numbers indicate the amino acid numbers from the start methionine. (B) Growth of ammonium transporter deficient yeast (31019b) transformed with control plasmid *pDR199*, AtAMT1;1 and the AtAMT1;1 mutants T460D and T460A on 3 mM ammonium as sole nitrogen source.

**FIGURE 4:** Effects of mutations in the carboxyl-terminus of AtAMT1;2 on NH$_4^+$ transport. (A) Growth of ammonium transporter deficient yeast transformed with AtAMT1;2 wild type and mutants on 3 mM ammonium as sole nitrogen source (upper
panel). The same growth assay for the mutants in an AtAMT1;2-GFP fusion protein (lower panel). (B) Fluorescence pattern of GFP-tagged AtAMT1;2 wild type and mutants in yeast (left panels) and bright field images (right panels). Scaling bars: 4 µm.

**FIGURE 5: A homology model of AtAMT1;2 predicts interactions by the carboxyl-terminus.** (A) Side view of a homology monomer model of AtAMT1;2 based on EcAmtB and AfAMT-1. Deviations in the structure from its templates are colored in red and closely resembling structures are given in dark blue. The first 53 amino-terminal amino acids and the final 34 cytosolic amino acids have no correlation in the structure of AfAMT-1 and were removed for clarity. The transmembrane helices are numbered (M1-M11) and the two short carboxyl-terminal helices, CH1 and CH2 are indicated at the cytoplasmic side. (B) View from the cytosolic side on surface and cartoon representations of the trimeric homology model. Chain A (red), chain B (blue) and chain C (green) are shown with the C-terminus in darker color. (C) Side view from a different angle of monomer chain A in red and the C-terminus of the neighboring chain B in blue. T472 from chain B is explicitly shown.

**FIGURE 6: Co-expression of AMT-mutants and AtAMT1;2 wild type in oocytes.** Inward currents by 0.2 mM NH₄Cl at -100 mV from oocytes injected with equal amounts of cRNA (A) or mixtures of cRNAs (B). Data are from 3-6 oocytes. Similar data were obtained in 4 independent experiments.

**FIGURE 7: Temperature dependence of NH₄⁺ currents and model for the regulation of AMT transporters by the C-terminus.** (A) Normalized ammonium-induced currents (1 mM) at -100 mV by AtAMT1;2 (closed circles) and the T472A mutant (open circles). The data were fitted with the Arrhenius equation. (B) Each monomer contains an independent pathway for NH₄⁺, but the activation of all subunits within the trimer (e.g. by de-phosphorylation) is essential for transport function. Conformational transitions are predicted to occur independent of further modifications in the carboxyl-tail of the T472A mutant.
Supplementary Figure 1: C-terminal alignment of selected AMTs from different species.

Supplementary Figure 2: (A) View from the cytosolic side on surface and cartoon representation of the trimeric homology model. Chain A (red), chain B (blue) and chain C (green) are shown with their C-terminus in darker color. (B,C,D) Angular view on the cytosolic helices of monomer chain A (red). Amino acids in the vicinity of 4.0 Å of the respective residue are shown as sticks. (B) T472 is close to the M7-M8 linker of the neighboring subunit shown in blue. The hydroxyl group is solvent exposed and next to a glutamate. (C) S461 is freely accessible to solvent and located at the beginning of CH1. (D) G468 is positioned at the hinge region between CH1 and CH2 and is tightly packed with the M1-M2 linker of the same subunit.

Supplementary Figure 3: $^{14}$C-Methylammonium uptake experiments with AtAMT1;2 and mutants expressed in the triple-mepΔ yeast (strain 31019b). Yeast transformed with the empty plasmid (pDR199) served as controls. (A) Time dependence of uptake at 100 µM substrate concentration, pDR (open circles); AtAMT1;2 (closed circles); AtAMT1;2 mutant T472D (open triangles); AtAMT1;2 mutant T468D (closed triangles). (B) Data from a different experiment with pDR (open circles); AtAMT1;2 (closed circles); AtAMT1;2 mutant T472A (open triangles).
A

|     | 437 | 447 | 449 | 441 | 448 | 440 | 406 | 360 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| AtAMT1;1 | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKKMKLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ |
| AtAMT1;2 | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FYGLHKMNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ |
| AtAMT1;3 | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ |
| AtAMT1;4 | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ |
| AtAMT1;5 | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ | FFILKRLNLLRISSEDEMAFMMDMTRHGGFAYMYFDDDESHKA IQ |
| AtAMT2;1 | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR | LLARIVFIPLRMAEEELGIG- DDAAHGEAAYALWG DGEKF DATR |
| AfAMT-1 | 360 | AKAVDAAVGLRVSQEEYVGLDLSQHEEVA YT | 360 | AKAVDAAVGLRVSQEEYVGLDLSQHEEVA YT | 360 | AKAVDAAVGLRVSQEEYVGLDLSQHEEVA YT | 360 | AKAVDAAVGLRVSQEEYVGLDLSQHEEVA YT |

B

|       | pDR199 | wild type | T460D | T460A |
|-------|--------|-----------|-------|-------|
| pDR   |         |           |       |       |
| AtAMT1;1 |       |           |       |       |
