A twin histidine motif is the core structure for high-affinity substrate selection in plant ammonium transporters

Pascal Ganz, Toyosi Ijato, Romano Porras-Murillo, Nils Stührwohldt, Uwe Ludewig, and Benjamin Neuhäuser

From the Institute of Crop Science, Nutritional Crop Physiology, and Institute of Plant Physiology and Biotechnology, University of Hohenheim, 70593 Stuttgart, Germany

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Ammonium transporters (AMT), methylamine permeases (Mep), and the more distantly related rhesus factors (Rh) are trimeric membrane proteins present in all domains of life. AMT/Mep/Rh are highly selective membrane proteins required for ammonium uptake or release, and they efficiently exclude the similarly sized K⁺ ion. Previously reported crystal structures have revealed that each transporter subunit contains a unique hydrophobic but occluded central pore, but it is unclear whether the base (NH₃) or NH₄⁺ coupled with an H⁺ are transported. Here, using expression of two plant AMTs (AtAMT1;2 and AMT2) in budding yeast, we found that systematic replacements in the conserved twin-histidine motif, a hallmark of most AMT/Mep/Rh, alter substrate recognition, transport capacities, N isotope selection, and selectivity against K⁺. AMT-specific differences were found for histidine variants. Variants that completely lost ammonium N isotope selection, a feature likely associated with NH₄⁺ deprotonation during passage, substantially transported K⁺ in addition to NH₄⁺. Of note, the twin-histidine motif was not essential for ammonium transport. However, it conferred key AMT features, such as high substrate affinity and selectivity against alkali cations via an NH₄⁺ deprotonation mechanism. Our findings indicate that the twin-His motif is the core structure responsible for substrate deprotonation and isotopic preferences in AMT pores and that decreased deprotonation capacity is associated with reduced selectivity against K⁺. We conclude that optimization for ammonium transport in plant AMT represents a compromise between substrate deprotonation for optimal selectivity and high substrate affinity and transport rates.

Ammonium (this term designates the sum of NH₄⁺ and the weak base NH₃, pKₐ of 9.25) is selectively transported across membranes of almost all organisms through proteins of the AMT/Mep/Rh family (1). Ammonium serves as a preferred N source for growth in many organisms, such as bacteria, fungi, and plants. Excessive ammonium, however, is excreted by other organisms, such as mammals and fish, where it is released via the kidneys or gills, respectively (2). At a slightly basic cytosolic pH, only a minor fraction of ammonium is found in the uncharged NH₃ form, whereas the majority (~99%) is NH₄⁺, which is driven into the cell by the electrochemical gradient. The strongly negative membrane potential of up to ~250/300 mV across plant and fungal plasma membranes is built up by H⁺-ATPases (3,4). The gradient for NH₄⁺, in contrast, is opposite (outward) under most physiological conditions, so that the unrestricted flow of both solutes will lead to futile cycling of ammonium across the membrane that dissipates the vital pH gradient. As NH₄⁺ is by orders of magnitude more abundant at acidic pH than NH₃, it has been argued that ammonium uptake in bacteria, fungi, and plants must be primarily in the form of NH₄⁺ to meet nutritional demands (5,6).

Several AMT1 transporters mediate high-affinity electrogenic ammonium transport into plant roots (7-10). For the substrate analog methylammonium (MeA), transport of each isotope-labeled MeA was coupled to transfer of a single charge, suggesting that ammonium transport in AMT1s occurs in the form of NH₄⁺ or NH₃/H⁺. Because there was previously no technical or analytical tool to discriminate between these two mechanistically distinct but physiologically identical options, we termed this a net NH₄⁺ transport (7,8). In addition, plants possess the AMT2 subfamily, whose physiological function is still less clear (11-13). The plant AMT2s investigated to date are by sequence more closely related to bacterial, archaeal, or human rhesus-like transporters and mediate ammonium transport without coupledionic currents, suggesting transfer of the base NH₃ rather than the NH₄⁺ ion in their pore (14-16). Similar to plant AMT2 transporters, structural as well as functional data initially suggested that the bacterial EcAmtB transports the uncharged small solute NH₃ (17,18). In contrast, based on the capacity to accumulate ammonium in cells and based on ammonium-dependent currents by EcAmtB into liposomes, NH₄⁺ transport has been proposed for EcAmtB by others (19,20).

The high-resolution crystal structures of bacterial, archaeal, and fungal ammonium transporters share a highly similar overall structure that is fundamental for insight into the transport mechanism (17,21-24). Each subunit of the homotrimer contains an external NH₄⁺ recruitment site that attracts positive ions. This is followed by a dihydroxyalanine constriction region.
In contrast to, e.g., potassium channels (26), static AMT structures do not provide evidence of a continuous water-filled pore that is wide enough to pass solutes. After crossing 30%–50% of the membrane electric field, \( \text{NH}_4^+ \) reaches a saturable binding site (8, 27, 28), where, by the low dielectric milieu of the pore, the equilibrium between \( \text{NH}_4^+ \) and \( \text{NH}_3 \) should be severely shifted toward the base. In the hydrophobic funnel, two preferential ammonium occupancy sites are close to the imidazole rings of two adjacent, pore-facing, conserved histidines (17, 29) (Fig. 1A). This arrangement has been suggested to favor the passage of \( \text{NH}_3 \) but impair transport of \( \text{NH}_4^+ \), being the critical barrier for ionic charge movement (17). Molecular dynamics simulations also suggest deep entry of ammonium into the pore until close to the two pore-lining His (30, 31). The critical role of these imidazoles in EcAmtB was confirmed by mutational studies. Of 13 variants, only a single mutant, where the outer His was replaced with Glu, a good structural mimic of His and the respective residue in most MEP1 homologs from fungi, was functional and transported the analog methylammonium (32). The second, inner His initially appeared to be essential for transport activity (32), but further studies revealed that single replacements of both His with Asp or Glu were tolerated and resulted in partially active transporters. Double mutants in both His into acidic residues were only functional with a further compensatory mutation in AmtB (27). By replacing the inner or outer His with Asp, the selectivity of AmtB for ammonium was ultimately lost (33). This is in agreement with the crucial role of these residues in AMT selectivity. More recently, it was recognized that the acquired nitrogen of AMT/Mep/Rhs-expressing yeast was deprived of the heavy \(^{15}\text{N} \) isotope. \(^{14}\text{NH}_3^+ \) and \(^{15}\text{NH}_3^+ \) have different mass and spectral properties (34) and (rate-limiting) fragmentation of \( \text{NH}_3^+ \) into \( \text{H}^- \) and \( \text{NH}_3 \) during AMT passage has been suggested to be responsible for discrimination against the heavy isotope (35).

We hypothesized that the twin His is critical for transport, selectivity, and isootope selection in two functionally distinct (but structurally highly conserved) plant AMT. Mutants in the electrogenic AMT1;2 and the nonelectrogenic AMT2 from Arabidopsis were generated and functionally analyzed. To our surprise, the twin-His motif was dispensable for high transport activity in plant AMTs, but the residues had AMT-specific roles. Although the outer His mainly determined high affinity toward \( \text{NH}_3^+ \), the inner His was crucial for selectivity against \( K^+ \).

**Results**

**The twin-His motif in the pore is dispensable for high ammonium transport rates**

The conserved pore-lining residues and the constriction region (Phe\(^{147}\) and Phe\(^{217}\)) in plant ammonium transporters are shown in a homology model of the electrogenic AtAMT1;2 (Fig. 1A). The imidazole side chains of His\(^{219}\) and His\(^{386}\) face toward the hydrophobic pore, resembling the situation in the bacterial model transporter EcAmtB (17). The specific functions of the pore-lining His in AMT1;2 were addressed by exchange into amino acids with different side chains. The activity of the mutant transporters was then assayed in a yeast mutant that lacks all endogenous ammonium transporters (\( \Delta\Delta\Delta\text{mep} \)) under conditions where ammonium uptake is rate-limiting for growth. Replacement of His\(^{219}\) with the basic amino acid arginine did not yield functional transporters, as evidenced by lack of growth of yeast that expressed the mutants on selective plates (Fig. 1B). Reduced yeast growth was observed in His\(^{219}\) mutants to alanine or Asp. Yeast growth was surprisingly similar to WT AMT1;2—transfected control cells when either His\(^{219}\) or His\(^{386}\) was changed to Asp (Fig. 1B). This contrasted the situation in EcAmtB, where all mutations in these residues were associated with dramatic loss of ammonium transport activity (14–16).

We therefore analyzed single and double Glu/Asp replacements more systematically and expanded this analysis to the nonelectrogenic AtAMT2.

**Ammonium transport by single and double His mutants of AMT1;2 and AMT2**

When expressed in yeast, protein of all AtAMT1;2 versions was detectable by Western blotting. The protein amount of mutants was slightly reduced but could not be directly correlated with transport activity (Fig. S1). In both transporters, replacement of the outer His (His\(^{219}\) in AMT1;2 and His\(^{188}\) in AMT2) with Asp did not affect transport activity much, as evident from the yeast growth complementation on ammonium (Fig. 2 and Fig. S2). Replacement with Glu, which resem-
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Figure 2. Shown are growth complementation and functionality of AMT1;2 (left panel) and AMT2 (right panel) His mutants in ΔΔmep yeast growth assays. Cells expressing single and double AMT1;2 and AMT2 pore His mutants spotted in 5-fold dilutions on 3 mM ammonium are shown. The experiment was performed three times independently with two technical repetitions each. Shown are representative data of one experiment.

Figure 3. Shown is methylammonium inhibition of yeast growth by AMT1;2 (left panel) and AMT2 (right panel) His mutants expressed in ΔΔmep yeast. Cells expressing single and double mutants were spotted as 5-fold dilutions on plates with Arg-based medium supplemented with 120 mM methylammonium. The experiment was performed three times independently with two technical repetitions each. Shown are representative data of one experiment.

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Increased MeA transport capacity in AtAMT2 mutants

AMT1;2 has a high intrinsic transport capacity for the toxic MeA, whereas AMT2-expressing ΔΔmep yeast cells are quite resistant to MeA (11, 36) (Fig. 3 and Fig. S1). Consistent with the high NH₄⁺ transport activity of the H219D and H386D mutants in AMT1;2, yeast expressing these mutants was highly sensitive to MeA, resembling the situation in WT AMT1;2. Double mutants in this backbone lacked NH₄⁺ transport activity and consistently failed to transport MeA. In contrast, three of the AMT2 single mutants (H188E, H342D, and H342E) and the H188E/H342D double mutant conferred MeA sensitivity, suggesting substantial uptake of MeA (Fig. 3) that was not seen in the corresponding WT.

Mutations in both His reduced substrate affinity for ammonium

We then quantified the ammonium transport kinetics of individual functional His mutants (Fig. 4). The saturation of ammonium uptake of all mutant transporters was shifted to higher ammonium concentrations compared with the wild types, indicating that the affinity for their substrate was reduced by the mutations (Fig. 4). At 1 mM ammonium, the uptake rates corroborated the qualitative growth differences of the yeast observed on plates (Figs. 2 and 4, B and D). Interestingly, the reduction in affinity toward NH₄⁺ was most pronounced in mutants of the outer His. Replacement of His188 in AMT1;2 with Glu and Asp or exchange of His188 in AMT2 with Asp increased the Kₘ by more than 10-fold. The substrate affinity in the AMT2 H188E mutant, however, was only 3-fold lower than in AMT2. Nevertheless, the inner His, which is deeply buried in the pore and, thus, more distant from the external recruitment site and the constriction region, also contributed to ammonium affinity. The H342D mutant saturated at 2- to 3-fold higher ammonium, whereas the H342E mutant in AMT2 had completely lost high affinity for ammonium. The only double mutant showing substantial ammonium transport as well as MeA uptake in the growth assays (AtAMT2 H188E/H342D) had strongly reduced transport capacity and low substrate affinity (Table S1). Finally, we noted that the maximal uptake activity of the AMT2 H188E mutant was substantially larger than that of other single AMT2 mutants; it was similar to WT AMT1;2. This exceptional uptake activity, however, came at the expense of reduced ammonium affinity compared with WT AMT2 (Fig. 4, C and D).

Mutants in the twin-His motif that transport the K⁺ ion

The WT AMT and functional mutants were then expressed in the WΔ3 yeast strain, which lacks two endogenous potassium transporters, trk1 and trk2. This yeast mutant is unable to grow on low-K⁺ plates. As expected, expression of WT transporters could not rescue growth of the WΔ3 yeast on low K⁺ (Fig. 5), but the AMT1;2 mutant H386D enabled considerable yeast growth on low K⁺ medium, indicating permeability to K⁺ (Fig. 5). Furthermore, several AMT2 single and double mutants promoted growth on low K⁺. Mutant pores where the inner His was replaced with Asp were capable of conducting the K⁺ ion, pointing to this residue as the critical barrier for alkali cation passage in AMT. The Asp residue, however, was not essential for K⁺ transport in mutants of AMT2, as the double mutant in which both His were changed to Glu (a mutant with moderate ammonium but no MeA transport) also transported K⁺ (Fig. 5). Potassium uptake was little affected by adding low concentrations of ammonium to the medium (Fig. S3).

Selectivity of AMT1;2 in oocytes

To address the selectivity of AMT1;2 and mutants for monovalent cations, we expressed these transporters heterologously in Xenopus laevis oocytes and measured the associated ionic currents. Ammonium induced large inward currents in AMT1;2-expressing oocytes but not in oocytes expressing WT AMT2. Replacement of ammonium with 10-fold larger concentrations of the similarly sized monovalent cations Tl⁺, Cs⁺, Rb⁺, K⁺, Na⁺, and Li⁺ failed to induce any inward currents in AMT1;2 (Fig. S4) and did not substantially reduce ionic currents by NH₄⁺ when applied together with the cations in 10-fold
excess. Only Tl⁺ tended to moderately inhibit AMT1;2 currents, confirming the exceptional selectivity toward NH₄⁺ (and MeA⁺). Surprisingly and unfortunately, no ionic currents were recorded from oocytes expressing any mutant construct (neither with NH₄⁺ nor with K⁺), suggesting that these mutant proteins were not functional in oocytes, precluding their detailed analysis in that expression system.

**Loss and gain of ¹⁴N isotope selection in the twin-His mutants**

Selection against the naturally low-abundant heavy ¹⁵N isotope was recently identified as a core property of all tested AMT/Mep/Rhs, and this feature was interpreted as resulting from deprotonation of the substrate during passage (35). As certain AMT mutants apparently transported K⁺ (which cannot be fragmented) in addition to NH₄⁺, we questioned whether these mutants retained the isotope preference for ¹⁴NH₄⁺. Therefore, the isotopic dilution δ of ¹⁵N in ΔΔΔmep yeast expressing functional transporter variants was measured. In agreement with a previous analysis (35), yeast expressing the WT AMT1;2 and AMT2 transporters strongly selected the lighter ¹⁴N isotope (25), but unexpectedly, mutants in both backgrounds in which the outer His was mutated to Asp showed a substantially stronger isotope preference. Increased δ was also apparent for the AMT1;2 H219E mutant but not for the equivalent Glu mutant in AMT2, which mediated moderately reduced isotope selection (Fig. 6). The AMT2 H342E...
mutant was indistinguishable from the WT, whereas the isotope preference of H342D was only half (Fig. 6). Most interestingly, however, the mutants H386D in AMT1;2 and H188E/H342D in AMT2, which both substantially transported K⁺, entirely lacked isotope selection.

**Discussion**

The highly selective, regulated passage of ammonium across membranes is a key physiological function of AMT/Mep/Rh proteins. Although most AMT/Mep transporters facilitate uptake of ammonium that is subsequently assimilated, the distantly related Rh transporters are often involved in excretion of ammonia from catabolism (1, 13). Ammonium-selective proteins generally face the problem that the charge and size of the hydrated and dehydrated ammonium substrates (dominated by NH₄⁺) are very similar to K⁺, and under N-depleted conditions, potassium can be orders of magnitude more abundant. Many potassium channels, despite excellent selection against Na⁺, are somewhat permeable to NH₄⁺ (37).

In EcAmtB, mutational analysis suggested that the twin-His feature is essential for function and high conduction (29). However, in the two plant AMTs investigated in this study, the twin His was not essential, and mutants had high ammonium transport rates (Figs. 1–4). Interestingly, in AMT2, the replacement H188E yielded a transporter with higher maximal transport rates than the WT (Fig. 4). The outer His was most important for high affinity in plant AMT, and loss of the His residue strongly reduced the affinity for ammonium (Table S1). The inner His, which was more important for exclusion of K⁺, however, also contributed to the high affinity because replacement with an acidic residue further increased the concentration required for saturation (Fig. 7). Several AMT variants were permeable to K⁺ and ammonium, but these mutants had reduced ammonium transport rates and NH₄⁺ affinity. Interestingly, mutants that altered the N isotope preference were identified. Single mutants with the outer His replaced with Asp had increased ¹⁴N isotope preference that was associated with reduced transport, whereas the H188E mutant in AMT2 had reduced ¹⁴N isotope selection but transported at higher maximal rates (Figs. 2, 4, and 6). H386D in AMT1;2 and H342D in AMT2 transported K⁺ (Fig. 5) and either lost isotope selection or had massively reduced isotope selection, respectively (Fig. 6). These data strongly support the interpretation that deprotonation in AMT pores is responsible for isotope preference (35) and that decreased competence to de-protonate the substrate is associated with reduced selectivity against K⁺. Potassium transport, however, was also observed in Glu mutants of AMT2 and in double mutants in which the outer His was changed to Glu. Although the protonation status of acidic residues in the low dielectric protein environment remains unclear, a pore with more potentially anionic residues replacing His may more easily accommodate an ion such as K⁺. Overall, the situation is somewhat distinct to the situation in EcAmtB (33), where replacement of the outer His with Asp yielded a mutant that conducted methylammonium, ammonium, and K⁺, whereas an Asp mutant in the inner His transported only ammonium and K⁺ (33). Both plant AMTs mutants in which the outer His was replaced with Asp efficiently transported MeA and had reduced substrate affinity. An EcAmtB double mutant with the outer His replaced with Asp and the inner His replaced with Glu carried only K⁺ (33), which somewhat resembles the AMT2 H188E/H342E double mutant, whereas the H188E/H342D mutant transported NH₄⁺, CH₃NH₃⁺, and K⁺. In AmtB, the H318D mutant (29) transported only K⁺ but not NH₄⁺, which is distinct from the corresponding plant AMT mutants, indicating somewhat distinct roles of the His residues in each AMT. Interestingly, an attempt to address the function of the pore histidines in the mouse Rhbg transporter using the X. laevis oocyte system failed because not all histidine mutants were stably inserted into the membrane (17). This indicates that these His residues might also important for folding or stability of AMT/Rh proteins, at least in oocytes, which seems to also apply to the AMT mutants investigated here. The failure of the electrophysiological oocyte expression system therefore does not appear to be due to a lack of sensitivity of the assay but is rather based on specific protein instability of the mutants.

It is unusual that maximal transport rates at a physiological relevant concentration are optimized by a single mutation (as in AMT2 H188E) because long evolutionary selection is expected...
to yield “optimal” transporters. The improved ammonium uptake in that mutant, however, came at the expense of some K⁺ leakage (Figs. 4 and 5). Interestingly, native Mep I from yeast also has Glu instead of His at the respective position, and the H219E mutant in AMT1;2 was not associated with K⁺ leakage (Fig. 5). Transports with higher transport rates came at the expense of isotopic selection, selectivity against K⁺, and lower NH₄⁺ affinity, whereas increased isotope selection was associated with reduced transport rates (Fig. 7). Because K⁺ cannot be fragmented, ion transport through mutant pores implies that deprotonation is not absolutely required for efficient ammonium transport.

Mutants mediating K⁺ as well as NH₄⁺ uptake driven by membrane potential might be of specific agricultural relevance. The reduced affinity for ammonium, however, might preclude use of most transporter mutants. The capability to maintain K⁺ uptake rates under high NH₄⁺ supply was found to be a main factor alleviating ammonium toxicity (38). In high-input systems, simultaneous uptake of both substrates might reduce ammonium toxicity and enable higher ammonium fertilization rates, increasing nitrogen use efficiency.

This potential agricultural relevance of the mutants remains elusive. Here we showed that AtAMT establish fundamentally distinct mechanistic selection against undesired alkali cations compared with, e.g., potassium channels, which select conducted ions by dehydration/rehydration selection in combination with size exclusion (26). We conclude that optimization for ammonium transport constitutes a compromise between sub-

Figure 6. Negative δ¹⁵N/¹⁴N values indicate discrimination against the heavy isotope. ΔΔΔmep yeast transfected with WT and mutant transporters was grown with 3 mM ammonium chloride in liquid culture. The experiment was performed four times with two technical replications for MS. Red lines give means ± S.D. (n = 4). ***, p < 0.001 compared with pDR199 control; ###, p < 0.001 compared with pDR199 and WT AtAMT (one-way ANOVA (F-value = 585.3, p = 2.2 × 10⁻¹⁶ (A); F-value = 454.1; p > 2 × 10⁻¹⁶ (B)) with post hoc Tukey HSD analysis.

Figure 7. Importance of the Twin-His motif in ammonium transport by AtAMTs. A, schematic of an AtAMT subunit with the two pore lining histidines highlighted. δ, His⁳⁸⁶ is critical for exclusion of potassium passage through the pore. The H386D mutant has reduced substrate deprotonation and allows K⁺ passage through the pore. C, His²¹⁹ is critical for high ammonium affinity and high transport rates. D, summary of transporter characteristics. -, +, ++, and +++ indicate the functionality in ammonium transport, NH₄⁺ affinity, MeA transport, K⁺ transport, and selection against the heavier ¹⁵N isotope (preference for ¹⁴N).
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Strate deprotonation for optimal selectivity (inner His most important) as well as high substrate affinity (outer His most important) and high transport rates. The twin-His motif is the core structure responsible for this mechanistic feature because of its direct involvement in substrate deprotonation.

Experimental procedures

Electrophysiological measurements, preparation, and injection of oocytes

Briefly, oocytes were taken from adult females, dissected by collagenase treatment (2 μg/ml, 1.5 h), and injected with 50 nl of linearized cRNA (1 ng/ml) of mutant or WT AMT1;2 or AMT2, as described previously (8). Oocytes were kept in ND96 for 3 days at 18 °C and then placed in a small recording chamber containing the recording solution: 110 mM choline Cl, 2 mM CaCl₂, 2 mM MgCl₂, and 5 mM MES (pH adjusted to 5.5 with Tris). Ammonium and MeA were added as Cl salts. Oocytes were impaled with 3 M KCl–filled glass capillaries of around 0.8 M goesomth resistance connected to a two-electrode voltage clamp amplifier (Dagan CA-1). Transport was measured at 1 mM NH₄⁺ or 10 mM Tl⁺, Cs⁺, Rb⁺, K⁺, Na⁺, or Li⁺ and 1 mM NH₄⁺ and 10 mM of the alkali cation, respectively. Data were normalized from three different experiments with two to three oocytes per experiment. Data are given as means ± S.D.

Yeast transfection and growth

The constructs involving AMT1;2 (At1g64780) and AMT2 (At2g38290) were based on earlier constructs (8, 11). The mutations were verified by full-length sequencing of the gene coding sequence. The plasmids were heat shock–transformed into the ura– ammonium transporter–defective yeast strain (31019b, ΔΔΔmep) (39) and the potassium transporter–defective Saccharomyces cerevisiae strain WΔ3 (MATa, ade2, ura3, trpl, trkhΔ::LEU2, trkh2::HIS3) (40). Selection for transformed yeast was done on solid Arg medium: 1 mM NH₄⁺ or 10 mM Tl⁺, Cs⁺, Rb⁺, K⁺, Na⁺, or Li⁺ and 1 mM NH₄⁺ and 10 mM of the alkali cation, respectively. Data were normalized from three different experiments with two to three oocytes per experiment. Data are given as means ± S.D.

Yeast NH₄⁺ depletion assay

Cells were grown overnight in 50 ml of SD (0.17% YNB without amino acids and ammonium sulfate (Difco) supplemented with 5% glucose) medium with 0.1% arginine at 28 °C, washed twice in water, and resuspended in SD medium at an optical density of 1 at 595 nm with 30, 100, 300, 1000, and 3000 μM ammonium chloride, respectively. The cultures were incubated with shaking (200 rpm) at 28 °C in 30-ml volumes, and 0.5-ml samples were taken at 30, 60, 120, and 180 min. The cells were pelleted, and 40 μl of the supernatant was added to 760 μl of OPA solution (540 mg of O-phthalaldehyde, 10 ml of ethanol, 50 μl of β-mercaptoethanol, and 0.2 mM phosphate buffer (pH 7.3) at 100 ml) to quantify the remaining ammonium. After 20 min of incubation in the dark, extinction at 420 nm was measured. As a reference, 760 μl of OPA solution and 40 μl of water were used. The system was calibrated with ammonium chloride concentrations from 0–3 mM. Data are given as means from three independent experiments ± S.D. Significance was calculated by one-way ANOVA, followed by Tukey HSD test.

Protein extraction

Yeast was grown in arginine minimal medium as described before. Cells were harvested by centrifugation and washed twice with water. 10 ml (A₅₉₅ = 1) of cell suspension was pelleted and frozen in liquid nitrogen. Cells were ground four times for 30 s and 50 Hz in a tissue lyser. The yeast powder was then incubated in extraction buffer (100 mM NaCl, 50 mM Tris/HCl (pH 7.5), 0.5% (v/v) Triton X-100, and 10 mM 2-mercaptoethanol) supplemented with Protease Inhibitor Mixture P (SERVA Electrophoresis GmbH, Heidelberg, Germany). Samples were centrifuged for 10 min at 16,000 × g, and the supernatant was transferred into a new tube. Protein concentration was measured photometrically using ROTI®Quant (Carl Roth GmbH, Karlsruhe, Germany).

Immunodetection

7.5 μg of protein was incubated for 30 min at 37 °C in loading buffer (50 mM Tris/HCl (pH 6.8), 2.5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, and 10% (v/v) glycerol). Proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane by semidry blotting. The membrane was blocked for 1 h at room temperature in Tris-buffered saline with Tween20 (TBST) supplemented with 1% (w/v) casein. Incubation with the primary antibody (rabbit polyclonal, anti-AMT1;2, 1:1000, PhytoAB Inc.) was done overnight at 4 °C. Blots were washed three times with TBST and incubated with the secondary antibody (anti-rabbit polyclonal, 1:25,000, Carl Roth GmbH) for 1 h at room temperature. Prior to detection, membranes were washed twice in TBST and once in TBS. The enhanced chemiluminescent signal was detected using an Odyssey Fc imager (LI-COR Biotechnology GmbH, Bad Homburg, Germany) combined with SuperSignal™ West Dura Extended Duration Substrate (Fisher Scientific GmbH, Schwerte, Germany).

Isotope selection assay

Discrimination of the transporters against the heavy isotope was determined in ΔΔΔmep yeast transfected as described.
above. Overnight preculture in 50 ml of SD medium with 0.1% arginine at 28 °C was diluted to \( A_{695} \) 0.01 and resuspended in 150 ml of culture SD medium with 3 mM \( \text{NH}_4^+ \) as the sole nitrogen source. Cultures were incubated at 28 °C with shaking at 120 rpm and harvested when \( A_{695} \) reached approximately 0.3. Cells were washed twice, pelleted, and freeze-dried before the δ^{15}/δ^{14}N ratio was determined by isotope ratio MS (Delta Plus Advantage, Thermo Fisher Scientific, coupled to an Eurovector EA 3000 elemental analyzer). Data are given as means from four independent experiments ± S.D. Significance was calculated by one-way ANOVA followed by a Tukey HSD test.

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