FEBS Letters 580 (2006) 3931–3936

Mechanisms of ammonium transport, accumulation, and retention in ooyctes and yeast cells expressing *Arabidopsis* AtAMT1;1

Craig C. Wood[a,1], Fabien Porée[b], Ingo Dreyer[b], Gabriele J. Koehler[c], Michael K. Udvardi[a,*]

[a] *Max-Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany*
[b] *Institut für Biochemie und Biologie, Universität Potsdam, Karl-Liebknecht-Str. 24-25, Haus 20, 14476 Golm, Germany*
[c] *Lehrstuhl fuer Funktionelle Genomforschung der Mikroorganismen, Heinrich-Heine-Universitaet Duesseldorf, Universitaetsstr. 1, Gebaeude 25.02.U1.23, 40225, Duesseldorf, Germany*

Received 31 May 2006; accepted 6 June 2006

Available online 21 June 2006

Edited by Ulf-Ingo Flügge

Abstract **Ammonium is a primary source of N for plants, so knowing how it is transported, stored, and assimilated in plant cells is important for rational approaches to optimise N-use in agriculture. Electrophysiological studies of *Arabidopsis* AtAMT1;1 expressed in oocytes revealed passive, $\Delta\psi$-driven transport of NH_4^+ through this protein. Expression of AtAMT1;1 in a novel yeast mutant defective in endogenous ammonium transport and vacuolar acidification supported the above mechanism for AtAMT1;1 and revealed a central role for acid vacuoles in storage and retention of ammonia in cells. These results highlight the mechanistic differences between plant AMT proteins and related transporters in bacteria and animal cells, and suggest novel strategies to enhance nitrogen use efficiency in agriculture.**

Keywords: Ammonium transport; AMT family; NH_4^+ uniport; N-use efficiency

1. Introduction

It has been argued that the Haber–Bosch process, which produces ammonia from molecular di-nitrogen, was the most significant technological advance of the 20th century: without ammonia-based fertilizers for agriculture, two billion fewer people would be alive today [1]. Unfortunately, huge losses of fertiliser nitrogen from agricultural systems wreak havoc with natural ecosystems [2–4]. Therefore, it is important that we understand exactly how plants transport, store and assimilate ammonium and other nitrogen sources in order to pursue rational approaches to optimise N-use in agriculture and to reduce the environmental impact of N-fertilizers [5].

Numerous ammonium transporters of the AMT/MEP/AmtB family have been identified in all kingdoms of life [6]. Plant AMT transporters typically exhibit high affinity for ammonium [7–9] and are located on the plasma membrane of plant cells [9–11]. It was shown using an electrophysiological approach that LeAMT1;1 from tomato is an NH_4^+ uniporter [12], which facilitates passive accumulation of NH_4^+ in the cytoplasm of plant cells in response to the negative electrical potential ($\Delta\psi$) of the plasma membrane. Later structure/function studies of *E. coli* AmtB indicated that it functions as an NH_3 channel, and it was concluded that all related proteins, including the plant AMT transporters are also NH_3 channels [13]. However, neither mechanism alone, NH_4^+ uniport nor NH_3 diffusion through a channel in the plasma membrane, can account for the massive accumulation of ammonium that has been measured in eukaryotic cells [7,14–17]. Thus, it has been suggested that ammonium transport into plant cells, for instance, is an active process, possibly NH_4^+/H^+ co-transport [18,19]. When contemplating how plant cells accumulate and retain ammonia, it is necessary to consider not only the mechanism of ammonia transport across the cell/plasma membrane, but also mechanisms of transport into sub-cellular compartments, especially acidic compartments such as the vacuole [20]. We speculated that passive, $\Delta\psi$-driven transport of NH_4^+ at the plasma membrane coupled to diffusion and subsequent acid-trapping of NH_3 in vacuoles may be sufficient to account for several-thousand-fold accumulation of ammonium in yeast and plant cells. To test this idea, we first determined the electrophysiological features of *Arabidopsis thaliana* AtAMT1;1 in frog oocytes, before expressing the protein in yeast mutant strains lacking all endogenous ammonia transporters, and containing or lacking acidic vacuoles.

2. Materials and methods

Yeast strain YCW031a/α (Δ*vma16*/Δ*vma16* Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* Δ*mep3*/Δ*mep3 ura3*/*ura3 MAT*a/α) was created from haploid strains MLY131a and MLY131α [21] via Cre-mediated *loxP* deletion of *VMA16* [22]. Yeast strains were transformed with plasmid pYES3 or recombinant pYES3 containing *AtAMT1;1* [9,23] and selected for uracil prototrophy. Yeast cells were grown under standard conditions in batch cultures containing 2% galactose and 5 mM glutamate with no ammonium [9]. Transport studies employed the silicon-oil centrifugation technique [9], using ^{14}C-methylammonia hydrochloride (^{14}C-MA; Amersham-Pharmacia) at a final concentration of 100 μM. Efflux of ^{14}C-MA was initiated by the addition of 1 mM NH_4Cl. Uptake and efflux data are presented as mean and standard deviation of five independent experiments, and accumulation ratios were calculated using total pellet and excluded volumes estimated using ^3H_2O and ^{14}C-carboxy-inulin, respectively (Amersham-Pharmacia) [24]. Prior to transport experiments all cell cultures were adjusted to the same $A_{600\,nm}$ to allow comparison between treatments.

*Corresponding author. Fax: +49 331 567 8250.
E-mail address: Udvardi@mpimp-golm.mpg.de (M.K. Udvardi).

[1] Present address: Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, P.O. Box 2601, Canberra, ACT, Australia.

Abbreviation: MA, methylammonium

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.026

For electrophysiology, AtAMT1;1 was subcloned into pGEM-HE [25], transcribed in vitro using T7 polymerase (mMessage mMachine Ambion Europe Ltd., Huntingdon, UK), and the resulting cRNA (40 ng in 40 nl) was injected into stage V and VI oocytes of *Xenopus laevis* (H. Kähler, Hamburg, Germany) suspended in a medium containing 96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/NaOH (pH 7.4) at 18–20 °C. Control oocytes were injected with 40 nl of deionized water. Whole cell currents were measured with a two-electrode voltage-clamp amplifier (Turbo TEC-10CX, npi electronic, Tamm, Germany). Voltage control, data acquisition, and data analyses were performed using the program Pulse/PulseFit (HEKA, Lambrecht/Pfalz, Germany). External standard solution was composed of 100 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ (pH 7.0). To measure the permeability of potassium, ammonium, and methyl-ammonium, different concentrations of NH_4Cl, KCl, and CH_3NH_3Cl, respectively, were added as indicated in the figure legends. The pH was adjusted to 7.5 and 8.5 with 10 mM Tris/MES, and to 6.0 with 10 mM MES/Tris.

3. Results and discussion

When expressed in oocytes, AtAMT1;1 generated significant NH_4^+ and $CH_3NH_3^+$ currents at 100 μM substrate, while K^+ was not transported by AtAMT1;1 (Fig. 1A). In water-injected oocytes, no significant currents were detected, as described previously [12]. External pH had little effect on NH_4^+ transport by AtAMT1;1 in oocytes (Fig. 1B), consistent with data for ^{13}N-ammonium transport by AtAMT1;1 in yeast [9]. AtAMT1;1 exhibited a high affinity for NH_4^+ (K_M of 34 ± 14 μM) and a lower affinity for the analogue $CH_3NH_3^+$ (Fig. 1C). The K_m for NH_4^+ of AtAMT1;1 expressed in oocytes was similar to that obtained for AtAMT1;1 in yeast, using $^{13}NH_4^+$ as substrate (K_m of 25 μM; [9]). Similar electrophysiological results were obtained recently for AtAMT1;1 in independent experiments (Dr. Uwe Ludewig, personal communication). These data are consistent with an NH_4^+-uniport mechanism for AtAMT1;1, as proposed for tomato LeAMT1;1 [12]. However, it has been suggested that currents measured through plant AMT proteins may reflect 'NH_4^+-slippage' through an NH_3 channel, rather than a predominant NH_4^+-uniport mechanism [13]. The following experiments of AtAMT1;1 expressed in yeast largely resolve this issue.

Saccharomyces cereviseae has three homologous ammonium transporters, MEP1, MEP2, and MEP3, without which it is unable to grow on low concentrations of ammonium as the sole source of N [16,21]. Yeast mutants defective in all three MEP genes have been used to identify novel ammonium transporters from different organisms, and to carry out biochemical studies of these transporters [7,23,26]. ^{14}C-methylammonium (^{14}C-MA) is generally used as an analogue of ammonium in such studies because it is a stable radioactive molecule that is transported by AMT/MEP proteins but it is not metabolised, at least in *S. cereviseae* [15,20]. Like ammonium, MA is a weak base that exists in both the cationic ($CH_3NH_3^+$) and neutral (CH_3NH_2) forms at physiological pH.

To determine the contribution that vacuoles make to ammonium accumulation in yeast cells, we disrupted vacuolar acidification by creating a deletion mutation in the *VMA16* gene, which encodes an essential sub-unit of the vacuolar membrane H^+-ATPase [27]. The *VMA16* mutation was created in strain MLY131a/α, which has deletions in all three ammonium transporter genes, *MEP1*, *MEP2*, and *MEP3* [21]. The resulting strain, YCW031a/α, like its progenitor MLY131a/α, was unable to take up significant amounts of ^{14}C-MA at either pH

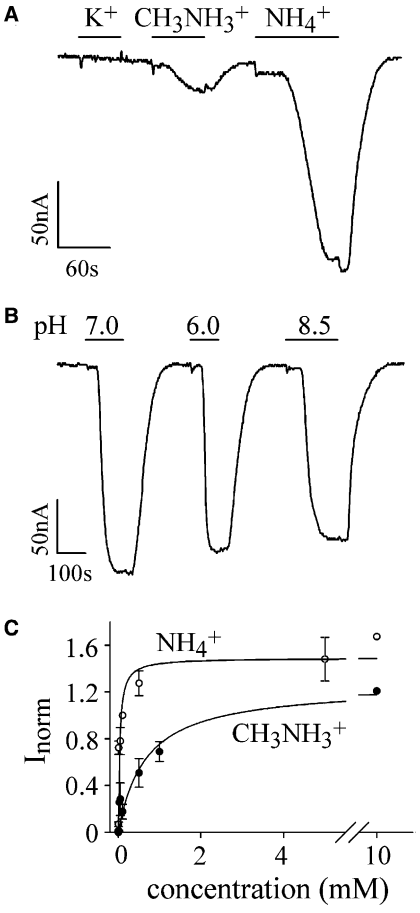

Fig. 1. AtAMT1;1 is a high-affinity, electrogenic NH_4^+ transporter. Representative currents obtained from oocytes injected with *AtAMT1;1* cRNA. Oocytes were voltage-clamped at −70 mV. (A) The bars indicate the time of perfusion with solutions containing 100 μM KCl, CH_3NH_3Cl, and NH_4Cl, respectively. In the other periods, the oocyte was perfused with standard external solution, which was free of potassium, ammonium, and methyl-ammonium. (B) The bars indicate the time of perfusion with solutions of the indicated pH values containing 100 μM NH_4Cl. In the other periods, the oocyte was perfused with standard external solution (free of NH_4Cl, pH 7.0). (C) NH_4Cl- and CH_3NH_3Cl-concentration dependence of the steady state current amplitude. To compare data measured on different oocytes, current amplitudes were normalized to the value measure in 100 μM NH_4Cl. Data are shown as means ± S.D. (n = 3–5 except for data collected at 10 mM where n = 2 in both cases). Data were fitted with a Michaelis–Menten kinetics yielding in a K_M of 34 ± 14 (S.E.) μM for NH_4Cl and of 670 ± 250 μM for CH_3NH_3Cl.

6.1 or 7.5 (Fig. 2). Next, we expressed AtAMT1;1 in MLY131a/α and YCW031a/α, which enabled both strains to take up MA (Fig. 2). The initial rate of MA uptake was similar in both transformed strains, consistent with previous observations that defects in the vacuolar H^+-ATPase do not directly affect transport at the plasma membrane [20]. However, a dramatic difference in long-term accumulation of MA by the two strains was observed: MA accumulation ratios (intracellular/final extracellular concentration) of 2000 and 60 were typical of strains MLY131a/α and YCW031a/α expressing AtAMT1;1, respectively, at an external pH of 6.1 (Fig. 2). Thus, vacuolar acidification via the vacuolar H^+-ATPase enables yeast cells to accumulate approximately 30-fold more MA than is possible otherwise. Wild-type yeast cells typically exhibit a ΔpH

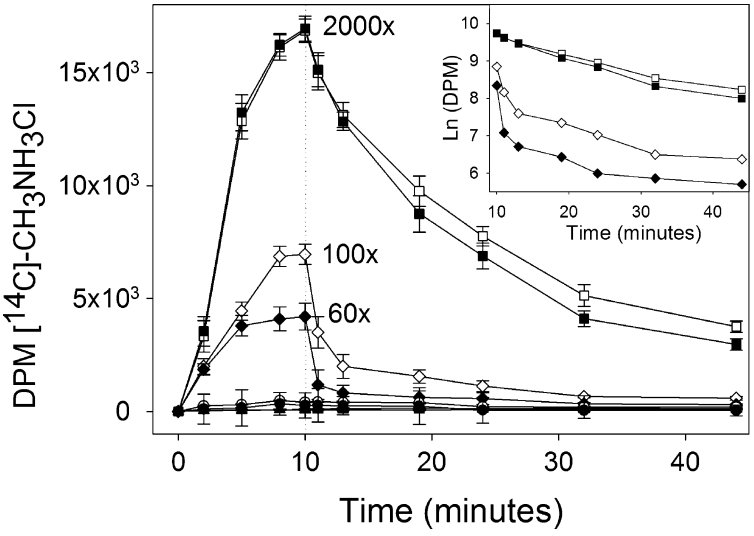

Fig. 2. Importance of vacuolar acidification for accumulation and retention of MA in yeast. Assay started by the addition of 100 μM $^{14}CH_3NH_3Cl$ and the dashed line at 10 min indicates when 1 mM NH_4Cl was added to the uptake assay to initiate efflux of $^{14}CH_3NH_3Cl$. Filled symbols, assays conducted at pH 6.1; open symbols, assays at pH 7.5; circles, MLY131 transformed with pYES3 (vector control); squares MLY131 expressing AtAMT1;1; triangles, YCW031 with pYES3; diamonds, YCW031 expressing AtAMT1;1. Data are the average and standard deviation of five independent experiments for each treatment. The near-equilibrium accumulation ratio (in/out) of $^{14}CH_3NH_3Cl$ at 10 min is indicated (i.e. 2000×, 100×, and 60×). Note that external ammonium concentration decreased while internal ammonium increased. Therefore, there is not a linear relationship between the amount of ^{14}C-MA accumulated by different strains and the MA accumulation ratio. The inset shows a semi-logarithmic plot of the averaged efflux data. Linear regression analysis of the log-transformed data was used to determine the half-time of efflux for the various phases.

of between 1 and 2 across the vacuolar membrane (interior acidic) [28,29], although the vacuolar H^+-ATPase is capable of generating a proton electrochemical gradient of 180 mV [30], which is equivalent to $\Delta pH = 3$ at $\Delta\psi = 0$. Given that vacuoles occupy approximately 50% of yeast cell volume [31,32], simple or facilitated diffusion of CH_3NH_2 across the vacuolar membrane and acid-trapping of $CH_3NH_3^+$ inside the vacuole could easily account for the difference in MA accumulation between strains MLY131a/α and YCW031a/α.

In the absence of vacuolar acidification, AtAMT1;1 expression resulted in a modest 60-fold accumulation of MA in strain YCW031a/α, consistent with $\Delta\psi$-driven uniport of $CH_3NH_3^+$, but not with the diffusion of CH_3NH_2 across the plasma membrane (Fig. 1). Changes in external pH had no effect on MA uptake by strain MLY131a/α, again consistent with uptake of $CH_3NH_3^+$ but not CH_3NH_2 via AtAMT1;1 (Fig. 2). Equilibrium levels of MA in yeast strain YCW031a/α increased less than twofold in response to an increase in external pH of 1.4 U, much less than would be expected if CH_3NH_2 rather than $CH_3NH_3^+$ was transported into cells via AMT1;1. As AMT1;1 activity is little changed between pH 6.1 and 7.5 (Fig. 1B), the increased accumulation of MA in strain YCW031a/α at higher external pH is consistent with the interpretation that CH_3NH_2 efflux from the cell declined in response to a lower ΔpH across the plasma membrane under these conditions (see below). Taken together with the electrophysiological data for AtAMT1;1 expressed in oocytes (Fig. 1), the results from the yeast experiments indicate that AtAMT1;1 is an electrogenic NH_4^+ transporter, and not an electroneutral NH_3 channel. A similar conclusion was reached for tomato LeAMT1;1, based on electrophysiological data [12,33]. Importantly, the mechanism of plant AMT transporters differs from that reported for homologues in bacteria and animals, which appear to be NH_3 channels [13,34]. Although plant AMT proteins transport NH_4^+, it remains unclear whether ammonium traverses the entire permeation pathway of AtAMT1;1 as NH_4^+, or whether it is deprotonated en-route and NH_3 and H^+ are transported separately for some distance. A comparison with the permeation process of potassium channels favours the latter mechanism. The passage of a K^+ ion through such channels is facilitated by carbonyl oxygen atoms in the selectivity filter, which replace the hydration shell of K^+ [35]. This ion coordination guarantees a high transport rate (>10^5 ions per second) and a high selectivity for K^+ over Na^+. However, the discrimination between K^+ and NH_4^+ in these channels is poor [36]. In contrast, AtAMT1;1 transports NH_4^+ but apparently not K^+ (Fig. 1A). Furthermore, the transport rate of NH_4^+ through AtAMT1;1 appears to be a factor of 100 lower than that of plant K^+ channels, indicating that the transport mechanism probably involves more than simply stripping of the hydration shell of NH_4^+.

Strains MLY131a/α and YCW031a/α provide a unique opportunity to delineate the role of acidic vacuoles in retention of ammonium in a model eukaryotic cell, *S. cerevisiae*. Addition of a 10-fold excess of ammonium to cells pre-equilibrated with ^{14}C-MA completely blocks uptake of MA via AtAMT1;1 [8], which reveals the kinetics of MA efflux from cells (Fig. 2). MA efflux analysis revealed a remarkable difference between strains MLY131a/α and YCW031a/α. MA efflux from strain MLY131a/α containing acidic vacuoles, exhibited simple, first-order decay kinetics, consistent with passive efflux of MA from a single cellular compartment, presumably the vacuole (Fig. 2 and inset). This efflux had a relatively long half-time ($t_{1/2}$) of 11 or 13 minutes at external pH 6.1 or 7.5, respectively, reflecting good retention of MA. In contrast, MA efflux from strain YCW031a/α lacking acidic vacuoles exhibited biphasic

kinetics with a rapid initial phase having a $t_{1/2}$ of efflux of 0.5 or 1.0 min at pH 6.1 or pH 7.5, respectively, followed by a second, slower phase with a $t_{1/2}$ of efflux of 11 or 12 min at pH 6.1 or 7.5, respectively. Clearly, these cells had difficulty not only in accumulating MA, but also in retaining it (Fig. 2). The efflux data from strain YCW031a/α is consistent with loss of MA from two cellular compartments, with the first phase of efflux representing the bulk of internal MA (~80%) and the second a much smaller pool (~20%). A comparison between MLY131a/α and YCW031a/α suggests that in yeast with acidic vacuoles, all the internal MA was contained within the vacuole. When vacuolar function was compromised, as in YCW031a/α, cellular MA was predominately found in another compartment, possibly the cytoplasm, from which it was more rapidly lost by the cell. Interestingly, the kinetics of MA efflux for the second, slower phase of YCW031a/α match the single efflux phase of MLY131a/α (Fig. 2, inset), indicating that the second, smaller pool of MA in YCW031a/α probably represented vacuolar MA. Finally, efflux of MA from YCW031a/α was substantially faster in media buffered at pH 6.1 compared to pH 7.5, consistent with the idea that most of the MA in these mutant cells was present in the cytoplasm, from which it was lost more rapidly across the cell membrane due to the steeper concentration gradient for uncharged MA across this membrane at lower external pH.

The results presented here have important implications for our understanding of ammonium transport, storage, and utilisation in plants, and for future efforts aimed at improving ammonium-use efficiency in agricultural species. First, transport and accumulation of ammonium in plant cells is a two-step process that involves $\Delta\psi$-driven transport of NH_4^+ across the plasma membrane via AMT proteins like AtAMT1;1 followed by NH_3 diffusion across the vacuolar membrane and subsequent acid-trapping of NH_4^+ (Fig. 3). Uptake of ammonium by plant cells leads to rapid alkalisation of vacuoles [37–40], a result consistent with NH_3 transport across the tonoplast and subsequent protonation and trapping of NH_4^+ inside the vacuole (Fig. 3). The central vacuole of plant cells is typically 1–2 pH units more acidic than the neutral cytoplasm, and given the fact that the vacuole may account for

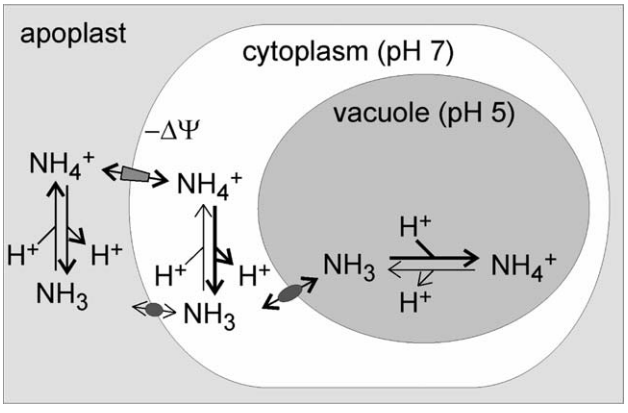

Fig. 3. Mechanisms of ammonia transport, accumulation, and retention in plant cells. Passive $\Delta\psi$-driven uniport of NH_4^+ at the plasma membrane coupled to facilitated NH_3 diffusion across the vacuolar membrane and subsequent acid-trapping of NH_4^+ leads to massive accumulation of ammonium within vacuoles. Relatively slow efflux of NH_3 across the vacuolar membrane ensures retention of ammonium until it is eventually assimilated in the cytoplasm.

90% or more of the cell volume, this compartment is likely to contain the bulk of cellular ammonium, trapped as NH_4^+. Recently, it was shown that tonoplast intrinsic proteins (TIPs) facilitate NH_3 transport into plant vacuoles [41], as predicted earlier [42], which suggests that transport of NH_3 across this membrane may be subject to regulation and not simply the result of un-controlled diffusion of NH_3 through the lipid bilayer (Fig. 3). Over-expression of TIP2;1 in transgenic Arabidopsis did not lead to over-accumulation of ammonium in roots, however, indicating that TIP activity is not normally rate-limiting to ammonium accumulation, nor an especially good target for enhancing plant N-use efficiency [41].

As in the case of yeast cells (Fig. 2), efflux of ammonia from the acidic vacuoles of plant cells is substantially slower than efflux from the cytoplasm [43,44]. Therefore, short-term storage of ammonium in acidic vacuoles limits the loss of intracellular ammonium to the environment. Slow-release of ammonium from vacuoles coupled to efficient assimilation of ammonium by cytosolic and plastidic glutamine synthetase (GS) presumably minimises loss of ammonium from plant cells, and may protect cellular processes that are inhibited by ammonium [42].

Finally, molecular breeding of plants that are able to increase proton pumping into vacuoles when fertiliser-ammonium is applied to the soil may increase transiently the ammonium-storage capacity of plants. Over-expression of the vacuolar H^+-pump, AVP1 in *Arabidopsis* enhanced cation uptake into vacuoles, presumably via increased proton-cation antiport activity across the tonoplast [45]. A concomitant increase in ΔpH in response to increased proton pumping into the vacuole would be expected to enhance ammonium sequestration there. It is tempting to speculate that if enhanced tonoplast proton pumping were coupled to an increased capacity to assimilate ammonium into organic N following application of N-fertiliser, a significant improvement in N-use efficiency in plants might be achieved. Over-expression of GS in plants has been associated with greater ammonium assimilation and enhanced growth [46,47]. On the other hand, plants have evolved regulatory mechanisms that limit ammonium uptake when external ammonium is consistently high [8,48,49]. Thus, there may be scope to re-program nitrogen regulation in plants in a way that maintains high ammonium uptake and assimilation rates in the face of unnaturally high and transient supplies of N-fertiliser. Interestingly, when we increased ammonium uptake in transgenic rice by over-expressing OsAMT1;1 without a concomitant increase in ammonium assimilation, tissue ammonium levels increased to toxic levels and plant growth was retarded [50]. We are now focussing our efforts on identifying regulatory proteins, such as transcription factors that could be used to enhance ammonium transport and metabolism in a coordinated manner to increase nitrogen-use efficiency in plants. Given that tens of millions of tonnes of fertiliser-N are lost to the environment each year, the aim of improving crop N-use efficiency is certainly an important one.

Acknowledgements: C.C.W. was funded by Alexander von Humboldt and Max Planck Postdoctoral Fellowships. We thank Ute Kraemer for valuable discussions and Christian Sohlenkamp for the AtAMT1;1 clone. We thank J. Heitman for providing various haploid yeast strains used in the construction of the diploid YCW031.

References

[1] Smil, V. (1999) Detonator of the population explosion. Nature 400, 415.
[2] Smil, V. (1999) Nitrogen in crop production: an account of global flows. Global Biogeochem. Cycles 13, 647–662.
[3] Giles, J. (2005) Nitrogen study fertilizes fears of pollution. Nature 433, 791.
[4] Beman, J.M., Arrigo, K.R. and Matson, P.A. (2005) Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. Nature 434, 211–214.
[5] Britto, D.T. and Kronzucker, H.J. (2004) Bioengineering nitrogen acquisition in rice: Can novel initiatives in rice genomics and physiology contribute to global food security? Bioessays 26, 683–692.
[6] Marini, A.M., SoussiBoudekou, S., Vissers, S. and Andre, B. (1997) A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 17, 4282–4293.
[7] Ninnemann, O., Jauniaux, J.C. and Frommer, W.B. (1994) Identification of a high-affinity NH_4^+ transporter from plants. EMBO J. 13, 3464–3471.
[8] Gazzarrini, S., Lejay, T., Gojon, A., Ninnemann, O., Frommer, W.B. and von Wiren, N. (1999) Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. Plant Cell 11, 937–947.
[9] Sohlenkamp, C., Wood, C.C., Roeb, G.W. and Udvardi, M.K. (2002) Characterization of *Arabidopsis* AtAMT2, a high-affinity ammonium transporter of the plasma membrane. Plant Physiol. 130, 1788–1796.
[10] Simon-Rosin, U., Wood, C. and Udvardi, M.K. (2003) Molecular and cellular characterisation of LjAMT2;1, an ammonium transporter from the model legume *Lotus japonicus*. Plant Mol. Biol. 51, 99–108.
[11] Ludewig, U., Wilken, S., Wu, B., Jost, W., Obrdlik, P., El Bakkoury, M., Marini, A.-M., Andre, B., Hamacher, T., Boles, E., von Wiren, N. and Frommer, W.B. (2003) Homo- and hetero-oligomerization of AMT1 NH_4^+-uniporters, J. Biol. Chem. 278, 45603–45610.
[12] Ludewig, U., von Wiren, N. and Frommer, W.B. (2002) Uniport of NH^{4+} by the root hair plasma membrane ammonium transporter LeAMT1;1. J. Biol. Chem. 277, 13548–13555.
[13] Khademi, S., O'Connell, J., Remis, J., Robles-Colmenares, Y., Miericke, L.J.W. and Stroud, R.M. (2004) Mechanism of ammonia transport by Amt/MEP/Rh: Structure of AmtB at 1.35 angstrom. Science 305, 1587–1594.
[14] Hackette, S.L., Skye, G.E., Burton, C. and Segel, I.H. (1970) Characterization of an ammonium transport system in filamentous fungi with methylammonium C-14 as substrate. J. Biol. Chem. 245, 4241.
[15] Roon, R.J., Even, H.L., Dunlop, P. and Larimore, F.L. (1975) Methylamine and ammonia transport in *Saccharomyces cerevisiae*. J. Bacteriol. 122, 502–509.
[16] Marini, A.M., Vissers, S., Urrestarazu, A. and Andre, B. (1994) Cloning and expression of the Mep1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. EMBO J. 13, 3456–3463.
[17] Britto, D.T., Siddiqi, M.Y., Glass, A.D.M. and Kronzucker, H.J. (2001) Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. Proc. Natl. Acad. Sci. USA 98, 4255–4258.
[18] von Wiren, N., Gazzarrini, S., Gojon, A. and Frommer, W.B. (2000) The molecular physiology of ammonium uptake and retrieval. Curr. Opin. Plant Biol. 3, 254–261.
[19] Wang, M.Y., Siddiqi, M.Y., Ruth, T.J. and Glass, A.D.M. (1993) Ammonium uptake by rice roots .1. Fluxes and subcellular-distribution of NH_4^+-N-13. Plant Physiol. 103, 1249–1258.
[20] Soupene, E., Ramirez, R.M. and Kustu, S. (2001) Evidence that fungal MEP proteins mediate diffusion of the uncharged species NH_3 across the cytoplasmic membrane. Mol. Cell. Biol. 21, 5733–5741.
[21] Lorenz, M.C. and Heitman, J. (1998) The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J. 17, 1236–1247.
[22] Güldner, U., Heinisch, J., Koehler, G.J., Voss, D. and Hegemann, J.H. (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucl. Acids Res. 30, art. no.-e23.
[23] Sohlenkamp, C., Shelden, M., Howitt, S. and Udvardi, M. (2000) Characterization of *Arabidopsis* AtAMT2, a novel ammonium transporter in plants. FEBS Lett. 467, 273–278.
[24] Wood, C.C., Ritchie, R.J. and Kennedy, I.R. (1998) Membrane potential, proton and sodium motive forces in *Azospirillum brasilense* Sp7-S. FEMS Microbiol. Lett. 164, 295–301.
[25] Liman, E.R., Tytgat, J. and Hess, P. (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 9, 861–871.
[26] Marini, A.M., Springael, J.Y., Frommer, W.B. and Andre, B. (2000) Cross-talk between ammonium transporters in yeast and interference by the soybean SAT1 protein. Mol. Microbiol. 35, 378–385.
[27] Hirata, R., Graham, L.A., Takatsuki, A., Stevens, T.H. and Anraku, Y. (1997) VMA11 and VMA16 encode second and third proteolipid subunits of the *Saccharomyces cerevisiae* vacuolar membrane H^+-ATPase. J. Biol. Chem. 272, 4795–4803.
[28] Greenfield, N.J., Hussain, M. and Lenard, J. (1987) Effects of growth state and amines on cytoplasmic and vacuolar pH, phosphate and polyphosphate levels in *Saccharomyces cerevisiae*: a ^{31}P-nuclear magnetic resonance study. Biochim. Biophys. Acta 926, 205–214.
[29] Preston, R.A., Murphy, R.F. and Jones, E.W. (1989) Assay of vacuolar pH in yeast and identification of acidification-defective mutants. Proc. Natl. Acad. Sci. USA 86, 7027–7031.
[30] Kakinuma, Y., Ohsumi, Y. and Anraku, Y. (1981) Properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. 256, 10859–10863.
[31] Nelson, N. and Harvey, W.R. (1999) Vacuolar and plasma membrane proton-adenosinetriphosphatases. Physiol. Rev. 79, 361–385.
[32] Cakar, Z.P., Sauer, U., Bailey, J.E., Muller, M., Stolz, M., Wallimann, T. and Schlattner, U. (2000) Vacuolar morphology and cell cycle distribution are modified by leucine limitation in auxotrophic *Saccharomyces cerevisiae*. Biol. Cell 92, 629–637.
[33] Mayer, M., Dynowski, M. and Ludewig, U. (2006) Ammonium ion transport by the AMT/Rh homologue LeAMT1;1. Biochem. J. 396, 431–437.
[34] Mayer, M., Schaaf, G., Mouro, I., Lopez, C., Colin, Y., Neumann, P., Cartron, J. and Ludewig, U. (2006) Different transport mechanisms in plant and human AMT/Rh-type ammonium transporters. J. Gen. Physiol. 127, 133–144.
[35] Doyle, D.A., Morais, C.J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280, 69–77.
[36] Dreyer, I., Mueller-Roeber, B. and Köhler, B. (2002) New challenges in plant ion transport research: from molecules to phenomena. Recent Res. Develop. Mol. Cell. Biol. 3, 379–395.
[37] Garbarino, J. and Dupont, F.M. (1988) NaCl Induces a Na^+/H^+ antiport in tonoplast vesicles from barley roots. Plant Physiol. 86, 231–236.
[38] Roberts, J.K.M. and Pang, M.K.I. (1992) Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear-magnetic-resonance spectroscopy. Plant Physiol. 100, 1571–1574.
[39] Yin, Z.H., Kaiser, W.M., Heber, U. and Raven, J.A. (1996) Acquisition and assimilation of gaseous ammonia as revealed by intracellular pH changes in leaves of higher plants. Planta 200, 380–387.
[40] Wilson, G.H., Grolig, F. and Kosegarten, H. (1998) Differential pH restoration after ammonia-elicited vacuolar alkalisation in rice and maize root hairs as measured by fluorescence ratio. Planta 206, 154–161.
[41] Loque, D., Ludewig, U., Yuan, L.X. and von Wiren, N. (2005) Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. Plant Physiol. 137, 671–680.
[42] Howitt, S.M. and Udvardi, M.K. (2000) Structure, function and regulation of ammonium transporters in plants. Biochim. Biophys. Acta-Biomembr. 1465, 152–170.
[43] Ryan, P.R. and Walker, N.A. (1994) The regulation of ammonia uptake in *Chara australis*. J. Exp. Bot. 45, 1057–1067.

[44] Kronzucker, H.J., Siddiqi, M.Y. and Glass, A.D.M. (1995) Analysis of (NH_4^+)-N-13 efflux in spruce roots – a test-case for phase identification in compartmental analysis. Plant Physiol. 109, 481–490.
[45] Gaxiola, R.A., Li, J.S., Undurraga, S., Dang, L.M., Allen, G.J., Alper, S.L. and Fink, G.R. (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc. Natl. Acad. Sci. USA 98, 11444–11449.
[46] Habash, D.Z., Massiah, A.J., Rong, H.L., Wallsgrove, R.M. and Leigh, R.A. (2001) The role of cytosolic glutamine synthetase in wheat. Annals Appl. Biol. 138, 83–89.
[47] Miflin, B.J. and Habash, D.Z. (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. 53, 979–987.
[48] Rawat, S.R., Silim, S.N., Kronzucker, H.J., Siddiqi, M.Y. and Glass, A.D.M. (1999) AtAMT1 gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J. 19, 143–152.
[49] Shelden, M.C., Dong, B., de Bruxelles, G.L., Trevaskis, B., Whelan, J., Ryan, P.R., Howitt, S.M. and Udvardi, M.K. (2001) Arabidopsis ammonium transporters, AtAMT1;1 and AtAMT1;2, have different biochemical properties and functional roles. Plant Soil 231, 151–160.
[50] Hoque, M.S., Masle, J., Udvardi, M.K., Ryan, P.R. and Upadhyaya, N.M. (2006) Over-expression of the rice OsAMT1-1 gene increases ammonium uptake and content, but impairs growth and development of plants under high ammonium nutrition. Funct. Plant Biol. 33, 153–163.