Aquaporin homologues in plants and mammals transport ammonia

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Abstract **Using functional complementation and a yeast mutant deficient in ammonium (NH_4^+) transport (Δ*mep1–3*), three wheat (*Triticum aestivum*) *TIP2* aquaporin homologues were isolated that restored the ability of the mutant to grow when 2 mM NH_4^+ was supplied as the sole nitrogen source. When expressed in *Xenopus* oocytes, *TaTIP2;1* increased the uptake of NH_4^+ analogues methylammonium and formamide. Furthermore, expression of *TaTIP2;1* increased acidification of the oocyte-bathing medium containing NH_4^+ in accordance with NH_3 diffusion through the aquaporin. Homology modeling of TaTIP2;1 in combination with site directed mutagenesis suggested a new subgroup of NH_3-transporting aquaporins here called aquaammoniaporins. Mammalian AQP8 sharing the aquaammoniaporin signature also complemented NH_4^+ transport deficiency in yeast.**
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1. Introduction

The ammonium ion (NH_4^+) and its conjugated base (NH_3) are the primary substrates for the synthesis of amino acids, and are important for all living organisms. NH_4^+ can accumulate to millimolar levels within cells [1]. However, in humans, high levels of exogenous NH_4^+ inhibit insulin release [2,3], cause metabolic acidosis and renal failure [4,5], and have been linked to Alzheimer's disease [4] and hepatic encephalopathy [6]. In plants, high levels of cytoplasmic NH_4^+ result in a futile recycling across the plasma membrane [1] and NH_3 volatilization from leaves [7].

Preston et al. [8] demonstrated that expression of CHIP28, now called aquaporin 1 (AQP1), in frog oocytes created pores in the plasma membrane, which specifically increased water permeability. Diffusion through the lipid bilayer seems not to be the only pathway for gaseous compounds either, given that membranes in several tissues and cell types have low permeabilities for CO_2 and NH_3 [9]. This view has very recently been supported by the finding that CO_2 limited growth correlated with *NtAQP1* expression in tobacco [10].

NH_4^+ uptake at low extracellular concentration in plants and yeast is catalyzed by members of the ammonium transporter/methylammonium permease (AMT/Mep) family [11,12]. These transports have K_m values ranging from 0.5 to 40 μM NH_4^+ [13]. Yeast (*Saccharomyces cerevisiae*) mutants, defective in Mep homologues, were earlier used to clone and characterize AMT homologues from plants and humans by functional complementation [11,14]. Yet, no specific NH_4^+/NH_3 transporter, operating at elevated NH_4^+/NH_3 concentrations, has been isolated or characterized in any organism.

Simultaneous deletion of *MEP1*, *MEP2* and *MEP3*(Δ*mep1–3*) renders yeast dependent on relatively high concentrations of NH_4^+ (>5 mM at pH 5.5) when supplied as the sole nitrogen source [15]. This prompted us to use this yeast mutant in an approach to identify other transporters potentially involved in NH_4^+ acquisition.

Here, we have identified members of the aquaporin superfamily in plants and human that transport NH_4^+/NH_3, hence aquaammoniaporins. We show that substrate specificity of the aquaammoniaporins is correlated with substitutions within the constriction region of the channels providing a larger pore diameter. These substitutions are conserved in both plant and mammalian isoforms.

2. Materials and methods

2.1. Yeast strain and growth

The yeast strain *Saccharomyces cerevisiae* 31019b (*MAT*a, *ura3*, *mep1*Δ, *mep2*Δ*::LEU2 mep3*Δ*::kanMX2*) [15] was transformed with a wheat root cDNA library in pYES2 [16] by electroporation as described (http://www.agr.kuleuven.ac.be/dp/logt/protocol/yeastelectroporation.htm). Transformants were grown on synthetic medium with 2% glucose or galactose, 50 mM succinic acid/Tris base, pH 5.5 (if not indicated otherwise), 0.7% yeast nitrogen base w/o amino acids and NH_4^+ (Difco) supplemented with 0.1% proline or different concentrations of $(NH_4)_2SO_4$ as the sole nitrogen source. Results shown from complementation analysis in yeast are representatives from more than 15 independent assays.

2.2. Oocyte expression and uptake of radiotracer

TaTIP2;1 and *HsAQP1* cDNAs were cloned into an oocyte expression vector containing the 5′- and 3′-untranslated regions for

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Abbreviations: AMT, ammonium transporter; AQP, aquaporin; GlpF, glycerol facilitator; Mep, methyl ammonium permease; MIP, major intrinsic protein; NIP, nodulin-like intrinsic protein; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein

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Xenopus laevis beta-globin and a poly-A segment. RNA preparation and injection of oocytes were as described earlier [17]. After injection of mRNA, the oocytes were kept at 19 °C for 5–6 days in Kulori (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM HEPES-Tris, pH 7.4) before further procedures. Measurements of extracellular pH were done with an extracellular pH-electrode (Radiometer, Copenhagen, Denmark) in 70 mM Na^+ and 20 mM NH_4^+ in 5 mM HEPES/Tris. For each measurement, 20 oocytes were added abruptly to a well stirred bathing medium while recording the pH. Uptake studies were done essentially as described [17]. The uptake medium contained 20 mM methylammonia or formamide supplemented with ^{14}C-methylammonia (Pharmacia) or ^{14}C-formamide (American Radiolabeled Chemicals, St. Louis, MO) to a final activity of 4 μCi/ml. Uptakes were performed at room temperature. Oocytes were subsequently washed in ice-cold Kulori and radiotracer uptake was measured in a scintillation counter (Packard Tri-Carb).

2.3. Modeling

Homology modeling of TaTIP2;1 was performed using the structure of bovine AQP1 (Protein Data Bank Accession No. 1j4n.pdb) as the template [18] and the program MODELLER6v.2 [19]. Graphics were generated using RasWin Molecular Graphics, Windows version 2.6-ucb, ©1993–1995 R.Sayle and software from SYBYL® 6.9 Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.

2.4. Mutagenesis of TaTIP2;1

Mutations in *TaTIP2;1* were introduced by whole plasmid PCR using complementary primers, including the respective mutations. The H64F mutation was introduced using primers GCC ATA TGC TTC GGG TTT GGG CTG T and GCC CAA ACC CGA AGC ATA TGG CCA C, the I184H mutation using primers GGC GCC AAC CAC CTC GTG GCC and GCC GGC CAC GAG GTG GTT GGC, and the G193C mutation using primers TTC TCC GGC TGC AGC ATG AAC CCT GCA C and CAG GGT TCA TGC TGC AGC CGG AGA AGG G. The PCR reactions were digested with *Dpn*I prior to transformation into *Escherichia coli* to digest the methylated template plasmid DNA. All sequences were verified by plasmid sequencing.

3. Results and discussion

3.1. Cloning of ammonium transporters

The yeast strain 31019b (Δmep1–3) [15] was transformed with a wheat root cDNA library in pYES2 [16] and around 100 000 primary transformants were selected. Transformants were plated on media with 2 mM NH_4^+ as the sole nitrogen source. Out of a total of about 100 yeast transformants analyzed, 70% of the clones were yeast mutants transformed with two different wheat *AMT* homologues. The remaining 30% were all transformed with one of the three highly similar wheat tonoplast-intrinsic protein homologues (*TIP2*), members of the aquaporin super-family in plants.

After re-transformation, yeast cells expressing *TIP2*s grew well at concentrations of 2 mM NH_4^+ and above, while at 0.2 mM NH_4^+ almost no difference was seen compared to the control transformed with an empty vector (Fig. 1A). At an NH_4^+ concentration of 10 mM, however, the yeast cells transformed with wheat *TIP2*s grew better than those transformed with a high-affinity transporter of the *AMT* family,

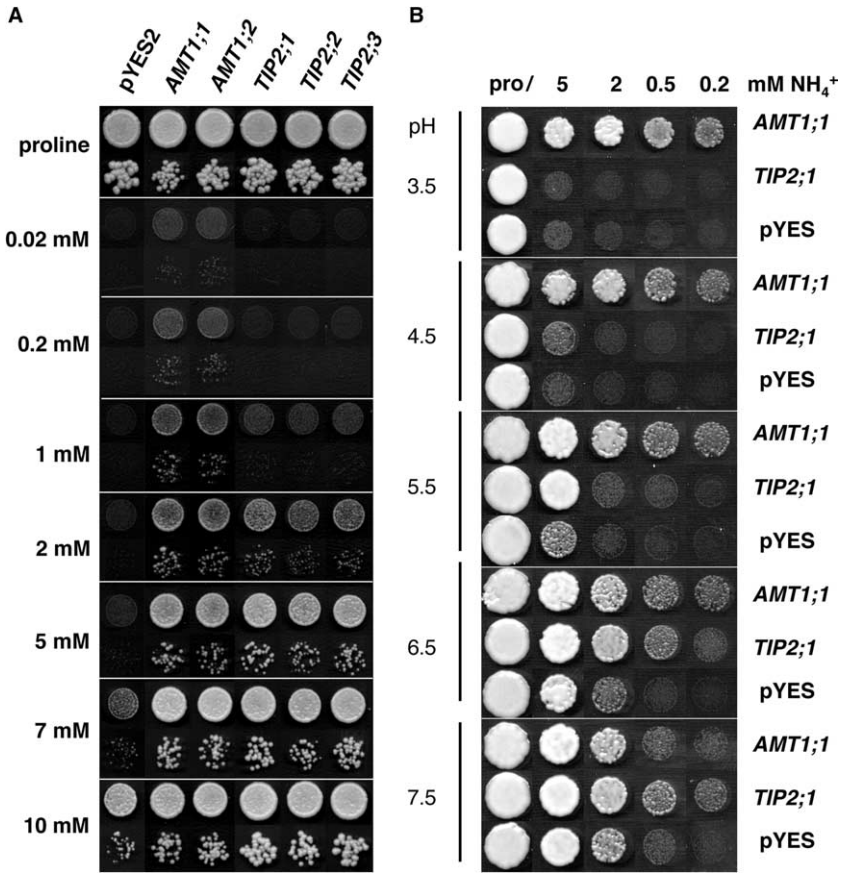

Fig. 1. Complementation by *AMT1* and *TIP2* of the Δ*mep1–3* deletion in yeast. (A) Yeast mutant Δ*mep1–3* (31019b) transformed with *TaAMT* and *TaTIP2* homologues (Accession Nos. AY525637, AY525638, AY525639, AY525640, and AY525641) were spotted onto galactose-containing medium with either proline or different concentrations of NH_4^+ as nitrogen source (10 μl $OD_{600\,nm}$ 10^{-2} and 10^{-5}; upper and lower spot, respectively). Growth was recorded after 6 days at 30 °C. (B) 31019b transformed with either *TaAMT1;1*, *TaTIP2;1* or empty pYES2 (10 μl OD_{660} 10^{-2}) were spotted onto galactose-containing medium with either proline or different concentrations of NH_4^+ at various pH. Growth was recorded after 5 days at 30 °C.

suggesting that NH_4^+/NH_3 specific transporters with low affinity but high capacity had been identified.

3.2. TIP2 facilitates diffusion of NH_3

Growth of yeast expressing *TaTIP2;1* on NH_4^+/NH_3 was strongly pH-dependent, with progressively better growth at increasing pH (Fig. 1B). The same was true for the control 31019b transformed with an empty vector pYES2. However, at all pH values tested, the strain expressing *TaTIP2;1* grew much better than the control at comparable NH_4^+ concentration. Increasing the pH by one unit strongly reduced the requirement of NH_4^+ in the medium, both on solid media (Fig. 1B) and in liquid cultures (data not shown). The data thus strongly suggested that NH_3, rather than NH_4^+, was the nitrogen species being transported by the TIP2s.

Addition of *Xenopus* oocytes to a well stirred bathing medium containing 20 mM NH_4^+ resulted in a continuous acidification of the medium, in line with the interpretation that NH_3 diffused into the oocyte, leaving H^+ in the external medium (Fig. 2A). The pH of the buffer solution was stable in the absence of oocytes and within the time of measurements presented, in line with the interpretation that pH changes were due to NH_3 diffusion into the oocytes rather than its evaporation from the buffer solution (not shown). Acidification was significantly increased after injection with *TaTIP2* mRNA compared to control oocytes injected with water (Fig. 2A; Table 1). Expression of human *AQP1* did not increase NH_4^+ induced acidification compared to water injected controls, although water transport could be demonstrated for both *TaTIP2;1* and *HsAQP1* mRNA injected oocytes (Table 1). Recordings of cytoplasmic pH, used earlier in attempts to measure NH_3 transport into oocytes [20], were strongly dependent on the particular position of the electrode within the oocyte and therefore less reproducible than pH measurements of the bathing medium (data not shown).

To directly demonstrate uptake as dependent on *TaTIP2*s, *Xenopus* oocytes injected with either water or *TaTIP2;1* mRNA were exposed to 20 mM ^{14}C-methylammonium or 20 mM ^{14}C-formamide (Fig. 2B and C). Methylammonium is a well-known analogue of NH_4^+, which has been used extensively in uptake studies to characterize Mep/AMT homologues [13]. Both methylammonia and formamide influx into *Xenopus* oocytes were increased following expression of *TaTIP2;1*. The initial flux of formamide increased to about 250%. Influx of methylammonia was less affected by expression of *TaTIP2;1*, but still evident (Fig. 2C).

3.3. Homology modeling of TaTIP2;1

Aquaporins form a super-family of channel proteins with a common primary structure characterized by a tandem repeat of two similar halves. Each half contains three transmembrane helices and a membrane embedded sequence with an absolutely conserved asparagine involved in formation of the channel pore (reviewed in [21]). Aquaporin homologues with different substrate specificities have recently been crystallized and structural data are available from X-ray diffraction for AQP1, a mammalian aquaporin [18], and the glycerol facilitator (GlpF) [22] and the aquaporin (AqpZ) [23] from *E. coli*, all at high resolution. All structures are surprisingly similar. However, the aqueous pore of GlpF is slightly wider and key hydrophobic residues allow the passage of the carbon backbone of glycerol [24].

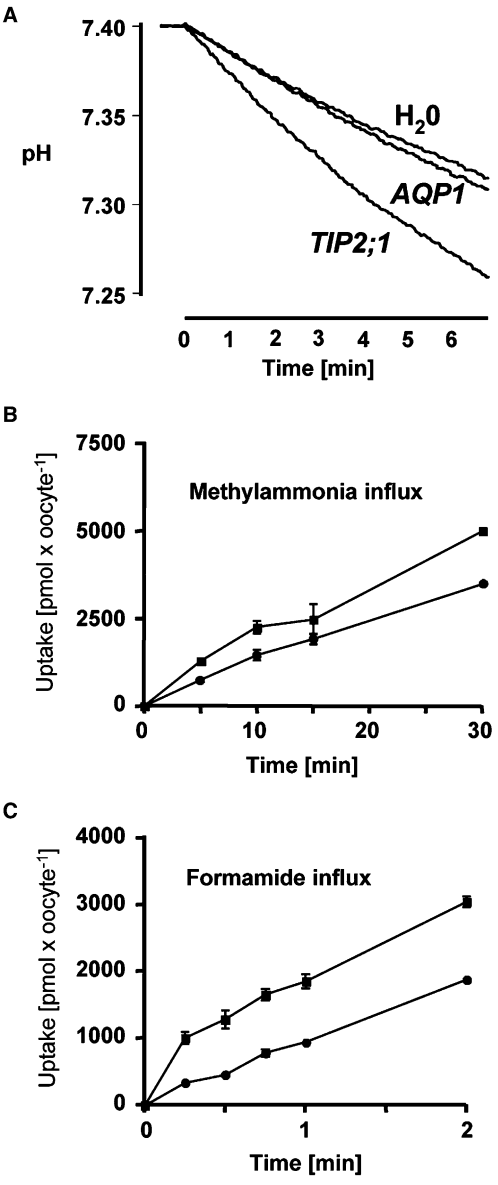

Fig. 2. Addition of NH_4^+/NH_3 and their radiotracer analogues ^{14}C-methylammonia and ^{14}C-formamide to *Xenopus* oocytes expressing *TaTIP2;1* and *HsAQP1*. (A) Oocytes injected with either water, *TaTIP2;1* mRNA or *HsAQP1* mRNA were bathed in a weakly buffered medium and the pH was recorded in the presence of 20 mM NH_4Cl. Proton fluxes are given in Table 1. (B, C) Oocytes injected with either water (●) or *TaTIP2;1* mRNA (■) were bathed in a medium containing either (B) ^{14}C-methylammonium or (C) ^{14}C-formamide. Results from $n = 15$ independent measurements are presented.

Based on a classification by Heymann and Engel [21], TIPs are structurally closer related to water-specific aquaporins than aquaglyceroporins. We used the X-ray structure of bovine AQP1 to create a homology model of TaTIP2;1. The overall sequential homology between AQP1 and TaTIP2;1 is 31%. The model was overlaid with the AQP1 structure and the overlay was subsequently rotated in order to examine differences in the constriction region of the channels (Fig. 3A and B), which are most likely to affect substrate specificity [18]. In the model of TaTIP2;1, F58 of AQP1 is substituted by

Table 1
Medium acidification expressed as H^+ fluxes (J_{H+} [10^{-11} mol s^{-1} oocyte^{-1}]) for oocytes bathed in solutions with different NH_3 concentrations and pH

NH_3 [mM]	pH_e	Ta tip2;1	Hs aqp1	Native
0.036	6.5	0.61 ± 0.046 (7)*	0.36 ± 0.025 (6)	0.37 ± 0.025 (6)
0.28	7.4	3.13 ± 0.22 (7)*	2.30 ± 0.17 (3)	2.06 ± 0.09 (20)
3.6	8.5	22.7 ± 1.8 (7)*	15.8 ± 1.6 (5)	12.6 ± 0.83 (3)
L_p [10^{-5} cm s^{-1} Osm^{-1}]		7.2 ± 0.6 (12)	5.3 ± 0.5 (10)	0.31 ± 0.05 (3)

J_{H+} were calculated from the changes in pH obtained in experiments as those shown in Fig. 2A and the buffer capacity of the bathing medium. pH_e, external pH; L_p, hydraulic conductivity.
* Significantly different from water injected oocytes at the 0.001 probability level. NH_3 concentrations were calculated from the p*K*a of NH_4^+.

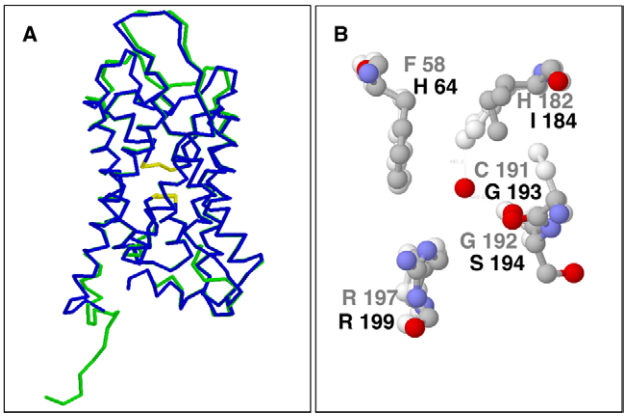

Fig. 3. (A) Overlay of the structure of bovine AQP1 (blue) with the model of TaTIP2;1 (green). The highly conserved NPA sequences are shown in yellow. (B) View through the constriction region of the channels from the extra-cytoplasmic face; residues from TaTIP2;1 are in front and labelled black. Residues from AQP1 are pale and in the background and labelled in grey. The position of the water molecule coordinated by H182 and the carbonyl oxygen of G192 in the structure of AQP1 is indicated as a red ball.

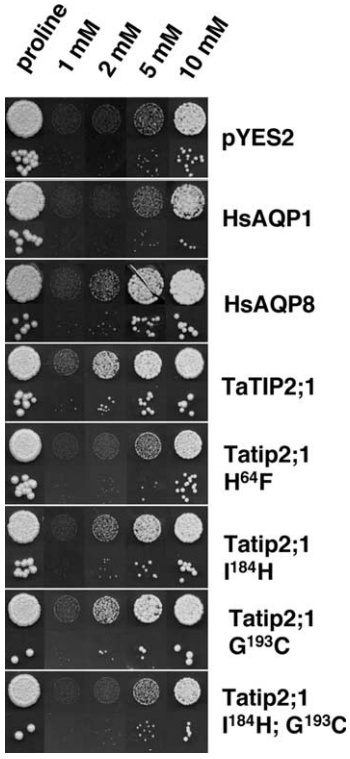

Fig. 4. Substitutions within the constriction region appear to be critical for NH_3 transport through aquaporins. Mammalian *HsAQP1* and *8* and plant aquaporin *TaTIP2;1* as well as mutants of *TaTIP2;1* were expressed in 31019b. The empty vector pYES2 was used as control. Transformants ($OD_{600\,nm}$ 10^{-2} and 10^{-5}; upper and lower spot, respectively) were spotted onto galactose-containing medium with either proline or different concentrations of NH_4^+ as nitrogen source. Growth was recorded after 6 days at 30 °C.

histidine, H182 is substituted by isoleucine and C191 by glycine. These three substitutions cause a widening of the constriction region. Taking into consideration that it is the carbonyl oxygen of C191 (bovine AQP1) [18] that participates in the coordination of the water molecule, the substitutions also increase the overall hydrophobicity of the constriction region.

3.4. *Mutating H64, I184 and G193 by site directed mutagenesis negatively affects yeast complementation*

Human AQP1 was earlier suggested to transport NH_3 when expressed in *Xenopus* oocytes [20] but failed to do so in our study. We therefore expressed in yeast *HsAQP1* and *HsAQP8*. Both isoforms are only about 30% homologous to TaTIP2;1. However, AQP8 shares the identical residue substitutions in the constriction region with TaTIP2. HsAQP1 did not complement the growth defect, while HsAQP8 supported growth on NH_4^+ to a similar extent like TaTIP2s (Fig. 4). In order to investigate the importance of H64, I184 and G193 in the transport of NH_4^+/NH_3, site directed mutagenesis was employed (Fig. 4). When H64 was changed into phenylalanine, the corresponding residue of mammalian AQP1, the resulting mutant *Tatip2;1* $H^{64}F$ no longer supported growth of the yeast mutant on NH_4^+. Substituting I184 by histidine significantly reduced growth of the yeast mutant on NH_4^+ compared to *wt* TaTIP2;1. Mutating G193 into cysteine did not affect yeast complementation. However, yeast transformed with the double mutant *Tatip2;1* $I^{184}H, G^{193}C$ displayed a similar phenotype as the yeast transformed with *Tatip2;1* $H^{64}F$ or the empty vector pYES2 (Fig. 4). Interestingly, H182 together with the carbonyl oxygen of C191 has been shown to coordinate a water molecule in AQP1 [18] and may therefore be crucial to restrict for the passage of H_2O molecules through the channel pore in AQP1. Point mutations employed here are unlikely to interfere with either the folding or the targeting of the mutant enzymes compared to *wt* TaTIP2;1 when expressed in yeast, because these mutations substitute residues in TaTIP2;1 with residues well conserved in other aquaporin homologues in the respective positions. This is further supported by the obser-

vation that neither of the single mutations I184H and G193C prevent ammonia transport. However, the double mutation I184H; G193C strongly impaired complementation on NH_4^+ when expressed in yeast. The data therefore strongly suggest that H64, I184 and G193 in TaTIP2 are important for the transport of NH_4^+/NH_3.

4. Conclusion

In addition to water, members of the aquaporin superfamily have been shown to transport different substrates. These have been classified into orthodox aquaporins transporting H_2O with high specificity and aquaglyceroporins that in addition to H_2O also transport glycerol and urea [25,26]. AQP9 was recently shown to transport an exceptionally wide range of different substrates [27] and may thus represent a member of a separate group. Data presented here provide evidence for another sub-family within the aquaporin superfamily that facilitates diffusion of NH_3 across biomembranes.

A database search revealed that many other MIPs, in particular in plants (TIPs and nodulin-like intrinsic proteins (NIPs)), contain isoleucine or valine and glycine or alanine in the respective positions (182 and 191 in bovine AQP1). In the corresponding position of F58 (bovine AQP1), NIPs are substituted by tryptophan, leucine or alanine, which would render the constriction region even more hydrophobic as compared to TIP2 and AQP8. Both the dipole moment of the NH_3 molecule (1.49 vs. 1.85 D) and the dielectric constant of liquid NH_3 (22 vs. 80) are considerably lower than the corresponding values for water. A more hydrophobic environment at the constriction region may therefore allow the transport of NH_3 and the slightly larger size of the ammonia molecule may require a larger pore diameter. The exchange of His from position 182 to position 58 (in the sequence of bovine AQP1) affects the arrangement of hydrogen bond acceptors and donors in the restriction region and is likely to be a major factor in selectivity.

TIPs represent a group of aquaporin homologues in plants, which are expressed in vacuolar membranes [28]. Recently, *AtTIP2;1* expression in *Arabidopsis* roots was shown to be upregulated in response to nitrogen starvation [29]. One possible explanation is that TIP2 functions in remobilization of NH_4^+/NH_3 from vacuoles under starvation. However, TIP2 or other TIPs may also be important for NH_4^+/NH_3 compartmentalization.

The major intrinsic protein (MIP) family in *Arabidopsis* has recently been classified into eight different groups according to residues lining the constriction region of the channel pore [30], a classification partially overlapping with the traditional classification grouping plant MIPs according to localizations such as TIP for tonoplast [31] and PIP for plasma membrane [32] localized isoforms. For instance, all *Arabidopsis* PIPs share a F/H/T/R signature, suggesting water specificity, while NIPs and especially TIPs are characterized by a number of various different signatures. Screening a wheat root cDNA library, here, only TIP2s characterized by a H/I/G/R signature were identified that are able to transport ammonia. The present paper therefore supports the view that the constriction region of MIPs represents a major factor for selectivity of the channel proteins and that a functional classification may be more appropriate than classification according to localization, as suggested earlier [33].

Interestingly, TIP2 was recently identified by mass-spectrometry in purified peribacteroid membranes from *Lotus* [34]. Thus, TIP2, but also NIPs, may be responsible for the transport of NH_3 from nitrogen-fixing symbionts to the plant. Ammonia permeablility through the peribacteroid membrane was earlier shown to be reversibly sensitive to the channel blocker mercury [35].

AtTIP2;1 was recently identified by mass spectrometry in plasma membrane preparations of *Arabidopsis* (Erik Alexandersson, personal communication) and was found to be localized to the plasma membrane when transiently overexpressed with a GFP tag in *Arabidopsis* protoplasts [29]. It is therefore tempting to speculate that TIP2 may be involved in low affinity and high capacity uptake at the plasma membrane.

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