FEBS Letters 583 (2009) 2833–2838

FEBS Letters

journal homepage: www.FEBSLetters.org

Channel-like NH_3 flux by ammonium transporter AtAMT2

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ARTICLE INFO

Article history:
Received 19 June 2009
Accepted 1 July 2009
Available online 25 July 2009

Edited by Julian Schroeder

Keywords:
Methylamine
Methylammonium
Gas channel
H^+-coupling

ABSTRACT

Prokaryotes, plants and animals control ammonium fluxes by the regulated expression of ammonium transporters (AMTs) and/or the related Rhesus (Rh) proteins. Plant AMTs were previously reported to mediate electrogenic transport. Functional analysis of AtAMT2 from Arabidopsis in yeast and oocytes suggests that NH_4^+ is the recruited substrate, but the uncharged form NH_3 is conducted. AtAMT2 partially co-localized with electrogenic AMTs and conducted methylamine with low affinity. This transport mechanism may apply to other plant ammonium transporters and explains the different capacities of AMTs to accumulate ammonium in the plant cell.

1. Introduction

Ammonium (this term designates the sum of NH_4^+ and NH_3) is an important nutrient and ubiquitous intermediate in nitrogen metabolism. Its transport and distribution across cellular membranes depends on AMT/Rh ammonium transporters [1,2]. In plants and microorganisms, the AMT/Rh-mediated ammonium transport is critical for providing sufficient nitrogen for optimal growth [1,2].

The crystal structures of several AMT/Rhs, including AmtB from *Escherichia coli*, showed that these proteins are arranged as (homo-) trimers and each subunit forms a hydrophobic pore in its center [3–5]. There is compelling evidence that NH_4^+ is recruited by AMT/Rhs at the external pore entrance, but the structures suggested that the ion is de-protonated, and NH_3 passes the channel [3–5]. This mechanism is supported by molecular simulation studies on EcAmtB, although the site of de-protonation is disputed [6–8]. A central phenylalanine has recently been experimentally determined to be critical [9]. Although some functional evidence supports this mechanism [3], several studies questioned whether "equilibrative" NH_3 transfer occurs in EcAmtB [9–11]. The structurally related plant AMT1s, in contrast, function as net transporters for NH_4^+ [12–16].

In the model plant *Arabidopsis*, three ammonium transporters (AtAMT1;1, AtAMT1;2 and AtAMT1;3) were responsible for ~90% of the total high affinity uptake in roots [17]. The more distant AtAMT2 was also expressed in roots, but did not contribute to the uptake [17,18]. In general, the molecular function and physiological role of plant AMT2 proteins is much less evident than those of AMT1s, although their potential relevance is highlighted by their abundance in plant genomes [1]. While *Arabidopsis* has only a single *AMT2* gene, other plants have multiple *AMT2* sequences in their genomes, e.g. rice has seven *AMT2*-like isoforms [1]. In this study, AtAMT2 was functionally analyzed in yeast and oocytes. The results suggest that AtAMT2 recruited NH_4^+, but mediated electroneutral ammonium transport, probably in the form of NH_3.

2. Materials and methods

2.1. Generation of constructs

The cDNA from *AtAMT2* (At2g38290) was amplified from a Landsberg cDNA library by PCR using Phusion polymerase (New England Biolabs, Ipswich, MA) with the following primers: AMT2-Fw: CAGGGATCCATGGCCGGAGCTTACGATCCAAG AMT2-Rv: GAGCTCGAGTCATAGAACAATGGTGACACCTCTAG. This and other PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) and were fully sequenced. The *AtAMT2* open reading frame was identical to the *AtAMT2* sequence from the *C24* ecotype (*AAF37192*) and contained two nucleotide exchanges compared to the *Col-0* sequence. The translated protein sequences differ at the non-conserved position 95: in *Col-0* that residue is aspartate, while in *C24* it is asparagine. Two independent clones confirmed that this amino acid exchange compared to *Col-0* did not result from a PCR error. The sequence was further subcloned into pOO2

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.07.039

(oocyte) and pDR199 (yeast) expression vectors using the BamHI and XhoI sites.

The 1.7 kB promoter fragment 5′ upstream of the *ATG* of *AtAMT2* was amplified from genomic *Arabidopsis* (ecotype *Col-0*) DNA using the forward primer AMT2-Prom-Fw: GAGAGGTACCAATGATTCGATCTTTTGTCTTCTCATAG and the reverse primer AMT2-Pr-Rv: CTTGGATCGTAAGCTCCGGCCATTTTGTTATTC. By a recombinant PCR of the *AtAMT2* ORF and the promoter fragment with the primers AMT2-Prom-Fw and AMT2-GFP-Rv: GAGATCTAGATAGAACAATGGTGACACCTCTAGCAC, a 3.1 kB *pAMT2:AtAMT2* fragment was obtained. This was cloned into *pCR-BluntII-TOPO*, sequenced and subcloned into the plant transformation binary vector *pTkan^{+}GFP* using the KpnI and XbaI restriction sites. The reverse primer was designed to eliminate the STOP codon and to generate a translational fusion with GFP.

2.2. 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 to achieve homogeneity was performed on MS plates containing 1X Murashige and Skoog salt (Duchefa), 1% sucrose, 0.8% agar and 50 μg/ml kanamycin. 6 to 30-day old fluorescent plants, grown on standard medium (0.8% Agar, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 100 μM $NaSiO_3$, 1.25 mM $CaSO_4$, 50 μM Na–Fe–EDTA, 50 μM H_3BO_3, 3 μM $MnSO_4$, 1 μM $ZnSO_4$, 1,3 μM $CuSO_4$, 0,03 μM $Mo_7O_{24}(NH_4)_6$ and 20 mM MES, with the pH adjusted to 6.0 using Tris) supplemented with 0–200 μM NH_4NO_3 as nitrogen source were analyzed by a Leica confocal microscope (Wetzlar, Germany) using the 488 nm Ar laser excitation beam line and a 505–530 nm band pass filter.

2.3. Yeast transformation

The plasmids containing the respective open reading frames were heat shock-transformed in the *ura*$^{-}$ wild type (23344c), and the *ura*$^{-}$ ammonium transporter defective yeast strain (31019b; *triple-Δmep*) [19]. Selection for transformed yeast was done on solid arginine medium (2% Agar, 0.17% YNB w/o amino acids and ammonium sulfate (Difco), supplemented with 3% glucose and 0.1% arginine (Arg) as nitrogen source, buffered with 20 mM MES/Tris, pH 6.1.

2.4. Yeast growth assays

Yeast was grown in liquid Arg medium until OD_{595} (optical density at 595 nm) reached 0.6–0.8. Cells were harvested, washed and resuspended in water to a final OD_{595} of 2. 10 μl of OD_{595} 2 cells and 5-fold dilutions were spotted on Arg medium with or without MeA (pH 6.0), or media containing no Arg, but 1 mM NH_4Cl as sole nitrogen source, as well as 10 mM $MgCl_2$ and 100 mM KCl.

2.5. Yeast uptake

Yeast was grown in liquid arginine medium until the OD_{595} reached 0.6–0.8. Cells were harvested, washed and resuspended in uptake buffer (50 mM potassium phosphate buffer supplemented with 0.6 M sorbitol, pH 6) to a final OD_{595} of 5. Before the uptake, cells from the data shown were energized by adding 10 μl of 1 M glucose to 100 μl cells OD_{595} 5 and incubated for 7 min at 30 °C. As controls, uptakes were also done with non-energized cells (by adding 10 μl uptake buffer and incubation), which gave qualitatively the same results. The uptake was started by adding 110 μl of uptake buffer containing ^{14}C-MeA (2.11 Gbq/mmol, Amersham Bioscience). 50 μl samples were taken after 30, 60, 120 and 300 s and washed three times in 4 ml ice-cold washing solution (uptake buffer containing a 100-fold excess of non-labled MeA). Immediately after taking the sample, the solution was filtered through glass fiber filters (GF/C, Whatman). The filters containing the yeast were measured by a liquid scintillation counter.

2.6. Electrophysiological measurements, preparation and injection of oocytes

These methods have been described in more detail elsewhere [20]. Briefly, oocytes were taken from adult females, dissociated by collagenase treatment (2 μg/ml, 1.5 h) and injected with 50 nl of cRNA (1 ng/nl). Oocytes were kept in ND96 for 3 days at 16 °C and then placed in a small recording chamber containing the recording solution (in mM): 110 CholineCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 N-morpholinoethane sulfonate (MES), pH adjusted to 6.0 with tris(hydroxymethyl) aminomethane (TRIS). Ammonium and methylammonium (MeA) were added as Cl salts.

2.7. Homology modeling and display of plant AMTs

This was carried out using the MODELLER software package (version 9v3, http://salilab.org/modeller/modeller.html). The models were based on three high-resolution structures of EcAmtB (PDB ID: 1U7G, 1XQF, 2NS1) and the high-resolution structure of AfAmt-1 (PDB ID: 2B2H). 42 amino acids (AA) of the N-terminus and 33 AA of the C-terminus of the primary sequence of AtAMT1;2 were truncated. In AtAMT2, 13 AA of the N-terminus and 38 AA of the C-terminus of the primary sequence were removed. Multiple sequence alignments were performed using the clustalW method with standard parameters from MegAlign. The sequence alignments were inspected visually, minor adjustments were made and these were then used for homology modeling. Graphical representations were prepared using the software 'Pymol' (http://www.pymol.org).

3. Results

3.1. AtAMT2 partially co-localized with AtAMT1 transporters

AtAMT2 expression in roots has been reported, but AtAMT2 did not contribute to the ammonium uptake by the roots [17]. To resolve this discrepancy, the cell specificity of AtAMT2 expression was analyzed. The gene was tagged with green fluorescent protein and expressed from the endogenous promoter. The rhizodermal cells of lateral roots were labeled with AtAMT2-GFP, and a weaker expression in inner root tissues was observed (Fig. 1). AtAMT2 localized to the plasma membrane of the root hairs (Fig. 1). This expression in the plasma membrane of rhizodermal cells is shared by AtAMT1;1 and AtAMT1;3 [14,21]. In photosynthetic tissues, AtAMT2 expression was most significant in the pith of the stem, the petioles and leaf hydathodes (Supplementary Fig. S1). A slightly distinct pattern had been reported using a reporter construct with a shorter promoter [18]. The AtAMT2-GFP fluorescence was lower in roots than in shoots and was slightly up-regulated by nitrogen starvation, in accordance with the mRNA and protein levels [17].

3.2. AtAMT2 is functionally distinct from AtAMT1 transporters when expressed in yeast

Since the localization data conflicted the lack of AtAMT2 activity in roots, the transporter was expressed in yeast and further functionally characterized. In accordance with earlier data [18], AtAMT2 improved the growth of a yeast strain lacking endogenous ammonium transporters (*triple-Δmep*) on selective media (Fig. 2A).