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AtTIP1;3 and *AtTIP5;1*, the only highly expressed Arabidopsis pollen-specific aquaporins, transport water and urea

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Abstract **Pollination includes processes where water and/or solute movements must be finely regulated, suggesting participation of aquaporins. Using information available from different transcriptional profilings of *Arabidopsis thaliana* mature pollen, we showed that the only aquaporins that are selectively and highly expressed in mature pollen are two TIPs: *AtTIP1;3* and *AtTIP5;1*. Pollen exhibited a lower number and more exclusive type of aquaporin expressed genes when compared to other single cell transcriptional profilings. When characterized using Xenopus oocyte swelling assays, *At*TIP1;3 and *At*TIP5;1 showed intermediate water permeabilities. Although they displayed neither glycerol nor boric acid permeability they both transported urea. In conclusion, these results suggest a function for *At*TIP1;3 and *At*TIP5;1 as specific water and urea channels in Arabidopsis pollen.**

Keywords: Arabidopsis; Tonoplast aquaporin; Pollen tube; Solute transport; Urea; Pollen hydration

1. Introduction

Pollination is a vital process for flowering plants, ensuring not only species maintenance but also enhancing genetic diversity. The whole process begins when pollen lands on a stigma, continues as it germinates and grows a pollen tube through style intercellular spaces and ends upon reaching the ovary where fertilization occurs.

In angiosperms, mature pollen grains are dehydrated when released, and accordingly have an almost inactive metabolism [1]. The first step for an efficient pollination is the hydration of pollen grains, a process that in dry stigmas only occurs if a compatible communication between the pollen and the pistil is established. After hydration, water increases the internal hydrostatic pressure of pollen, a key point in the development of the pollen tube. To provide the ability for both spatial and temporal regulation, pollen hydration is a finely regulated process. Natural candidates involved in water or solute movement are aquaporins, membrane proteins that specifically regulate the flow of large amounts of water across cellular membranes [2]. Plant aquaporins are classified into four main subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs) and small and basic membrane intrinsic proteins (SIPs) [3]. Many of the TIPs and PIPs are water channels although some of the isoforms additionally transport solutes such as glycerol, urea or boric acid [3]. Considering that pollen hydration is the first step for pollination, our hypothesis is that aquaporins might be present in mature pollen grains and potentially involved in water and/or solute uptake from the stigma or/and style.

Analysis of gene expression has helped to identify genes responsible for pollen development. In particular the *Arabidopsis thaliana* genome has been an important source of information for the study of gene expression patterns in pollen through the use of commercial oligonucleotide microarrays [4–9]. Even though the Arabidopsis genome contains 35 loci for aquaporins, functional analyses of many of them are still missing. In this paper, we show the functional characterization of the aquaporins genes that are highly and selectively expressed in mature pollen of *A. thaliana*. Interestingly, we found only two *TIP* loci, *AtTip1;3* and *AtTip5;1*. Using Xenopus oocyte swelling assays, we established that *At*TIP1;3 and *At*TIP5;1 are bifunctional aquaporins with intermediate levels of permeability for water and high permeability for urea.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis (*A. thaliana*) ecotype Col-0 plants were used. Growth conditions were light/dark cycle 16 h/8 h, light intensity of 150 μmol m^{-2} s^{-1}, and temperature of 22–25 °C.

To collect pollen, inflorescences from 200 plants were cut off, shaken in a conical tube with 25 ml of TE (Tris–EDTA) buffer and filtered using several layers of cheesecloth. Pollen was later pelleted by centrifugation at 3500 × g for 10 min.

2.2. RNA extraction and RT-PCR

Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. For pollen extraction, initially, 100 μl of 0.5 mm glass beads were added to the resuspended pollen and vortexed for 10 min [10]. Samples of 200 ng total RNA isolated from roots, leaves and pollen were reverse-transcribed

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in a 20 μl reaction using MMLV-RT (Promega, Madison, WI, USA) following the manufacturer's instructions. For PCR amplification, 2 μl of RT reaction was used. The PCR reactions were carried out in 25 μl with 0.8 μM of each primer (tip5;1f 5′GGACTAGTGAAATTGATGAGAAGAATGATTCC3′; tip5;1r 5′GGGAATTCAGTCATTACACACCAATGGCATC3′; tip1;3f 5′GGGAATTCCCTAACTTAGAAATCATTAGAAGG3′; and tip1;3r 5′GGACTAGTAACGATGCCTATCAACAGAATTGCG3′).

2.3. Cloning and expression of A. thaliana pollen aquaporins

cDNAs of *A. thaliana* aquaporin genes (*AtTIP1;3* and *AtTIP5;1*) were amplified by PCR from pollen cDNA, subcloned into pSGEM, a pGEM-derived vector and sequenced [11]. *AtPIP2;3* and *AQP1* cRNAs were used as controls. cRNAs were synthesized using T7 RNA polymerase and purified following the manufacturer's instructions (Promega, Madison, WI, USA). cRNAs were suspended in water and diluted to a final concentration of 1 μg μl^{-1}.

2.4. Oocyte preparation, cRNA injection and swelling assays

Xenopus laevis mature oocytes (stage V–VI) were isolated and incubated in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.4) supplemented with 1 μg ml^{-1} gentamicin (Gibco-Invitrogen, Carlsbad, CA, USA) at 18 °C as described previously [12]. Defolliculated oocytes were microinjected using an automatic injector (Drummond Scientific Co., Broomall, PA, USA) with 50 nl of in vitro cRNAs (50 ng). Distilled water was used as negative control. Typically, 3 days after the injection, single oocytes were individually transferred from Barth's solution (200 mosmol kg^{-1}) to a Barth's solution diluted 1:5 with water (final osmolarity 40 mosmol kg^{-1}). Changes in cell volume were monitored and videocaptured as described [13]. To determine the kinetics of oocyte volume (V) change, the area of each oocyte was measured for each experimental time. The osmotic water permeability (P_f) was calculated according to the following equation:

$$P_f = \frac{V_0\left(\frac{dV}{dt}\right)}{S * V_a * (\Delta Osm)}$$

where V_0 is the initial oocyte volume (9×10^{-4} cm^3), S is the initial oocyte surface, V_a is the molar volume of water (18 cm^3 mol^{-1}) and ΔOsm is osmolarity difference between oocyte cytosol and external media [14].

2.5. Solute measurement

Oocytes were transferred into iso-osmotic Barth's solution where iso-osmolarity was achieved by adding a solute (boric acid, glycerol or urea) to a five-fold diluted Barth's solution [15,16]. A combination of 25 ng cRNA of *AtTIP5;1* or *AtTIP1;3* alone or together with 25 ng of *AtPIP2;3* cRNA were injected in individual oocytes. Water and cRNA from *AtPIP2;3* alone were used as controls. Solute permeabilities were compared analyzing the initial swelling rates ($d(V/V_0)/dt$).

2.6. Statistical analysis

Water permeability results were log-transformed and then retro-transformed to show the values in the figure. Confidence intervals (CI) are reported. Solute results are reported in the form of mean ± S.E.M. Experiments were always repeated at least three times. Significant differences between treatments were calculated using t-test.

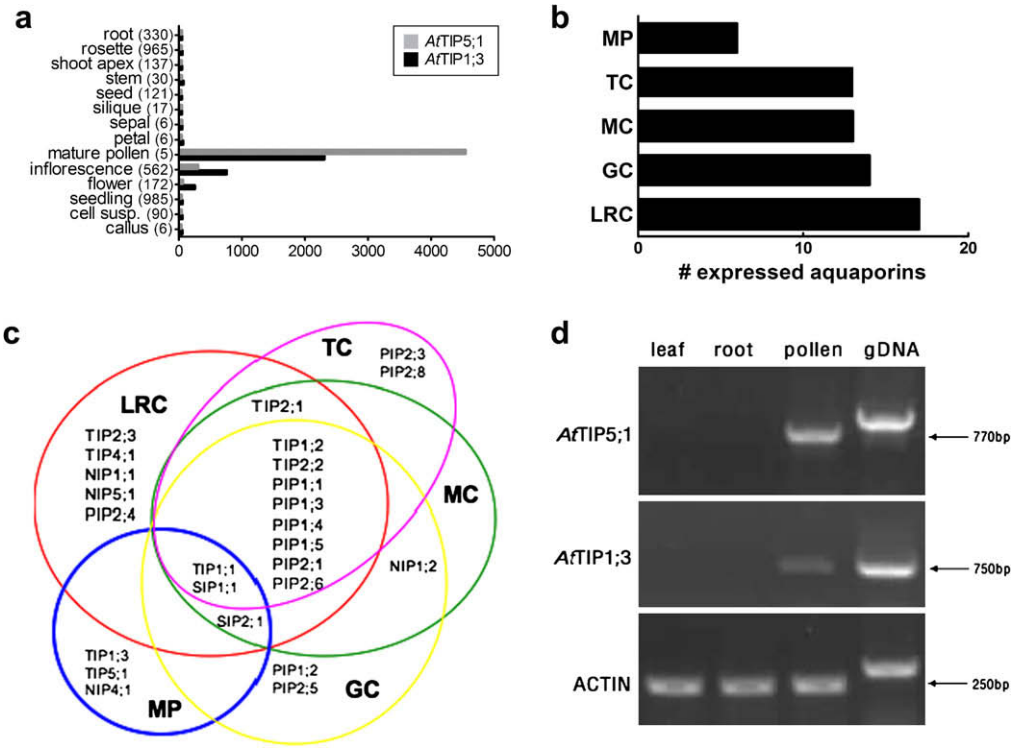

Fig. 1. (a) Expression profiles of *AtTIP1;3* and *AtTIP5;1* in various Arabidopsis organs. The *x*-axis represents the signal intensity (arbitrary units). Data used to create the Digital Northern were obtained from the public GENEVESTIGATOR data set (http://www.genevestigator.ethz.ch) [17] together with other expression analyses [4,5,8]. Parentheses indicate the number of different array experiments done for each tissue. (b) and (c) Comparison of aquaporin gene expression between mature pollen and different single cell types. (b) The *x*-axis represents the number of putative aquaporins present in different single cell-type microarrays (according to the authors criteria). Mature pollen grains (MP); trichome cells (TC); mesophyll cells (MC); stomatal guard cells (GC) and lateral root cap stage I (LRC). (c) Venn diagram showing the overlap and differences of aquaporin expressed genes in mature pollen (MP) compared to trichome cells (TC), mesophyll cells (MC), stomatal guard cells (GC) and lateral root cap cells stage I (LRC). (d) *AtTIP1;3* and *AtTIP5;1* are selectively expressed in mature Arabidopsis pollen. RT-PCR analysis of transcript levels of *AtTIP1;3* and *AtTIP5;1* from leaf, root and mature pollen of *Arabidopsis thaliana*. Control reaction was performed with actin-specific primers [30]. Arabidopsis genomic DNA (gDNA) was included as a control.

3. Results

In order to characterize the aquaporin genes that are expressed in mature pollen, we used information available from different transcriptional profilings of *A. thaliana* mature pollen [4–9]. We selected all aquaporin homologs that were selectively expressed in pollen and also ranked within the top 50% of pollen-expressed genes (according to [7]). Using these criteria only two of the 35 aquaporin loci present in the Arabidopsis genome were selected. Interestingly, they both belonged to the TIP subfamily: *AtTIP5;1* (GeneID: 823898, locus tag: At3g47440) and *AtTIP1;3* (GeneID: 828051, locus tag: At4g01470). *AtTIP5;1* and *AtTIP1;3* are also both ranked within the top 10% of Arabidopsis mature pollen-expressed genes [4–9]. Microarray data available at GENEVESTIGATOR (http://www.genevestigator.ethz.ch) showed for *AtTIP5;1* and *AtTIP1;3* the highest expression level in mature pollen, moderate in flower, very low in inflorescence and non-detectable in any other plant tissue (Fig. 1a) [17]. We also performed a comparative analysis of aquaporin expression between mature pollen (MP) [7] and single cell types such as trichome cells (TC) [18], mesophyll cells (MC) [19], stomatal guard cells (GC) [19] and lateral root cap stage I (LRC) used as a representative of the transcriptomic analysis obtained for three developmental stages in five different domains of Arabidopsis root [20]. We found that trichome cells (TC), mesophyll cells (MC), stomatal guard cells (GC) and lateral root cap cells stage 1 (LRC), or any other root cell type (data not shown), all expressed a larger number of aquaporin genes (13, 13, 14 and 17, respectively) compared to Arabidopsis mature pollen grains that only expressed 6 (Fig. 1b). Venn diagram analysis confirms the big divergences in aquaporin gene expression of mature pollen when compared to their expression in these other single cell vegetative tissues (Fig. 1c). Three of the six mature pollen aquaporins (*AtSIP1;1*, *AtSIP2;1* and *AtTIP1;1*) are also expressed in most of these vegetative tissues while *AtTIP1;3*, *AtTIP5;1* and *AtNIP4;1* are the pollen-selective genes. These comparative expression analyses show a lower number of aquaporin pollen-expressed genes compared to the sporophytic tissues analyzed, reaffirming the repressive state of the Arabidopsis mature pollen transcriptome. Pollen transcriptional profilings also show that while *AtNIP4;1* is expressed within the lowest 5% of the mature pollen genes, *AtTIP1;3* and *AtTIP5;1*, are also among the most highly expressed genes in mature pollen, suggesting a specialized function in post-pollination events [4–9].

To confirm the in silico expression data, RT-PCR for *AtTIP5;1* and *AtTIP1;3* genes was performed in leaves, roots and mature pollen of *A. thaliana*. In concordance with the array results, expression of *AtTIP5;1* and *AtTIP1;3* genes was found in mature pollen but not in roots and leaves (Fig. 1d).

To functionally characterize *At*TIP5;1 and *At*TIP1;3, their cRNAs were individually expressed in Xenopus oocytes. In swelling assays using 1:5 diluted Barth's solution, *At*TIP5;1 and *At*TIP1;3 both induced oocyte swelling (Fig. 2). For oocytes expressing *At*TIP1;3, the water permeability (P_f: 12.9×10^{-4} cm s^{-1}, CI between 11.8 and 15.6×10^{-4} cm s^{-1}, $n = 26$) was two times higher than the P_f of water-injected oocytes (P_f: 6.6×10^{-4} cm s^{-1}, CI between 5.9 and 7.3×10^{-4} cm s^{-1}, $n = 13$), whereas *At*TIP5;1 expression led to a threefold increase (P_f: 18×10^{-4} cm s^{-1}, CI between 15.8 and

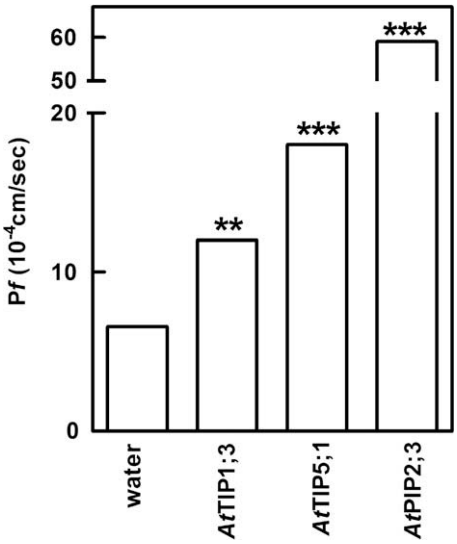

Fig. 2. *At*TIP5;1 and *At*TIP1;3 both show intermediate levels of water permeability. Xenopus oocytes were injected with water and *AtPIP2;3* (controls), or with *AtTIP1;3* or *AtTIP5;1* and then tested for water permeability (P_f). P_f values were estimated from the rate of cell volume change upon hypo-osmotic shock. Data obtained from independent experiments including different batches of oocytes and different *TIP* mRNA extractions, were log-transformed for statistical analysis. P_f means, but not their standard errors, were retro-transformed to show the values in the figure. Confidence intervals (CI) are indicated in the text. Statistically significant differences are indicated by asterisks (*t*-test, $^{***}P < 0.001$ and $^{**}P < 0.01$).

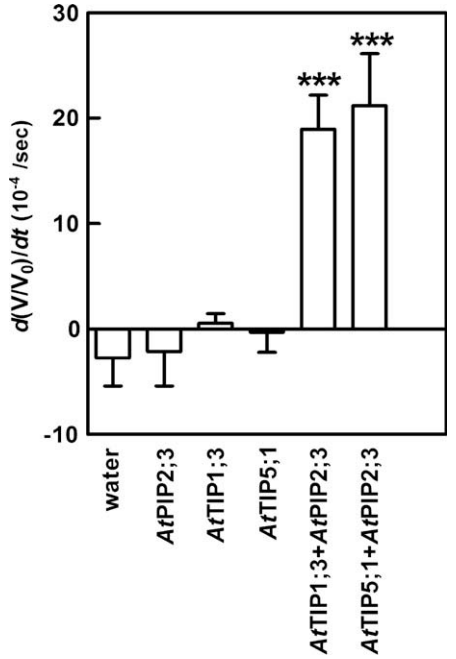

Fig. 3. *At*TIP1;3 and *At*TIP5;1 are both urea transporters with high levels of permeability. *Xenopus* oocytes were injected with water, or alone with *AtPIP2;3*, *AtTIP1;3* or *AtTIP5;1* or co-injected with *AtPIP2;3* + *AtTIP1;3* or with *AtPIP2;3* + *AtTIP5;1* and tested for urea permeability. Bars represent the initial swelling rates ($d(V/V_0)/dt$) in experiments run up to 80 s in 10^{-4} s^{-1}. All values are mean ± S.E.M. ($n = 15$–18). Statistically significant differences are indicated by asterisks (*t*-test, $^{***}P < 0.001$).

21.5×10^{-4} cm s^{-1}, $n = 30$). *At*PIP2;3 was included as a positive control and showed its typical high water permeability (P_f: 59.4×10^{-4} cm s^{-1}, CI between 56.8 and 62.0×10^{-4} cm s^{-1}, $n = 13$). Therefore, *At*TIP5;1 and *At*TIP1;3 are aquaporins that show moderate water permeability.

We then tested whether *At*TIP5;1 or *At*TIP1;3 are involved in the transport of small, uncharged solutes across the membrane. First, we analyzed urea permeability in an isoosmotic swelling assay with a inwardly directed gradient of urea [15]. Xenopus oocytes were transferred from Barth's solution (200 mosmol kg^{-1}) to a five-fold diluted Barth's solution supplemented with urea up to 200 mosmol kg^{-1}. Thus, this final solution behaves as isotonic unless the aquaporin is permeable to urea. Under these conditions, the increase in oocyte volume would be a consequence of water influx driven by the osmotic gradient caused by the initial urea uptake (chemical gradient). It is therefore important that the water permeability of the tested aquaporin is sufficiently high not to become restrictive for the final swelling process [15].

Oocytes injected with *AtTIP5;1* or *AtTIP1;3* alone showed no significative swelling rates (Fig. 3). To discard if their moderate water permeabilities were not sufficiently high for the subsequent swelling response [16] and test if indeed *At*TIP5;1 and *At*TIP1;3 transport urea, oocytes were injected with cRNA of either *AtTIP5;1* or *AtTIP1;3* together with *AtPIP2;3* an aquaporin that displayed high water permeability [21] (Fig. 2). *AtPIP2;3* alone (Fig. 3) or co-injected with *AQP1*, a water but not urea transporter [16], does not uptake urea (data not shown). Therefore, the co-injection of *AtPIP2;3* with any of these two TIPs will favor the subsequent urea swelling process if any of them are permeable to urea. Under these conditions, oocytes showed high swelling rates, increasing about 21-fold for *At*TIP5;1 and 18-fold for *At*TIP1;3 (Fig. 3 and Table 1). Similar results were obtained using AQP1 instead of *At*PIP2;3 as the selective water transporter (data not shown). This result in Xenopus oocytes clearly demonstrates that *At*TIP5;1 and *At*TIP1;3 display high urea permeability. Interestingly, the solute transport is restricted to urea, as glycerol or boric acid were not transported by *At*TIP5;1 or *At*TIP1;3 (Table 1).

As mentioned before, no other TIPs or PIPs are expressed in pollen. However, by RT-PCR we found that *AtNIP4;1* and *AtSIP1;1* are both expressed in Arabidopsis mature pollen (data not shown). *AtNIP4;1* is selectively expressed in pollen but expressed within the lowest 5% of the mature pollen genes [4–8]. We found that *At*NIP4;1 had moderate levels of water permeability (2.5-fold compared to the water control), but did not transport urea when was co-injected with *At*PIP2;3 (data not shown). *AtSIP1;1* is also expressed in mature pollen but also in sporophytic tissues [4–8]. In conclusion, the only highly pollen-expressed genes *At*TIP5;1 and *At*TIP1;3 are the best candidates for water and urea transport in mature Arabidopsis pollen.

4. Discussion

Because of the implication of the process of water transport during pollen hydration and pollen tube development, the study of mature pollen-specific aquaporins is of great interest for plant reproductive biology. In this work, we show that *At*TIP5;1 and *At*TIP1;3, two TIPs selectively expressed in mature pollen of *A. thaliana* within the top 10% of mature pollen-expressed genes, are both water and urea transporters. According to the information obtained from several transcriptional profiling of *A. thaliana* mature pollen, PIPs are not significantly expressed in Arabidopsis mature pollen [4–8]. Even though *AtPIP2;8* expression is high during Arabidopsis pollen development, it declines to basal levels in mature pollen [4–8]. Without discarding that other aquaporins (besides *AtNIP4;1, AtSIP1;1*, *AtSIP2;1* and *AtTIP1;1*) are expressed in mature pollen, still TIPs seems to be the most relevant subtype of aquaporins expressed in Arabidopsis mature pollen. It is also possible that some aquaporin genes are induced to higher levels upon germination as it seems to occur with *AtNIP4;1* that increases its expression level from almost non detectable levels in mature pollen (35 relative units) to the top 5% of the pollen tubes expressed genes (6200 relative units) [8]. In addition, recently Borges et al., found that *AtTIP5;1* is highly expressed in Arabidopsis sperm cells suggesting a new and appealing role for *AtTIP5;1* during sperm cell development [5]. Taken together, our comparative expression analyses emphasize the special arrangement of the pollen transcriptome in comparison with single cell sporophytic tissues.

On the whole, pollen tube growth is a dynamic process where pollen tube tip growth is supported by the fusion of secretory vesicles with the apical plasma membrane. At the same time, endocytosis of small vesicles is used to balance the amount of membrane incorporated to the tip. Using membrane-tracking dyes such as FM4-64, it was shown in pollen tubes that after being internalized, FM4-64 appeared in some vacuolar subapical structures, before being possibly redistributed back to the tube tip [22]. This dynamic subcellular process suggests that the plasma membrane of the pollen tube is constantly recycled to vacuolar cytoplasmic structures. This observation was supported by the fact that FM4-64 colocalized in pollen tubes with ectopically expressed *At*TIP2;1-GFP, a tonoplast marker [23]. All these could suggest why, at least in part, PIPs, mostly specifically localized at the plasma membrane and not in endomembrane systems, are not expressed in mature

Table 1
Solute permeability of pollen aquaporins. Xenopus oocytes were injected with water, with *AtPIP2;3*, or co-injected with *AtPIP2;3* + *AtTIP1;3* or with *AtPIP2;3* + *AtTIP5;1* and tested for urea, boric acid and glycerol permeability. Units for solute transport are 10^{-3} s^{-1}. Data are expressed as mean ± S.D. for 15–18 oocytes (three independent experiments) for urea transport and 5–6 oocytes (two independent experiments) for boric acid and glycerol.

Material injected	Urea transport	Boric acid transport	Glycerol transport
Water	−2.76 ± 2.66	1.53 ± 2.31	−0.68 ± 1.12
cRNA AtPIP2;3	−2.12 ± 3.30	1.60 ± 3.25	−1.4 ± 1.06
cRNA (*AtPIP2;3* + *AtTIP1;3*)	18.94 ± 3.29	2.08 ± 3.30	−1.05 ± 1.00
cRNA (*AtPIP2;3* + *AtTIP5;1*)	21.18 ± 4.97	2.01 ± 3.02	−0.99 ± 1.90

Table 2
Aromatic/arginine selectivity filter residues in aquaporins. Aromatic/arginine selectivity filter residues in Arabidopsis TIP proteins and substrate specificity. H2: residues from helices 2; H5: residues from helices 5; LE1 and LE2 are two residues from loop E. ND, not determined; *Hs*, *Homo sapiens*; *At*, *Arabidopsis thaliana*.

Protein	H2	H5	LE1	LE2	Transport	Reference
*Hs*AQP1	F	H	C	R	Water	[31]
*At*PIP2;3	F	H	T	R	Water	[21]
*At*TIP1;1	H	I	A	V	Urea, water and hydrogen peroxide	[12,27,28]
*At*TIP1;2	H	I	A	V	Urea and hydrogen peroxide	[27,28]
*At*TIP1;3	H	I	A	V	Urea and water	This work
*At*TIP2;1	H	I	G	R	Urea and ammonium	[27,28,32,33]
*At*TIP2;2	H	I	G	R	ND	
*At*TIP2;3	H	I	G	R	Ammonium and hydrogen peroxide	[33,34]
*At*TIP3;1	H	I	A	R	ND	
*At*TIP3;2	H	I	A	R	ND	
*At*TIP4;1	H	I	A	R	Urea	[27,32,33]
*At*TIP5;1	N	V	G	C	Urea and water	This work

pollen. In animals, regulatory cycling between membranes is well documented for several aquaporins [24], whereas in plants, TIP relocalization has been proposed as a regulatory mechanism [25]. Therefore it is possible that TIP relocalization, if it occurs, would depend on specifically hydrodynamic signals that direct pollen tube growth.

In the present work, we study the intrinsic transport properties of *At*TIP5;1 and *At*TIP1;3 by means of their heterologous expression in Xenopus oocytes. Our results show that both TIPs are able to transport water and urea but no glycerol or boric acid. Unlike PIPs, TIPs show high diversity within the putative pore regions, with three different aromatic/arginine (ar/R) selectivity subgroups [26]. Table 2 shows that the ar/R regions of *At*TIP5;1 and *At*TIP1;3 differ from typical pores of aquaporins that exclusively transport water. *At*TIP1;3 shows the same restriction of *At*TIP1;1, which transports urea, water and hydrogen peroxide [12,27,28], and of *At*TIP1;2, which transports urea and hydrogen peroxide [27,28]. On the contrary, the ar/R region of *At*TIP5;1 is unique. Because of this, *At*TIP5;1 would have a larger apparent pore aperture and a reduced ability to form hydrogen bonds with transported solutes [26]. Therefore, *At*TIP5;1 may have transport properties that are unlike conventional aquaporins, although we showed here that *At*TIP5;1 transports water and specifically urea, since no transport of glycerol or boric acid was observed. Our results agreed with a previous report that showed that when the ar/R residues of *At*PIP2;1 are exchanged by the *At*TIP5;1 or *At*TIP1;3 residues and introduced into a mutant yeast strain unable to uptake urea, yeast cells that bear the chimeric aquaporins grew under selective conditions [29].

Because the final destination of *At*TIP5;1 and *At*TIP1;3 in pollen tubes is still tentative, it is difficult to speculate the need to transport urea or urea-like compounds into pollen tubes. If these TIPs were tonoplast-localized, would the urea transiently accumulate in the tonoplast then be remobilized and used as a nitrogen source? Or is it directly used in the cytoplasm if the TIPs were plasma membrane-localized? Is urea used as one of the modulators of the osmotically driven force that adjust pollen tube tip volume, regulating the balance of endocytosis and exocytosis and finally pollen tube growth? All these questions raised speculations about the role that urea or any of its derivatives could have during pollen tube growth. Although functional analysis in planta will be needed to define precisely the function of *At*TIP5;1 and *At*TIP1;3, our results suggest a role during pollen tube development and growth.

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