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TIP5;1 is an aquaporin specifically targeted to pollen mitochondria and is probably involved in nitrogen remobilization in *Arabidopsis thaliana*

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SUMMARY

In plant sexual reproduction, water and solute movement are tightly regulated, suggesting the involvement of aquaporins. We previously identified *TIP5;1* and *TIP1;3* as the only Arabidopsis aquaporin genes that are selectively and highly expressed in mature pollen, and showed that they can transport both water and urea when expressed in *Xenopus* oocytes. Here, we show that TIP5;1 has unusual characteristics, as its water transport activity is regulated by pH. Analysis of the water transport activity of a mutant version of TIP5;1 (TIP5;1-H131A) and amino acid alignment with other plant aquaporins regulated by pH suggested that a conserved motif is involved in pH sensing. GFP–TIP5;1 is located in the mitochondria of pollen tubes. The single mutants *tip1;3* and *tip5;1*, as well as the *tip1;3 tip5;1* double mutant, are fertile, but all mutants had shorter than normal pollen tubes when germinated *in vitro* in the absence of exogenous nitrogen. Thus, we propose that TIP5;1 and TIP1;3 are involved in nitrogen recycling in pollen tubes of *Arabidopsis thaliana*.

Keywords: aquaporins, urea, nitrogen metabolism, pH sensing, mitochondria.

INTRODUCTION

In flowering plants, pollen germination and pollen tube growth are critical stages during sexual reproduction. During pollen development, pollen grains represent a significant sink for carbon and nitrogen, which must be imported from source organs such as leaves (Schneidereit *et al.*, 2003; Lee and Tegeder, 2004). Additionally, during pollen tube growth, the tubes are symplasmically isolated (Scott *et al.*, 1991; Yuan *et al.*, 2009), so import of nitrogen from female tissues is essential to ensure efficient growth and reproductive success.

Urea is a small, neutral and polar molecule that is an important metabolic intermediate in plants produced by nitrogen re-assimilation. There are at least three key enzymes involved in nitrogen recycling in plants: arginase, glutamine synthetase and urease. Arginase specifically converts arginine into ornithine and urea (Brownfield *et al.*, 2008), which is later hydrolyzed by urease into ammonia and carbonic acid (Goldraij and Polacco, 2000). The arginase ARGAH1 (*At4g08900*) is an active enzyme localized to pollen mitochondria (Flores *et al.*, 2008), but the presence of glutamine synthetase and urease has not been reported in pollen. However, urease activity is ubiquitous, and is responsible for the use of external or internally generated urea as a nitrogen source (Kojima *et al.*, 2006). Because plant ureases are cytoplasmic enzymes (Faye *et al.*, 1986), use of urea derived from the action of the mitochondrial arginase (Zonia *et al.*, 1995) would require transport of urea from mitochondria (Goldraij and Polacco, 1999).

Thus, for urea to serve as a nitrogen source for pollen, it would have to be moved across biological membranes, a

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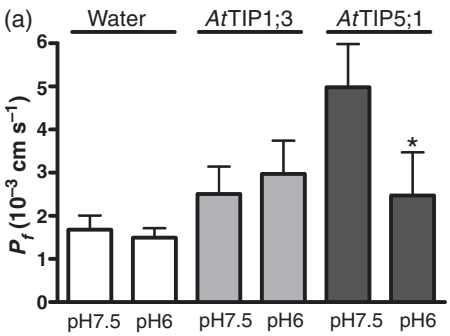
task that requires specific transport proteins (Wang *et al.*, 2008). As the only known Arabidopsis urea transporter, DUR3, is not expressed in mature pollen grains or pollen tubes (https://www.genevestigator.com/gv/index.jsp), TIP aquaporins are candidates to transport urea across pollen membranes. Aquaporins are integral membrane proteins that facilitate bi-directional transfer of water and small solutes across plasma and intracellular membranes. In plants, plasma membrane intrinsic proteins (PIP) and tonoplast intrinsic proteins (TIP) aquaporins are the most abundant sub-groups, each with many members. In Arabidopsis, all TIPs tested, except TIP2;3, can transport urea (Klebl *et al.*, 2003; Liu *et al.*, 2003; Loque *et al.*, 2005; Soto *et al.*, 2008). Most tested members of the TIP sub-group were tonoplast-localized (Wudick *et al.*, 2009), but TIP1;1 and TIP2;1 were also found in chloroplasts (Ferri *et al.*, 2003), and the *Mesembryanthemun crystallinum* protein *Mc*TIP1;2 was found in endosomes (Vera-Estrella *et al.*, 2004). No aquaporins or other urea transporters have been found to be localized to plant mitochondria. Nevertheless, mammalian aquaporins that transport urea, such as AQP8 (Ma *et al.*, 1997) and AQP9 (Tsukaguchi *et al.*, 1998), have been detected in inner mitochondrial membranes (Amiry-Moghaddam *et al.*, 2005; Calamita *et al.*, 2005). Although the participation of AQP8 and AQP9 in adjusting mitochondrial volume was suggested, their actual function within this organelle remains a matter of discussion (Yang *et al.*, 2006). Due to the high sequence identity between mammalian AQP8 and TIPs, it was proposed that plant TIPs might also be located in mitochondria (Wudick *et al.*, 2009). However, there is no evidence to date to support that localization.

We previously identified and characterized Arabidopsis aquaporins that are expressed in mature pollen, and found that TIP1;3 (*At4g01470*) and TIP5;1 (*At3g47440*) are bi-functional pollen-specific aquaporins that transport both water and urea (Soto *et al.*, 2008). TIP5;1 was also found to be expressed in dry seeds (Vander Willigen *et al.*, 2006). Here we used an experimental approach in order to more fully understand the physiological functions of TIP1;3 and TIP5;1. We show that water transport by TIP5;1, but not TIP1;3, is inhibited by acidic pH. Amino acid alignments of TIP5;1 with plant PIPs that are regulated by pH showed the presence of a conserved amino acid motif that could participate in pH regulation of water transport. TIP5;1 is localized to pollen mitochondria, whereas TIP1;3 is localized to endomembranes. Physiological analyses *in planta* suggested that TIP5;1 may be a urea transporter for pollen mitochondria.

RESULTS

TIP5;1 is regulated by external acid pH

We previously showed that TIP1;3 and TIP5;1 are bi-functional TIP aquaporins with intermediate levels of water permeability and high permeability for urea (Soto *et al.*, 2008). To further characterize the regulatory properties that affect channel activity, we investigated the effect of external acid pH on the osmotic water permeability (P_f) of *Xenopus* oocytes expressing TIP5;1 and TIP1;3. Figure 1(a) shows that the P_f of oocytes expressing TIP1;3 was not affected by external acidification, but the water transport activity of TIP5;1 was significantly inhibited 60%. All other assayed TIP



(b)

Motif		–	μ	H	φ	φ	φ	
*At*TIP5;1	(NP_190328)	E	Q	**H**	V	**P**	I	134
*At*PIP2;2	(AAD18142)	D	S	**H**	V	**P**	V	200
*At*PIP1;2	(AAC28529)	D	S	**H**	V	**P**	V	209
*At*PIP2;1	(CAB67649)	D	S	**H**	V	**P**	I	202
*At*PIP2;2	(AAD18142)	D	S	**H**	V	**P**	V	200
*At*PIP2;3	(AAD18141)	D	S	**H**	V	**P**	V	200
*Nt*PIP2;1	(AAL33586)	D	S	**H**	V	**P**	V	199
*Os*PIP2;1	(EAY86778)	D	S	**H**	I	**P**	V	205
*Gh*PIP	(ABK60195)	D	S	**H**	V	**P**	V	201
*Cf*PIP	(ABN11917)	D	S	**H**	I	**P**	V	200
*Hv*PIP	(BAE02729)	D	S	**H**	I	**P**	V	206

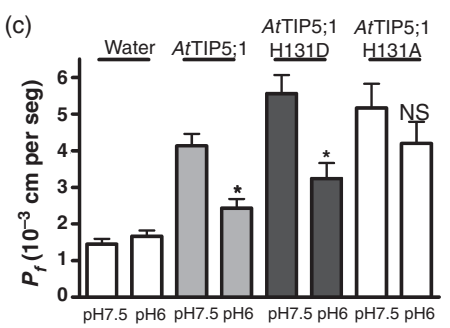


Figure 1. Effect of pH on TIP1;3 and TIP5;1 water permeability.
(a) Effect of external acidic pH on the water transport activity of aquaporin TIPs in *Xenopus* oocytes. Oocytes were injected with water (negative control) or with TIP1;3 or TIP5;1, and then tested for water permeability (P_f). Data were obtained from three independent experiments. All values are means ± SEM (n = 9). Statistically significant differences between pH levels are indicated by asterisks (t test, $^*P < 0.05$).
(b) Conserved motif potentially involved in pH sensing. –, acidic residue; μ, polar non-charged residue; H, histidine; φ, hydrophobic residue. *At*, *Arabidopsis thaliana*; *Nt*, *Nicotiana tabacum*; *Os*, *Oryza sativa*; *Gh*, *Gossypium hirsutum*; *Cf*, *Cucurbita ficifolia*; *Hv*, *Hordeum vulgare*. Numbers on the right indicate the position of the last residue of the motif in each sequence. Numbers in parentheses indicate accession numbers.
(c) Effect of pH on water permeability in *Xenopus* oocytes expressing wild-type or site-directed mutants of TIP5;1. Water was used as a negative control. Data were obtained from two independent experiments. All values are means ± SEM (n = 6–8). Statistically significant differences between pH levels are indicated by asterisks (t test, $^*P < 0.05$). NS, not significant.

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aquaporins showed water transport activities that were insensitive to pH changes (Liu *et al.*, 2003; Tournaire-Roux *et al.*, 2003). In addition, in *Xenopus* assays, TIP5;1 sensed changes in oocyte external pH, in contrast to the intracellular perception of pH described for PIPs (Tournaire-Roux *et al.*, 2003).

In all Arabidopsis PIP2s, a histidine residue (His197 in *At*PIP2;2), the two acidic residues upstream of that histidine and the three consecutive hydrophobic residues downstream of that histidine are conserved. We postulated that His131 in TIP5;1 might be a pH-sensing amino acid, as it is located in extracellular loop C and is not present in other Arabidopsis TIPs. Moreover, the region flanking His131 showed high amino acidic conservation when it was aligned with the His197 region of PIP2 and other pH-regulated PIPs (Figure 1b), suggesting that there is a conserved motif in all pH-regulated plant aquaporins.

To examine the relevance of His131 in sensing pH changes, we mutated His131 to aspartic acid (H131D), a negatively charged amino acid, or alanine (H131A), a nonpolar amino acid that is incapable of establishing hydrogen bonds. TIP5;1-H131D showed the same acidic pH sensitivity as TIP5;1, while TIP5;1-H131D was insensitive to acid treatment (Figure 1c). This result suggests that His131 is involved in pH modulation of TIP5;1 water transport activity.

Subcellular localization of AtTIP1;3 and AtTIP5;1

In order to evaluate the subcellular location of TIP1;3 and TIP5;1, we first used SUBA, a subcellular location database for Arabidopsis proteins (http://www.suba.bcs.uwa.edu.au) (Heazlewood *et al.*, 2007). As expected for a TIP aquaporin, TIP1;3 was predicted to be targeted to the tonoplast, and also to plastids and the plasma membrane (Figure S1). Unexpectedly, TIP5;1 was predicted to be targeted to the mitochondrion membrane (Figure S1). We also used MITOPROT software, which predicts the probability of export to mitochondria (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html). Figure 2(a) shows that TIP5;1 is the only Arabidopsis aquaporin that is predicted to be exported to mitochondria, with a high probability score of 93%, comparable to the scores for *bona fide* mitochondrial proteins such as Arabidopsis thaliana magnesium transport protein 5 (AtMGT5) (Li *et al.*, 2008), Arabidopsis thaliana phosphatidylserine decarboxylase 1 (AtPSD1) (Rontein *et al.*, 2003) and Arabidopsis thaliana oxidase assembly 1 (AtOXA1) (Sakamoto *et al.*, 2000).

In order to investigate whether there is a mitochondrial export signal in TIP5;1, we first identified TIP5;1 orthologous genes in the monocots *Zea mays* and *Oryza sativa* and the dicot *Vitis vinifera* (Figure S2). Of the 10 *Arabidopsis thaliana* TIP aquaporins, only TIP5;1 showed a canonical φχχφφ mitochondrial export signal (Figure 2b), where φ represents any hydrophobic residue and χ any amino acid (Glaser *et al.*, 1998). As for most membrane mitochondrial proteins, the TIP5;1 export signal is located upstream of the first transmembrane domain. In plant mitochondrial proteins, the amino acid sequence located upstream of the export signal is rich in serine, arginine, alanine and leucine, but low in cysteine, histidine, tryptophan, tyrosine, glutamic acid and aspartic acid (Glaser *et al.*, 1998). As shown in Figure 2(b),

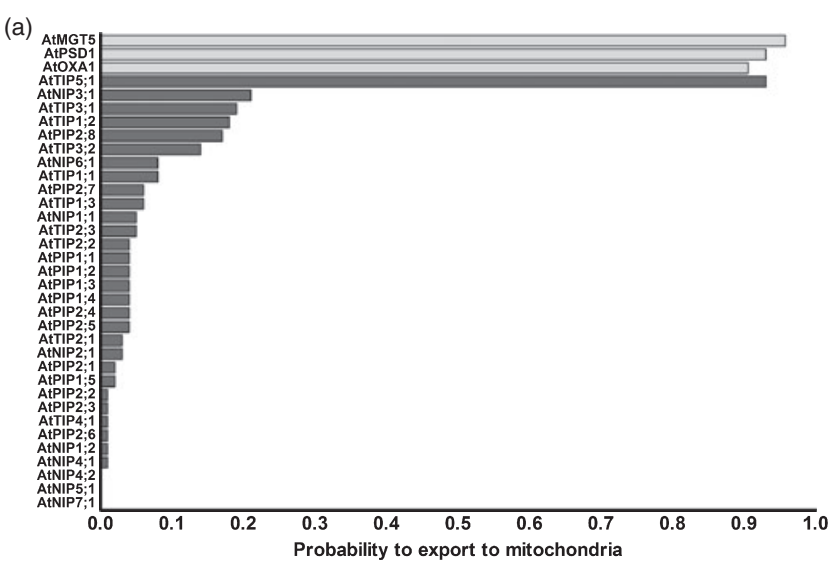

Figure 2. Probability of mitochondrial export and phylogenetic analysis of *At*TIP5;1.
(a) MITOPROT probability of export to mitochondria for all Arabidopsis aquaporins. Arabidopsis OXA1, PSD1 and MGT5, which are transmembrane internal mitochondrial membrane proteins, were used as positive controls.
(b) N-terminal sequence alignment of TIP5;1 from Arabidopsis and its orthologous genes. Alignment is shown up to the first transmembrane domain. The box indicates the mitochondrion export consensus. Black shading indicates hydrophobic residues. 'φ' indicates a hydrophobic residue and 'χ' indicates any amino acid. Numbers in parentheses indicate accession numbers. *At*, *Arabidopsis thaliana*; *Zm*, *Zea mays*; *Vv*, *Vitis vinifera*; *Os*, *Oryza sativa*.

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the four TIP5;1 protein sequences are especially rich in serine and arginine residues, but cysteine, histidine, tryptophan and glutamic acid residues are absent.

To confirm the subcellular localization of TIP1;3 and TIP5;1 in pollen tubes, we constructed full-length N-terminal GFP fusions under the control of the LAT52 pollen-specific promoter (pLAT52) (Twell *et al.*, 1990). pLAT52::GFP was used as a control. As expected, the GFP control was homogeneously distributed in the pollen tube cytoplasm (Figure 3a). Figure 3(b,c) shows that both GFP–TIP5;1 and GFP–TIP1;3 were found at defined internal membrane structures, characteristic of endomembrane proteins (Saito *et al.*, 2002; Yoon *et al.*, 2006; Beebo *et al.*, 2009).

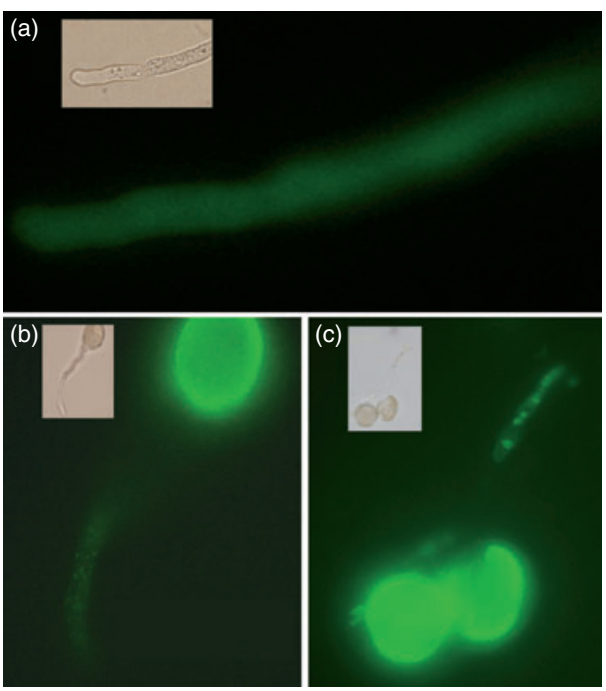

Figure 3. Pollen tube localization of TIP5;1 and TIP1;3.
(a) GFP, (b) GFP–TIP5;1 and (c) GFP–TIP1;3 were stably expressed in Arabidopsis transgenic plants under the control of the LAT52 pollen-specific promoter. In (b) and (c), the focus was set to visualize the internal particles. Insets show the same pollen tubes under white light. All images are representative of at least three independent transgenic lines.

To analyze whether TIP5;1 is associated with membrane secretion and/or endosomal recycling in pollen tubes, we germinated transgenic pollen in the presence of FM4-64. FM4-64 is efficiently internalized by endocytosis, followed by membrane recycling from the endosomal system to the pollen tube apex (Parton *et al.*, 2003). Figure 4 showed that FM4-64 labeled endocytic membranes, while the GFP–TIP5;1 fluorescence was associated with punctate cytoplasmic structures that did not coincide with the FM4-64 pattern.

We then incubated GFP–TIP5;1 pollen with MitoTracker, a fluorescent dye that specifically labels mitochondria. Figure 4 shows that the MitoTracker fluorescence overlapped with GFP fluorescence, confirming that TIP5;1 specifically localizes to mitochondria. For TIP1;3, no specific subcellular localization was identified.

Isolation of TIP1;3 and TIP5;1 homozygous T-DNA insertion lines

To investigate the function of *TIP1;3* and *TIP5;1* in Arabidopsis pollen, we obtained T-DNA mutant alleles for *TIP1;3* (SALK_088276) and *TIP5;1* (GABI_041E09) in Columbia-0 *Arabidopsis thaliana* (Figure 5a). According to the Arabidopsis Information Resource (TAIR), the T-DNA insertions are located in the 5′ UTR region of *TIP1;3* and the third exon of *TIP5;1*. Homozygous lines for each T-DNA insertion were identified by PCR analyses (Figure S3). RT-PCR with mature pollen RNA showed that the homozygous lines contained no *TIP1;3* or *TIP5;1* transcripts (Figure 5b).

That we recovered viable homozygous plants for *tip1;3* and *tip5;1* suggests that no single pollen aquaporin is essential for fertilization under our experimental conditions. Homozygous *tip1;3* and *tip5;1* plants were indistinguishable from wild-type plants (data not shown). The mutant lines were analyzed for pollen phenotypic differences. No significant differences from wild-type pollen were observed when pollen development, pollen size or abundance were compared (data not shown). No differences in pollen morphology between genotypes were found upon germination on solid medium (Figure S4a). Furthermore, we found no significant reduction in fertility, as homozygous *tip1;3* and *tip5;1* plants yielded normal seeds in numbers similar to those of wild-type plants (Figure S4b). They also showed the

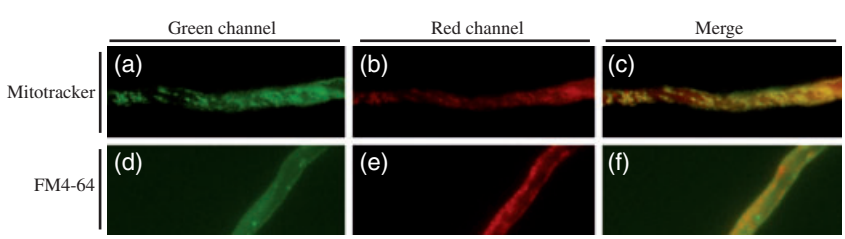

Figure 4. TIP5;1 is expressed in pollen tube mitochondria.
Co-localization of GFP with endocytic (FM4-64) and mitochondrial (MitoTracker) markers in pollen tubes expressing GFP–TIP5;1. (a, d) GFP (green channel), (b) MitoTracker (red channel) or (e) FM4-64 (red channel) fluorescence, (c) GFP/MitoTracker merged image, (f) GFP/FM4-64 merged image. Co-localization is observed as yellow fluorescence. All images are representative of at least two independent transgenic lines.

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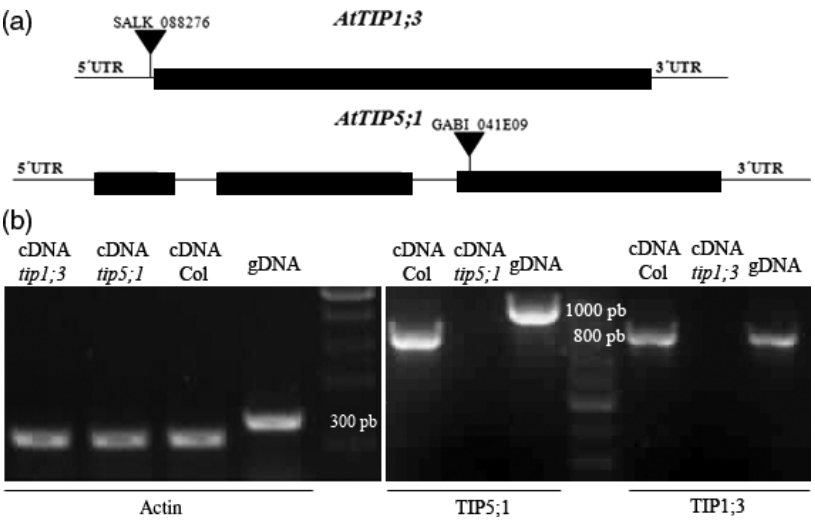

Figure 5. T-DNA insertion mutants for TIP5;1 and TIP1;3.
(a) Positions of T-DNA insertion mutants. Black boxes represent exons, black lines represent genomic regions including introns, 3′ UTRs and 5′ UTRs.
(b) RT-PCR analysis of wild-type and mutant pollen. Actin was used as a positive control gene; genomic DNA (gDNA) and wild-type Columbia pollen cDNA (cDNA Col) were used as controls for PCR reactions. A 1 kb ladder was used as a size marker.

Table 1 Inheritance in *tip1;3* and *tip5;1* mutant plants

		Genotypes (%)		
Natural self-cross	Number of progeny	+/+	+/–	–/–
Expected		25	50	25
tip1;3+/–	150	24	48	28
tip5;1+/–	205	23	51	26

Statistically significant differences were evaluated using Chi-squared test. None of the values were significantly different from expected values.

expected Mendelian segregation of mutant alleles when crossed with wild-type plants (Table 1).

To investigate whether the presence of either isoform can substitute for the lack of the other in single mutant lines, we generated a *tip1;3 tip5;1* double mutant by manual crossing. Double homozygous lines were identified by PCR analyses (Figure S3). The *tip1;3 tip5;1* plants had no obvious phenotypes compared with wild-type or single mutant plants (data not shown). No differences from wild-type were found in terms of the number of seeds or the Mendelian segregation ratios (five of 78 plants obtained from a double heterozygous cross were double homozygotes).

Pollen *tip* mutants are sensitive to nitrogen limitation

As both TIP1;3 and TIP5;1 are urea transporters (Soto *et al.*, 2008), we measured the *in vitro* pollen tube length of both single and double mutants in germination medium with or without nitrogen (Figure 6). Only under nitrogen-limited conditions were the pollen tubes shorter in the *tip1;3* and *tip5;1* single mutants and the *tip1;3 tip5;1* double mutant. These observations that TIP1;3 and TIP5;1 are required for normal pollen tube elongation under nitrogen-deficient conditions.

TIP5;1 is probably involved in pollen nitrogen recycling

To assess which enzymes might be involved in nitrogen recycling in pollen, we first performed an *in silico* search to analyze the expression of glutamine synthetases and urease in pollen, using the software eFP Browser (Winter *et al.*, 2007). Table S1 shows that two glutamine synthetases, *GLN1;3* (*At3g17820*) and *GLN1;5* (*At1g48470*), and the urease gene *At1g67550* are expressed in pollen. As the expression signal for the urease gene was very weak, we used RT-PCR followed by cDNA sequencing to confirm its expression in mature pollen (Figure 7a). Figure 7(b) shows that pollen tubes that germinated in the presence of phenylphosphordiamidate (PPD), an inhibitor of urease (Polacco *et al.*, 1985), were shorter. This result suggests the involvement of urease activity during pollen tube growth.

The enhancement of nitrogen re-mobilization correlates with increased activity of cytosolic glutamine synthetase (Masclaux *et al.*, 2000; Witte *et al.*, 2005; Kojima *et al.*, 2006). We therefore evaluated whether the absence of TIP5;1 in the *tip5;1* mutant affects the expression of *GLN1;5* in pollen. Figure 7(c) shows that expression of *GLN1;5* in *tip5;1* mutant pollen was 87% lower than its expression level in wild-type Arabidopsis pollen.

In order to study whether TIP5;1 is involved in mitochondrial homeostasis in pollen, we performed a comparative mitochondria volumetric analysis using wild-type and *tip5;1* pollen tubes germinated in medium with or without a nitrogen source. As confocal fluorescence microscopy and electron microscopy have disadvantages for measuring mitochondrial volume (Kaasik *et al.*, 2007), we combined confocal microscopy with 3D deconvolution analysis (Safiulina *et al.*, 2006). We found a 40% decrease in the mitochondrial volume of *tip5;1* pollen tubes relative to the mitochondrial volume in wild-type pollen tubes in medium containing nitrogen (data not shown), and a more marked

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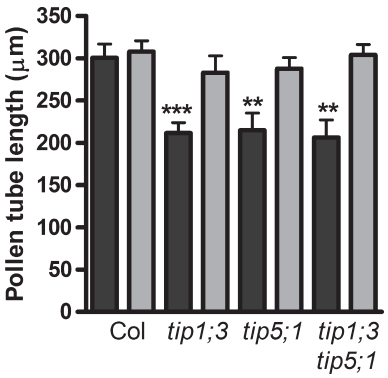

Figure 6. The influence of nitrogen on pollen tube elongation in *tip* mutants. Pollen was germinated *in vitro* in medium with nitrogen (black bars) or without nitrogen (gray bars). All values are means ± SEM (n = 9). Statistically significant differences between the nitrogen conditions are indicated by asterisks (ANOVA: $^{**}P < 0.01$, $^{***}P < 0.001$).

decrease (80%) when pollen grains were germinated in medium without nitrogen (Figure 7d). Taken together, these results suggest a connection between the mitochondrial localization of TIP5;1 and the involvement of TIP5;1 in pollen nitrogen metabolism.

DISCUSSION

Here we show that the water transport activity of TIP5;1 is significantly inhibited when the external pH is lowered from pH 7.5 to pH 6 (Figure 1a). PIPs aquaporins have been shown to sense cytoplasmic acidification (Tournaire-Roux *et al.*, 2003), and several mammalian aquaporins are regulated by external pH (Yasui *et al.*, 1999; Zeuthen and Klaerke, 1999; Nemeth-Cahalan and Hall, 2000). The Arabidopsis PIP2;2-H197A mutant lost pH sensitivity for water transport activity, and the PIP2;2-H197D mutant showed similar sensitivity to TIP5;1. In yeast spheroplasts expressing tobacco PIP2;1, water permeability decreased when the pH was lowered from 6.46 to 6.13 (Fischer and Kaldenhoff, 2008). For the tobacco PIP2;1-H196A mutant, the pH sensitivity was lost, with transport rates similar to the lowest rate obtained under acidic conditions (pH 6.13). Here we shod that TIP5;1-H131A also lost pH sensitivity but had a water transport rate as high as the rate obtained under high pH (pH 7.5). For TIP5;1-H131D, pH sensitivity was still observed, suggesting that polarity and/or the ability to establish hydrogen bonds are both important for pH regulation of the water transport activity of TIP5;1. Similar behavior was reported for the antiporter NhaA (Rimon *et al.*, 1995). We postulate that His131 is a pH-sensing site, although it is possible that other residues are also involved. When we aligned His131 of TIP5;1 with the corresponding histidines of the 11 PIPs that are also regulated by pH, we found a conserved motif present in all pH-regulated aquaporins (Figure 1b), suggesting that this motif is involved in pH sensing. In PIPs, the pH-sensing motif is located in intracellular loop D, but in TIP5;1 it is located in loop C, which is topologically arranged externally of the *Xenopus* oocyte. This finding is consistent with the fact that TIP5;1 senses external pH changes in this heterologous expression system.

With regard to TIP aquaporin localization, it has been reported that TIPs are predominantly located at the

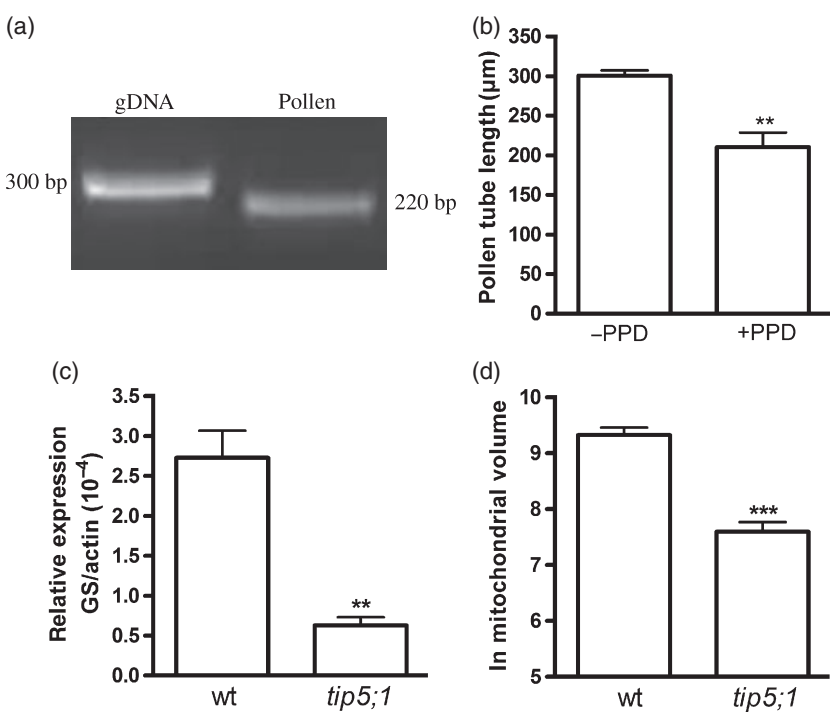

Figure 7. TIP5;1 is potentially involved in pollen nitrogen recycling.
(a) RT-PCR analysis of expression of urease in wild-type mature pollen. Genomic DNA (gDNA) is used as a positive control. Molecular weight is indicated.
(b) Pollen was germinated *in vitro* in medium with or without 100 μM phenylphosphordiamidate (PPD). All values are means ± SEM (n = 9). Asterisks indicate a statistically significant difference (ANOVA: $^{**}P < 0.01$).
(c) Real-time RT-PCR of glutamine synthetase (GLN1;5) levels in wild-type and *tip5;1* pollen. All values are means ± SEM (n = 3). Asterisks indicate a statistically significant difference (ANOVA: $^{**}P < 0.01$).
(d) Reduction of pollen mitochondrial volume in *tip5;1* pollen tubes grown in germination medium without nitrogen. Data were log-transformed and are expressed as means ± SEM. Asterisks indicate a statistically significant difference (t test: $^{***}P < 0.001$).

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tonoplast (Wudick *et al.*, 2009). TIP1;3 showed a spotted distribution (Figure 3c) with a typical pattern of endomembrane localization (Beebo *et al.*, 2009). Our co-localization experiments showed that, under our experimental conditions, TIP1;3 did not localize to the plasma membrane, mitochondria or the vesicular recycling machinery in pollen tubes (data not shown). Phylogenetic and signal peptide analyses suggested that TIP5;1 is an ancestral aquaporin localized in the mitochondria (Figure 2b and Figure S2). Complementary bioinformatic approaches (Figure 2a and Figure S1) and GFP fusion experiments (Figure 4) confirmed that *At*TIP5;1 is an aquaporin with mitochondrial localization. In rat liver, AQP8 was found in the inner mitochondrial membrane (Ferri *et al.*, 2003; Calamita *et al.*, 2005). Despite its high water conductance, it is still not clear whether AQP8 moves water across the mitochondrial membrane (Calamita *et al.*, 2006; Yang *et al.*, 2006; Gena *et al.*, 2009). It has been speculated that AQP8 may be involved in the generation of reactive oxygen species (Bienert *et al.*, 2007) and in mitochondrial ammonia detoxification via ureagenesis (Soria *et al.*, 2010).

We found that both single *tip5;1* and *tip1;3* mutants and the *tip5;1 tip1;3* double mutant had slightly shorter pollen tubes in germination medium without nitrogen when compared to the lengths of wild-type tubes (Figure 6). This result suggests that TIP5;1 and TIP1;3 are important for pollen tube elongation under low-nitrogen conditions. No additive or synergistic effects on the phenotype in the double mutant were observed, suggesting that both TIPs are involved in the same pathway.

The observed reduction of *in vitro* pollen tube growth did not cause a substantial decrease in pollen fertility (see Table 1). A possible explanation could be that, in nature, the pollen germinates and elongates through female reproductive tissues that are rich in nitrogen compounds. Another reason could be functional redundancy among other pollen aquaporin genes. The candidates in Arabidopsis are NIP4;1, a pollen-specific aquaporin, and SIP1;1, SIP1;2 and TIP1;1, aquaporins that are expressed in mature pollen and other sporophytic tissues. In this regard, it is noteworthy that most aquaporin null mutants have no obvious phenotypes in either animals or plants (Maurel, 2007; Gomes *et al.*, 2009). For instance, the Arabidopsis *tip1;1 tip1;2* double mutant does not show any detectable phenotype, presumably because other TIP homologues can compensate for their loss (Schussler *et al.*, 2008). Given that robustness of the sexual reproduction process is essential to ensure the maintenance of species, it is reasonable to postulate that pollen can usually compensate for any such loss except under stress conditions such as nitrogen deficiency.

The reduced mitochondrial swelling in mutant *tip5;1* pollen tubes (Figure 7d) may have a number of consequences for many cellular functions, such as the movement of mitochondria to the place where ATP synthesis is required. As *tip5;1* pollen tubes have a reduced ability to elongate in germination medium without nitrogen, we speculate that the absence of *tip5;1* diminishes the availability of cellular energy necessary for proper pollen tube growth.

Here we show that the only Arabidopsis urease gene is expressed in mature pollen (Figure 7a), and that PPD, an inhibitor of the urease activity, inhibitspollen tube growth (Figure 7b). This results suggests that the nitrogen mobilization pathway described in seeds (Goldraij and Polacco, 2000) is also present in pollen. In order to be hydrolyzed, urea must cross the mitochondrial membrane by an unknown transporter. DUR3 must be excluded as a urea transporter candidate because it is localized in the plasma membrane (Kojima *et al.*, 2006) and is not expressed in pollen (http://www.genevestigator.com). Thus, the results shown here suggest that TIP5;1 is involved in the transport of mitochondrial urea to the cytoplasm, where it can be hydrolyzed by urease or stored in transient intracellular reserves.

EXPERIMENTAL PROCEDURES

Phylogenetic analysis of sequence data

Sequence searches were performed using BLASTP tools. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.0 (Kumar *et al.*, 2004). Protein sequences were aligned using the ClustalW program. Phylogenetic trees were constructed using the neighbor-joining method. Prediction of transmembrane helices was performed using TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Plant materials

Arabidopsis thaliana (Col-0) was used as wild-type. Seeds of the *tip1;3* T-DNA insertion line SALK_088276 were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/), and the *tip5;1* T-DNA insertion line GABI_041E09 was supplied by the Max Planck Institute for Plant Breeding Research (http://www.GABI-Kat.de) (Li *et al.*, 2007). Genomic DNA for genotyping analysis was isolated as described previously (Mazzella *et al.*, 2005). Genotyping of the *tip1;3*, *tip5;1* and *tip1;3 tip5;1* insertional mutants was performed by PCR (Figure S3) and RT-PCR (Figure 5b). The primers used are given in Table S2.

Growth conditions

Plants were grown under light/dark cycles of 16 h/8 h, with light intensities of 150 μmol m^{-2} sec^{-1} at 22–25°C. Seeds were vernalized for 2 days at 4°C before planting. To collect pollen, inflorescences from 200 plants of each genotype were cut off, shaken in a conical tube with 25 ml of TE (Tris/EDTA) buffer, and filtered using several layers of cheesecloth (grade 50) then collected by centrifugation at 3500 *g* for 10 min.

RNA extraction and RT-PCR amplification

Total RNA was extracted using an RNeasy plant mini kit (Qiagen, http://www.qiagen.com/) according to the manufacturer's instructions. For pollen extraction, 100 μl of 0.5 mm glass beads were

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added to the resuspended pollen and vortexed for 10 min. Samples of 200 ng total RNA isolated from pollen were reverse-transcribed in a 20 μl reaction using MMLV reverse transcriptase (Promega, http://www.promega.com/) according to the manufacturer's instructions. For PCR amplification, 2 μl of the reverse transcription reaction mixture was used. The PCR reactions were carried out in 25 μl volumes using 0.8 μM of each primer.

Cloning of pollen TIP aquaporins in binary and expression vectors

Plasmids pLAT52::GFP-TIP1;3 and pLAT52::GFP-TIP5;1 carrying the LAT52 promoter were constructed as follows. *TIP1;3* and *TIP5;1* cDNA clones were amplified from Arabidopsis pollen by RT-PCR. The amplified fragments were cloned into pENTR1A (Invitrogen, http://www.invitrogen.com/). Cloned PCR products were confirmed by DNA sequencing. Cloning steps were performed using the Gateway system according to the manufacturer's instructions (Invitrogen). The destination vector (kindly donated by Dr Sheila McCormick, Plant Gene Expression Center and Department of Plant and Microbial Biology, U.S. Department of Agriculture/Agricultural Research Service and University of California at Berkeley, California) was pZYO3 for GFP N-terminal fusions. This vector carries the *bar* gene, which confers ammonium glufosinate resistance.

cDNAs of Arabidopsis TIP aquaporin genes (*TIP1;3* and *TIP5;1*) were cloned into pSGEM, and cRNAs were synthesized as described previously (Soto *et al.*, 2008). TIP5;1 mutants were generated by oligonucleotide-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, http://www.stratagene.com/). The identity of all constructs was verified by sequencing.

Culture of *Agrobacterium tumefaciens* and Arabidopsis transformation

Agrobacterium tumefaciens strain GV3101 (Koncz and Schell, 1986) carrying binary plasmids was used to stably transform Arabidopsis plants. Protocols for bacterial culture were as described previously (Clough and Bent, 1998). Arabidopsis transformation was performed by floral dipping as described by Zhang *et al.* (2006).

Plant selection

Plant selection was performed as described previously (Harrison *et al.*, 2006). Briefly, seeds were sown onto 1% agar with ammonium glufosinate at a concentration of 12.5 mg L^{-1} (Sigma, http://www.sigmaaldrich.com/). Seeds were stratified for 2 days in the dark at 4°C, then transferred to a growth chamber and incubated for 6 h at 22°C in continuous white light in order to stimulate germination. The plates were then wrapped in aluminum foil and incubated for 2 days at 22°C. The foil was removed and seedlings were incubated for 4 days at 22°C in continuous white light.

Oocyte preparation, cRNA injection and swelling assays in Xenopus

Oocyte preparation, cRNA injection, swelling assays and P_f determination were performed as previously described (Soto *et al.*, 2008).

Pollen tube elongation

The protocol for Arabidopsis pollen germination was as described previously (Boavida and McCormick, 2007). In summary, 15 flowers were transferred to 5 ml glass tubes and immersed in 100 μl of standard germination medium (0.01% boric acid, 1 mM $MgSO_4$, 2 mM $CaNO_3$, 18% sucrose pH 6.5). Tubes were agitated for 3 min to release pollen, and then incubated for 4 h in a growth chamber under controlled light and temperature. For germination medium without nitrogen, $CaNO_3$ was replaced by $CaCl_2$. Pollen tube length was determined using ImageJ software (Abramoff *et al.*, 2004).

Microscopy

Pollen was visualized using an epifluorescence microscope BX41 (Olympus, http://www.olympus-global.com/) with appropriate filters. For MitoTracker and FM4-64 experiments, pollen was germinated in 100 μl of standard germination medium. Immediately before observation, 10 μl MitoTracker orange CMTMROS (100 pM final concentration) (Molecular Probes, http://www.invitrogen.com/) or 1 μl FM4-64 (1 μM final concentration) (Molecular Probes) was added to the medium. DAPI staining was performed as described previously (Johnson-Brousseau and McCormick, 2004).

Statistical analysis

Water permeability measurements were repeated at least three times. Significant differences between treatments were calculated using Student's *t* test. Pollen tube length assays were repeated at least five times. Significant differences were calculated using ANOVA. Mendelian segregation deviations were evaluated using the Chi-squared test.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bioinformatics of pollen aquaporins.

Figure S2. Phylogenetic analysis of *Arabidopsis thaliana* TIPs and TIP5;1 orthologous genes.

Figure S3. Genotyping of mutant insertional plants by PCR.

Figure S4. Physiological analysis of *tip* mutants.

Table S1. Expression of key enzymes of nitrogen recycling in mature pollen of Arabidopsis.

Table S2. Primers used in this study.

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