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Ectopic expression of *Arabidopsis thaliana* plasma membrane intrinsic protein 2 aquaporins in lily pollen increases the plasma membrane water permeability of grain but not of tube protoplasts

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Summary

- To investigate the role of aquaporin-mediated water transport during pollen grain germination and tube growth, *Arabidopsis thaliana* plasma membrane intrinsic proteins (PIPs) were expressed in pollen of *Lilium longiflorum* (lily).
- Successful expression of AtPIPs in particle-bombarded lily pollen grains was monitored by co-expression with fluorescent proteins and single-cell RT-PCR, and by measuring the water permeability coefficient (P_{os}) in swelling assays using protoplasts prepared from transformed pollen grains and tubes.
- Expression of AtPIP1;1 and AtPIP1;2 in pollen grains resulted in P_{os} values similar to those measured in nontransformed pollen grain protoplasts (6.65 ± 2.41 μm s^{-1}), whereas expression of AtPIP2 significantly increased P_{os} (AtPIP2;1, 13.79 ± 6.38; AtPIP2;2, 10.16 ± 3.30 μm s^{-1}). Transformation with combinations of AtPIP1 and AtPIP2 did not further enhance P_{os}. Native pollen tube protoplasts showed higher P_{os} values (13.23 ± 4.14 μm s^{-1}) than pollen grain protoplasts but expression of AtPIP2;1 (18.85 ± 7.60 μm s^{-1}) did not significantly increase their P_{os} values. Expression of none of the tested PIPs had any effect on pollen tube growth rates.
- The ectopic expression of AtPIP2s in lily pollen increased the water permeability of the plasma membrane in pollen grains, but not in pollen tubes. The measured endogenous water permeability does not limit water uptake during tube growth, but has to be regulated to prevent tube bursting.

Keywords

aquaporin; fluorescent protein; pollen; protoplast; tip growth; water transport

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Introduction

A mature pollen grain landing on a perceptive stigma rehydrates by taking up water from its environment. The hydrated pollen grain then starts to grow a pollen tube that penetrates the stigma tissue and delivers the sperm cells to the ovary for fertilization. Water uptake is important during at least two stages of pollen development: rehydration on the stigma and growth of the pollen tube. After the formation of a continuous lipid bilayer during rehydration (Crowe *et al.*, 1989; Tiwari *et al.*, 1990; Hoekstra *et al.*, 1991), additional water has to be taken up across the plasma membrane to allow adjustment of cytosolic ion concentrations, for example hydrogen (H^+), calcium (Ca^{2+}) and potassium (K^+), and turgor pressure. This early water uptake was postulated to be an initial step in pollen grain germination (Feijó *et al.*, 1995). The hydration of *Brassica* pollen grains is mediated via plasma membrane intrinsic proteins (PIPs), and the PIP-dependent water permeability of papillar and subpapillar cells of the stigma, demonstrated by expression of *PIP1* genes in these cells, has been suggested to be involved in the self-incompatibility process in Brassicaceae (Sarker *et al.*, 1988; Ikeda *et al.*, 1997; Marin-Olivier *et al.*, 2000). These studies show the importance of aquaporins in the stigma tissue to control the supply of water. However, the situation on the male side, that is, water uptake by the pollen grain, remains less clear and contradictory results concerning the presence of PIPs in *Brassica* pollen have been published (Marin-Olivier*et al.*, 2000; Dixit *et al.*, 2001). Because of limitations in the availability of tools and genome sequences, the only comprehensive analysis of major intrinsic protein (MIP) gene expression was previously performed in *Arabidopsis thaliana*. However, Bock *et al.* (2006) could not detect any PIP transcripts using the ATH1 gene chip. Only tonoplast intrinsic proteins (TIP: AtTIP1;3, AtTIP5;1) and NOD 26-like intrinsic proteins (NIP: AtNIP4;1) were preferentially expressed in mature pollen. Both TIP genes had been found previously in an expression analysis of hydrated *A. thaliana* pollen grains together with a single PIP gene, AtPIP1;3 (Becker *et al.*, 2003). The findings of both analyses are corroborated by the AtGen-Express plant organ data (www.genevestigator.ethz.ch), which do not indicate substantial expression of any PIP gene in *A. thaliana* pollen. Using the more sensitive RT-PCR, Bots *et al.* (2005) were able to detect two PIP mRNAs, NtPIP1;1 and NtPIP2;1, in tobacco (*Nicotiana tabacum*) pollen grains and tubes, but again at a much lower level than the corresponding transcripts detected in complete reproductive tissues such as anthers and pistils. Thus, in *A. thaliana* pollen no substantial transcription of PIP and even most MIP genes was detected. In *Brassica* and tobacco pollen there is very low transcription of PIP genes, as revealed by molecular biology studies.

However, physiological studies have provided substantial evidence for water transport during pollen grain germination and tube growth. Pollen tubes are one of the fastest growing plant cells and sufficient water uptake has to accommodate this increase in volume. A water uptake rate of 9.33 $\mu m^3\ s^{-1}$ (*c.* 0.56 pl min^{-1}) was estimated in a study on the hydrodynamics of growing *Agapanthus umbellatus* pollen tubes (Malhó & Pais, 1992) and volume changes were observed in tip-growing tobacco pollen tubes (Zonia *et al.*, 2002, 2006). In addition, water uptake must be well regulated, as demonstrated by the finding that the turgor pressure of lily (*Lilium longiflorum*) pollen tubes adapted to the respective osmolality of the germination medium (Benkert *et al.*, 1997), whereas sudden concentration changes immediately arrested tube growth and prevented Ca^{2+} influxes (Pierson *et al.*, 1994). Despite this clear evidence for water uptake and osmoregulation during germination and tube growth, data on the water transport itself or on the specific role of aquaporin-mediated water transport are completely lacking to date.

To investigate a putative physiological role of aquaporins in water uptake and pollen tube growth, four well-characterized PIP genes of *A. thaliana* were transiently expressed in lily

pollen by particle bombardment. Their effects on the water transport properties of protoplasts obtained from transformed pollen grains and tubes were measured in swelling assays and their effects on pollen tube growth rates were also determined.

Materials and Methods

Plant material, *in vitro* germination and protoplast preparation

Pollen grains of *Lilium longiflorum* Thunb. grown in a glasshouse under normal environmental conditions were collected from dehydrated anthers and used immediately for experiments or frozen in liquid N_2 and stored at −80°C. No differences in germination frequency and tube morphology between fresh and frozen pollen grains were detected.

To measure tube growth rates, pollen grains were suspended in germination buffer (10% (w/v) sucrose, 1.6 mM H_3BO_3, 1 mM KCl, and 0.1 mM $CaCl_2$, pH 5.6). For germination and tube growth measurements of transformed pollen, the isolation medium was carefully exchanged in a stepwise manner with germination medium. Tube growth was monitored by taking images every 15 s with a video-equipped microscope (Axiovert 135; Zeiss, Oberkochen, Germany). Pollen tube lengths were measured using META IMAGING Series 6.3 software (Visitron Sytems, Puchheim, Germany).

Protoplasts from control and transformed lily pollen grains and tubes were prepared as described previously (Griessner & Obermeyer, 2003). Briefly, lily pollen grains from one anther were incubated in 2.5 ml of isolation medium (IsoMed: 680mM mannitol, 5mM $CaCl_2$, 10 mM KCl, 0.5 mM ascorbic acid and 10 mM 2-morpholino-ethanesulfonic acid (MES) adjusted with 1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP) to pH 6.0) containing the following enzyme concentrations: 24 U ml^{-1} cellulase (Onozuka; Serva, Heidelberg, Germany), 2 U ml^{-1} pectinase (Sigma, Taufkirchen, Germany) and 2.6 U ml^{-1} macerozyme (Serva). Pollen grains were incubated in the dark, with continuous gentle shaking at room temperature. Released protoplasts were pipetted into a Petri dish with fresh, ice-cold IsoMed without enzymes using a Pasteur pipette with a tip diameter of 200–300 μm. This manual washing procedure was repeated twice and protoplasts were always kept on ice. For preparation of pollen tube protoplasts, transformed, fluorescent pollen grains were manually collected, re-suspended in germination medium, and incubated at room temperature. Germinated pollen grains were then suspended in an enzyme solution (IsoMed with 22 U ml^{-1} cellulase and 11.2 U ml^{-1} macerozyme) and incubated at room temperature for 30 min. The fluorescent pollen tube protoplasts were washed like the pollen grain protoplasts. All collected protoplasts were kept on ice and used within 1–4 h for swelling experiments. No regeneration of cell walls could be observed under these conditions.

Construction of expression plasmids

To generate the plasmid pLAT52pA2, nucleotides −602 (*Sal*I) to −5 with respect to the ATG start codon of the tomato (*Lycopersicon esculentum*) LeLAT52 promoter (accession number X15855; Twell *et al.*, 1990) were fused in a pBluescript KS (+) vector background (Stratagene, La Jolla, CA, USA) with the *Sac*I–*Not*I vector cassette at the 5′-side of the promoter, a polylinker sequence 5′-ACCATGGGGATCCGAATTCGATATCAAGCTTATCGATTCTAGACTGCAG followed by the nopaline synthase polyadenylation sequence (NOS; 252 bp), a regenerated *Bgl*II site and the vector-derived *Xho*I–*Kpn*I cassette. The coding region of a synthetic green fluorescent protein (GFP) gene and the same NOS sequence (Chiu *et al.*, 1996) were fused with the LeLAT52 promoter fragment described above to create the plasmid pLat52::sGFP.

To obtain pSUC3::sGFP, pSUC3::ECFP, and pSUC3::EYFP the coding sequences of the three fluorescent proteins were amplified by PCR with a 5′-*Bsi*EI and a 3′-*Bam*HI adaptor

and ligated into the *Sac*II and *Bgl*II sites of pCL23 (Meyer *et al.*, 2004), removing all four restriction sites from the final construct; in addition the 5′-*Nco*I site (mutation in the oligo for PCR amplification) and the 3′-*Sac*I and *Xba*I sites (restriction with *Sac*I followed by T4 DNA polymerase and re-ligation) were eliminated.

Using 5′-GGGGACTCGAGCTTGGTAAGAAGAACTCTATCAC and 5′-GGGGAGAGCT CCCGACTAGTAACATAGATGAC the complete expression cassettes of pSUC3::sGFP, pSUC3::ECFP, and pSUC3::EYFP were amplified and ligated head-to-head into pLAT52pA2 digested with *Sac*I and *Sal*I to obtain plasmid vectors pSUC3::XFP-LAT52pA. These plasmid vectors harbor a polylinker for insertion of any open reading frame of interest under the control of the pollen-specific LeLAT52 promoter and a complete expression cassette for expression of either of the fluorescent proteins under the control of the pollen-expressed AtSUC3 promoter. In this study, the coding regions of AtPIP1;1 and AtPIP1;2 were amplified by PCR from cDNA clones, restricted with *Bam*HI/*Hin*dIII and *Nco*I/*Eco*RI, respectively, and ligated into pSUC3::ECFP-LAT52pA to generate pSUC3::ECFP-LAT52-AtPIP1;1 and pSUC3::ECFP-LAT52-AtPIP1;2. Similarly, AtPIP2;1 and AtPIP2;2 were amplified, and cut with *Bam*HI/*Hin*dIII and *Bsp*HI/*Hin*dIII to create pSUC3::EYFP-LAT52-AtPIP2;1 and pSUC3::EYFP-LAT52-AtPIP2;2.

Transformation of lily pollen

The pollen grains of one anther were rehydrated for 20 min in 1 ml of IsoMed, washed three times with the same medium and finally re-suspended in 50 μl of IsoMed. The pollen grain suspension was carefully deposited in the middle of a filter paper and placed at the bottom of a well (six- or 24-well tissue culture plate; Greiner, Kremsmünster, Austria). Usually, the pollen grains of at least two anthers were transformed by particle bombardment using a Helios Gene Gun (BioRad, Hercules, CA, USA). Gold particles were coated with plasmids according to the manufacturer's instructions. The DNA loading ratio was 2 μg of DNA per mg of gold beads, and the microcarrier loading quantity (MLQ) was 0.5 mg of microcarrier per shot. For double-coating experiments, a ratio of 1 : 1 (w/w) for the two plasmids was used. The optimal bombardment conditions for lily pollen grain transformation were 1.6-μm gold beads, a helium pressure of 200 psi and a distance of 2 cm between the gene gun spacer and the target. Immediately after the bombardment, the pollen grains were suspended in 2.5 ml of fresh IsoMed and incubated in small Petri dishes in the dark at room temperature for 16–18 h, unless otherwise stated. The transformation efficiency for each bombardment experiment was determined by counting the percentage of fluorescent pollen grains in each Petri dish.

Fluorescence microscopy

The transformed pollen grains were screened for the expression of the respective fluorescent protein using an inverted microscope (Axiovert 135) equipped with a CCD video camera (SPOT Insight; Visitron Systems, Puchheim, Germany) and the appropriate filter settings (AHF, Tübingen, Germany) for GFP (ex, 470 ± 20 nm; dichroic mirror, 495 nm; em, 525 ± 25 nm), yellow fluorescent protein (YFP) (ex, 500 ± 10 nm; dichroic, 515 nm; em, 535 ± 15 nm), and cyan fluorescent protein (CFP) (ex, 436 ± 5 nm; dichroic, 460 nm; em, 470 ± 15 nm).

Protoplast swelling assay and determination of P_{os}

To determine the permeability coefficient (P_{os}) of pollen grain and tube protoplasts obtained from control as well as transformed pollen, protoplasts were pipetted into hypoosmolar medium (same composition as the isolation medium but with osmolality set to 400–500 mOsmol), and images were acquired at a sampling rate of 0.25 Hz for 450–600 s with the video-equipped, inverted microscope, and finally analysed using the META IMAGING software. The

osmolalities of all media were determined with an osmometer (Gonotec, Berlin, Germany) for each experiment. The diameter D of a swelling protoplast was measured and the corresponding volume was calculated as $V = (\pi D^3)/6$. Data processing and the calculation of P_{os} and the relative nonosmotic volume β were performed as described previously (Sommer *et al.*, 2007). Briefly, the *measured volume* values were normalized and transformed to *relative osmotic volume* values by considering β, according to the following relation:

$$V_{rel}^{os}(t) = \frac{V_{rel}^{measured}(t) - \beta}{1 - \beta} \quad \text{Eqn 1}$$

with $V_{rel}^{measured}(t) = V^{measured}(t) / V_0^{measured}$ and $V_{rel}^{os}(t) = V^{os}(t) / V_0^{os}$. The subscripts '0' and 'f' (Eqns 2, 4) refer to initial (t = 0 s) and final (steady-state) values, respectively, and the superscript 'os' to osmotic variables as opposed to measured variables. The value of the relative nonosmotic volume β was inferred for each measured protoplast from the corresponding volume transient using the following equation:

$$\beta = \frac{\alpha - \frac{V_f^{measured}}{V_0^{measured}}}{\alpha - 1} \quad \text{Eqn 2}$$

In a second step, the values of the relative osmotic volume $V_{rel}^{os}(t)$ were fitted with Eqn 3 and the fit parameter k was finally used to calculate the osmotic water permeability coefficient P_{os} (Eqn 4; see also Sommer *et al.*, 2007):

$$\ln\frac{\alpha - V_{rel}^{os}(t)}{\alpha - 1} + \frac{V_{rel}^{os}(t) - 1}{\alpha} = -kt \quad \text{Eqn 3}$$

$$P_{os} = \frac{k\alpha V_0^{os}}{V_w A c_f} \quad \text{Eqn 4}$$

with the following notations for α: $c_0/c_f = V_f^{os}/V_0^{os} = \alpha$. Fitting routines were performed with SIGMAPLOT 9 software (Jandel Scientific, Erkrath, Germany).

Statistical analysis

Mean values and standard deviations (SDs) were calculated. Significance levels were determined using SIGMASTAT 3 or SIGMAPLOT 9 software (Jandel Scientific), performing either an unpaired Student's *t*-test or a Mann–Whitney rank sum test as indicated. Two data groups were designated as significantly different for $P < 0.01$.

Single-cell RT-PCR

Detection of the mRNAs of AtPIP1;1 in individual, transformed pollen grain protoplasts was carried out according to Gehwolf *et al.* (2002). First an AtPIP1;1-specific, digoxigenin-labelled probe was generated by amplification of a 403-bp region of the ATPIP1;1 plasmid with the following primer pair: AtPIP1_1_fwd (5′-TGCTGAGATCATTGGCACA-3′) and NOSpA_rev (5′-AAGACCGGCAACAGGATT-3′). The specificity of the probe and the hybridization conditions were optimized by hybridization of the probe with digested control plasmids containing AtPIP1;1 (*Bam*HI/*Hin*dIII), AtPIP2;1 (*Bam*HI/*Hin*dIII), and AtPIP1;2 (*Nco*I/*Eco*RI) sequences. Single protoplasts were collected after a swelling experiment, pipetted into PCR tubes with 1 μl of protoplast medium plus 20 U RNasin (Fermentas, St Leon-Rot, Germany), and frozen in liquid N_2 to break the protoplast. Reverse transcription

was performed with the specific reverse primer NOSpA-rev and products were subsequently amplified by PCR with the AtPIP1_1_fwd and NOSpA_rev primers. Amplified products were electrophoresed in a 1% agarose gel, transferred onto a membrane and detected with the digoxigenin-labelled probe as follows: hybridization at 60°C overnight, 2 × 5 min washing at room temperature with 2 × saline sodium citrate (SSC) + 0.1% sodium dodecyl sulphate (SDS) and washing at 72°C for 20 min twice with 0.1 × SSC + 0.1% SDS. After washing in maleate-Tween 20 buffer the hybridized probe was detected with anti-digoxigenin antibody conjugated with horseradish peroxidase (Roche Diagnostics, Vienna, Austria) by chemiluminescence (ECL; GE-Healthcare, Vienna, Austria).

Results

Optimization of the bombardment conditions

To determine the optimal conditions for the transient transformation of lily pollen, the particle bombardment parameters were changed by a step-by-step procedure to achieve a high transformation rate while leaving pollen germination unaffected and keeping loss of material to a minimum. In the first step, the gold particle size (1.6 μm) and the MLQ (0.25 mg per shot) were kept constant while the helium pressure (100, 150, 200 and 400 psi) and distance to the target (0 and 2 cm) were varied. At pressures higher than 200 psi and no additional distance (0 cm) between the gene gun and the pollen we noted high sample loss and considerable damage of the pollen grains for all the pressures used. In contrast, a helium pressure of 100 psi led to an unacceptably low transformation rate. Helium pressures of 200 and 150 psi and a distance of 2 cm to the target were tested while varying the size of the gold particles (1 and 1.6 μm) and the MLQ (0.25 and 0.5 mg per shot). The combination that gave the best and most reliable results was 0.5 mg of gold per shot, a particle size of 1.6 μm, a distance of 2 cm and a helium pressure of 200 psi, and this combination was therefore used throughout this study.

Transient expression of fluorescent proteins in lily pollen

To analyse the effect of particle bombardment and the transient expression of fluorescent proteins on water permeability, pollen grains were transformed with plasmids pLAT52::sGFP and pSUC3::sGFP containing the GFP sequence under the control of two promoters. The LeLAT52 (Twell *et al.*, 1990) and AtSUC3 (Meyer *et al.*, 2004) promoters had previously been shown to direct expression in pollen of tomato and *A. thaliana*, respectively. GFP-specific fluorescence could be observed 16–24 h after transformation, indicating that both promoters are also active in pollen grains of the monocot plant *L. longiflorum*, and the observed fluorescence intensity was independent of the promoter used (data not shown). Pollen grain protoplasts that expressed GFP under the control of the AtSUC3 and LeLAT52 promoters showed permeability coefficient P_{os} values of 5.41 ± 1.50 and 6.97 ± 2.23 μm s^{-1}, respectively, and were not significantly different ($P > 0.05$, Student's *t*-test) from pollen grain protoplasts bombarded with noncoated gold particles (Fig. 1, no plasmid: 7.44 ± 2.87 μm s^{-1}). The P_{os} values of all protoplasts presented in Fig. 1 ranged from 3.5 to 11 μm s^{-1}, giving a mean value of 6.65 ± 2.41 μm s^{-1}, which is similar to that of native, untreated lily pollen grain protoplasts (6.59 ± 2.26 μm s^{-1}; Sommer *et al.*, 2007). Therefore, neither the bombardment itself, nor the expression of a fluorescent protein, nor the subsequent incubation affected the water permeability of the plasma membrane of the protoplasts.

Expression of AtPIPs in lily pollen

To investigate the putative role of aquaporins during water transport across the plasma membrane and subsequently in pollen tube growth, pollen grains were bombarded with plasmids of the type pSUC3::xFP_LAT52::PIPy;z (see the Materials and Methods; xFP =

CFP or YFP and PIPy;z = PIP1;1, PIP1;2, PIP2;1 or PIP2;2) that allow the expression of a fluorescent protein under the control of the AtSUC3 promoter and the expression of the respective PIP under the control of the LeLAT52 promoter. The PIP genes were chosen because they are highly expressed in *A. thaliana* plants (Kammerloher *et al.*, 1994; Jang *et al.*, 2004) and were found to confer a slight (PIP1;1 and PIP1;2) or strong (PIP2;1 and PIP2;2) enhancement of water permeability in the heterologous oocyte expression system (Kammerloher *et al.*, 1994). Bright field and fluorescence images of the respective protoplasts, for which a volume transient was measured, were taken at the start (t_0) as well as at the end (t_{final}) of a swelling episode and clearly show the expression of the respective fluorescent protein and the increase in protoplast volume (Fig. 2). Typical time-dependent increases in the relative osmotic volume (V_{rel}^{os}) of pollen grain protoplasts bombarded with plasmids for the expression of AtPIP1;1/CFP and AtPIP1;2/CFP are shown in Fig. 2a and b, respectively. Although both protoplasts showed CFP fluorescence, indicating their transformation, their P_{os} values (7.65 μm s^{-1} for AtPIP1;1 and 7.87 μm s^{-1} for AtPIP1;2) were not different from those of control protoplasts. However, pollen grain protoplasts expressing PIP2;1/YFP or PIP2;2/YFP did show an increase in P_{os} values (16.72 and 12.37 μm s^{-1}, respectively), indicating an increase in the water permeability of the protoplast plasma membrane upon PIP2 expression (Fig. 2c,d). This result is confirmed by a statistical comparison of the means of the respective P_{os} values obtained from protoplasts expressing members of the PIP1 or PIP2 family (Fig. 3): pollen grain protoplasts transformed with PIP2;1 (13.79 ± 6.38 μm s^{-1}) and PIP2;2 (10.16 ± 3.30 μm s^{-1}) showed a significant increase in P_{os}, whereas the values for pollen grain protoplasts expressing PIP1;1 (6.93 ± 2.13 μm s^{-1}) and PIP1;2 (6.24 ± 1.32 μm s^{-1}) were within the range of the controls.

To confirm the transcription of AtPIP1;1 in cells showing the expression of the corresponding fluorescent reporter (CFP) without an effect on the swelling behaviour, single-cell RT-PCR experiments on individual pollen grain protoplasts transformed with AtPIP1;1/CFP were performed. A PIP1;1-specific signal of the expected size could be observed in the transformed, but not in the nontransformed, control pollen grain protoplasts (Fig. 4), indicating the successful transcription of AtPIP1;1. Immunological detection of the AtPIP1 protein was not possible because of the lack of an antibody recognizing AtPIP1 proteins specifically in lily protoplasts and in a microsomal fraction of lily pollen (data not shown). Therefore, no definite conclusion can be drawn regarding the effect of PIP1 proteins on water permeability.

Recent observations indicate that PIP1 and PIP2 proteins might interact (Fetter *et al.*, 2004; Zelazny *et al.*, 2007) and a synergistic enhancement of water permeability has been shown in the heterologous oocyte expression system (Fetter *et al.*, 2004; Temmei *et al.*, 2005). Co-expression analyses based on public array data indicated that AtPIP1;1 and AtPIP2;2 as well as AtPIP1;2 and AtPIP2;1 show the highest correlation factors for PIP gene expression (O. Da Ines & A. R. Schäffner, unpublished; *Arabidopsis* Co-expression Data Mining Tool ACT (Manfield *et al.*, 2006)). Therefore, these combinations were chosen for co-expression in lily pollen grains. For co-transformation, the gold microcarriers were double-coated with the respective PIP1/CFP and PIP2/YFP plasmids. It should be noted that the fluorescence of CFP was faint when CFP was co-expressed with YFP as long as equimolar ratios were used, an observation also made in transformed mammalian cells (pers. comm., Markus Ritter, Paracelsus Medical University, Salzburg, Austria). Indeed, the spectroscopic properties of the two fluorescent proteins are very different: the molar extinction coefficient of YFP is almost three times higher than that of CFP, and thus YFP has a higher quantum efficiency (Dixit *et al.*, 2006). At a weight ratio of 4 : 1 for CFP:YFP plasmids, the fluorescence intensities of the two proteins were comparable (data not shown). Nevertheless, a 1 : 1 plasmid ratio was used in the co-transformation of lily pollen grains, because we were interested in equal expression of PIP1 and PIP2 driven by the same promoter. Simultaneous

transformation of pollen grains with a PIP1 and a PIP2 did not further increase water permeability (Fig. 5). Typical swelling experiments for the combinations AtPIP1;1 with AtPIP2;2 and AtPIP1;2 with AtPIP2;1 resulted in P_{os} values of 10.57 and 11.44 μm s^{-1}, respectively (Fig. 5a,b). The P_{os} values were in the range of those measured in protoplasts expressing the respective PIP2 protein alone, as summarized in Fig. 3, which could be confirmed by statistical analysis using Student's *t*-test or the Mann–Whitney rank sum test.

Protoplasts obtained from transformed pollen tubes also showed fluorescence and could be subjected to swelling assays. The volume transient of a pollen tube protoplast transformed with AtPIP2;1 is shown in Fig. 6a; this gave a P_{os} value of 18.02 μm s^{-1}. The mean P_{os} value for transformed pollen tube protoplasts (18.85 ± 7.60 μm s^{-1}) was not significantly different ($P = 0.058$) from that of control pollen tube protoplasts (13.23 ± 4.14 μm s^{-1}; Fig. 3).

Growth of pollen tubes expressing AtPIPs

To determine whether there was an effect of the AtPIPs on pollen tube growth, the successfully transformed, fluorescent pollen grains were collected manually and carefully suspended in germination medium. Despite the long incubation time necessary for the expression of the fluorescent protein, the pollen grains started to germinate after a longer lag-phase (2–3 h) and transformed pollen tubes showed the same morphology as the control tubes (Supporting Information Fig. S1). The mean tube growth rates of pollen tubes transformed with either AtPIP1 or AtPIP2 plasmids ranged between 6 and 10 μm min^{-1} and were similar to that of control pollen tubes (9.05 ± 2.67 μm min^{-1}; Fig. 6b).

Discussion

Expression and activity of AtPIPs in lily pollen

Transformation of lily pollen grains by particle bombardment with control gold beads or gold beads coated with plasmids containing expression cassettes for fluorescent proteins (CFP, GFP and YFP) did not affect the germination of pollen grains, pollen tube morphology or the water permeability coefficient P_{os} of the plasma membrane. This finding is in agreement with other reports showing that GFP expression did not alter pollen germination frequency or pollen tube growth rates (Keller & Hamilton, 1998; Hudson & Stewart, 2004). Protoplasts prepared from pollen grains expressing only fluorescent proteins exhibited similar water permeability coefficient values (6.65 ± 2.41 μm s^{-1}) to protoplasts derived from nonbombarded pollen grains (6.59 ± 2.26 μm s^{-1}; Sommer *et al.*, 2007).

In contrast, expression of *A. thaliana* PIP2;1 and PIP2;2 increased water permeability in pollen grain protoplasts, whereas PIP1;1 and PIP1;2 did not. This result is in accordance with studies in heterologous, nonplant expression systems (*Xenopus* oocytes) in which no or lower water transport activity was detected when PIP1s were expressed (*A. thaliana* PIPs: Kammerloher *et al.*, 1994; tobacco PIPs: Biela *et al.*, 1999; Bots *et al.*, 2005; maize (*Zea mays*) PIPs: Fetter *et al.*, 2004; *Brassica* PIPs: Dixit *et al.*, 2001; *Samanea saman* PIPs: Moshelion *et al.*, 2002). Therefore, this study provides a proof-of-principle that pollen grain protoplasts are suitable for use as a plant-based expression system to test the functionality of putative aquaporins, in view of the simple handling procedures required, their ability to become transformed by particle bombardment and, most importantly, their low endogenous water permeability which was postulated as a prerequisite for 'homologous' expression systems for plant PIPs (Maurel, 2007).

As the functional activity of AtPIP2s observed in the pollen grain protoplasts is an unequivocal proof of their correct targeting to the plasma membrane, we did not investigate their localization in particular. PIPs can only contribute to water transport if they are

incorporated into the plasma membrane of pollen grain protoplasts. Pollen tubes, where ectopic AtPIP2 expression did not further increase water permeability, developed from transformed pollen grains, still representing the same cells observed 2 h later. Therefore, the capability to locate the ectopic AtPIP2 to the plasma membrane will probably be retained. Indeed, the plasma membrane localization of PIP2 proteins was previously reported in several cell types with PIPs of *Z. mays* expressed in *Xenopus* oocytes and *Z. mays* mesophyll cells (Fetter *et al.*, 2004; Zelazny *et al.*, 2007) and with PIPs of *A. thaliana* root cells (Boursiac *et al.*, 2005) by using GFP::PIP fusion proteins. However, we cannot exclude the possibility of post-translational modifications that may cause an inactivation of the PIPs in the plasma membrane of pollen tubes.

In contrast to *Xenopus* oocyte experiments, in which co-expression of PIP1s together with PIP2s from *Z. mays* further increased the water permeability of the oocytes (Fetter *et al.*, 2004), we observed that co-expression of AtPIP1;2 with AtPIP2;1 or AtPIP1;1 with AtPIP2;2 did not increase the P_{os} values of lily pollen grain protoplasts compared with pollen grain protoplasts expressing the respective PIP2 alone. Localization of the ZmPIPs expressed in maize mesophyll protoplasts had revealed an interaction between PIP1 and PIP2: under these conditions, the preferentially endoplasmic reticulum (ER)-localized ZmPIP1s re-localized to the plasma membrane in the presence of ZmPIP2s (Zelazny *et al.*, 2007). However, the effect on the water permeability of transformed maize mesophyll protoplasts was less clear: an increase in P_{os} was detected in mesophyll protoplasts expressing mCFP::ZmPIP2;1 whereas variable values were obtained upon co-expression with mYFP::ZmPIP1;2 (Zelazny *et al.*, 2007). In the present study, the lack of an additive effect on water permeability upon co-expression of PIP1s and PIP2s in pollen protoplasts has several possible explanations: (1) the two AtPIPs do not interact although they harbour the amino acid residues, at the expected positions, proposed by Fetter *et al.* (2004) to be responsible for the interaction between PIP isoforms in oocytes; (2) they do interact but plant-specific regulation may prevent a further increase in water permeability, or (3) an endogenous lily pollen PIP1 interacts with the expressed AtPIP2 and forms a functional water channel in the plasma membrane, increasing water permeability. Additional expression of AtPIP1 cannot increase the water permeability any further because of the limiting amount of expressed AtPIP2s necessary for functional water channels.

Nevertheless, the endogenous water permeability of the pollen grain plasma membrane increased during pollen grain germination and subsequent tube growth, as indicated by the twofold enhancement of the measured P_{os} values of tube protoplasts (*c.* 6.5 $\mu m\ s^{-1}$ for the pollen grain and 13 $\mu m\ s^{-1}$ for the pollen tube). We can only speculate on the molecular background of this observation: there may be an increase in the translation rate of endogenous aquaporins, or their activity might be increased by post-translational regulation, or synergisms between different endogenous lily PIP isoforms may result in higher water permeability.

Water transport and pollen tube growth

The P_{os} values measured in this study can be used to estimate whether the measured rate of water uptake may account for the volume increase during tube growth. Pollen tubes grow by tip growth, showing a constant tube diameter, and can reach a length of up to 10 cm depending on the respective style length. This dramatic increase in cell volume has to be complemented mainly by the uptake of water. The rate of volume increase corresponding to the average pollen tube growth rate of 10 $\mu m\ min^{-1}$ is 1.1 $pl\ min^{-1}$ as calculated from the geometry of a lily pollen tube with a representative diameter of 12 μm.

Using the measured P_{os} values, water influxes can be calculated according to the equations presented by Steudle (1989) and Zimmermann (1989), resulting in a maximal water influx

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of about 110 pl min^{-1} for a pollen grain with a 500-μm-long pollen tube. This water influx is more than sufficient to allow the observed volume increase that occurs during tube growth and has to be tightly regulated by adjustment of the osmotic potential gradient across the plasma membrane to prevent bursting of the growing pollen tube. However, net water uptake might be reduced by allowing water efflux at a region different from the sites of water influx, as postulated by Zonia *et al.* (2006) and Zonia & Munnik (2007). This is, however, only possible if one assumes reversed driving force directions for water flux at different regions of the large, longitudinal pollen tube, which have not been measured to date.

In addition to their role as water channels, aquaporins might have a role in osmoregulation by sensing the pressure and/or osmotic gradient across the plasma membrane (Hill *et al.*, 2004). Accordingly, in pollen, aquaporins may also function as osmosensors, preventing the growing tube from bursting, and thus, as part of the regulatory network, control pollen tube growth and contribute to the concerted action of the cellular processes necessary for tip growth (Feijó *et al.*, 1995; Holdaway-Clarke & Hepler, 2003).

In conclusion, expression of *A. thaliana* PIPs in lily pollen showed that members of the PIP1 subfamily did not confer enhanced water channel activity, whereas members of the PIP2 subfamily clearly did. The water permeability of the plasma membrane of unmodified pollen tubes is higher than the water permeability of the plasma membrane of the pollen grain and, interestingly, the ectopic expression of AtPIP2s did not further enhance the water permeability of pollen tubes. The molecular basis of the physiological increase in P_{os} during pollen grain germination and tube growth, for example, differential expression or regulation of endogenous lily PIPs, will be a matter for future investigations. Nevertheless, the calculated water uptake rates and the fact that expression of AtPIPs did not increase pollen tube growth rates suggest that water permeability and the resulting water influx across the plasma membrane are not limiting pollen tube growth, but have to be tightly regulated to prevent pollen tube bursting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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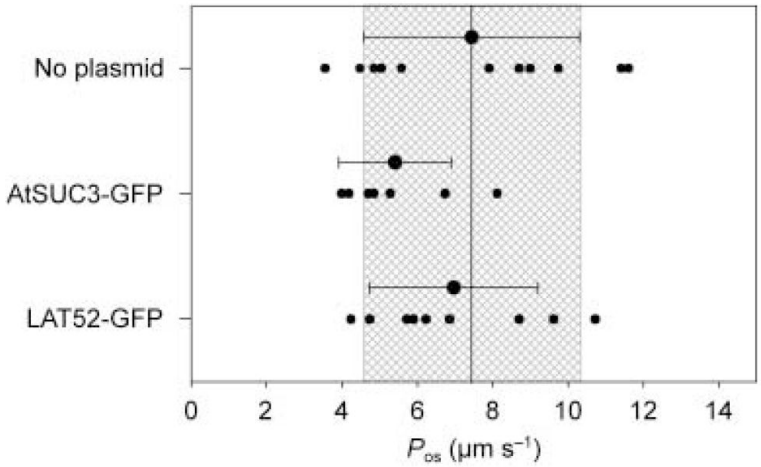

Fig. 1.

Water permeability coefficient (P_{os}) measured in lily (*Lilium longiflorum*) control pollen grain protoplasts (only gold particles, not plasmid coated) or protoplasts obtained from pollen grains bombarded with gold beads coated with AtSUC3::GFP and LeLAT52::GFP plasmids. Small data points indicate individual experiments. Large data points are mean ± SD. The mean P_{os} value of controls is indicated by the solid line; the grey box indicates the SD of control values.

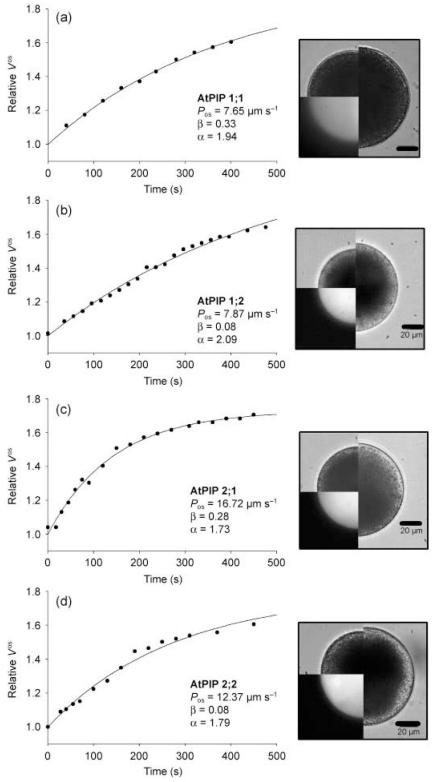

Fig. 2.

Typical volume transients (swelling curves) of protoplasts obtained from lily (*Lilium longiflorum*) pollen grains transformed with *Arabidopsis thaliana* plasma membrane intrinsic protein 1;1 (AtPIP1;1)/cyan fluorescent protein (CFP) plasmids (a), AtPIP1;2/CFP plasmids (b), AtPIP2;1/yellow fluorescent protein (YFP) plasmids (c), or AtPIP2;2/YFP plasmids (d). The calculated water permeability coefficient (P_{os}) and the respective parameters α and β (see Materials and Methods) are given for each individual protoplast. The images show the protoplast at $t = 0$ min as bright field images (upper left quarter) and fluorescence images (lower left quarter). The right half of the image shows the protoplast at the end of the swelling curve (t_{final}). Bars, 20 µm.

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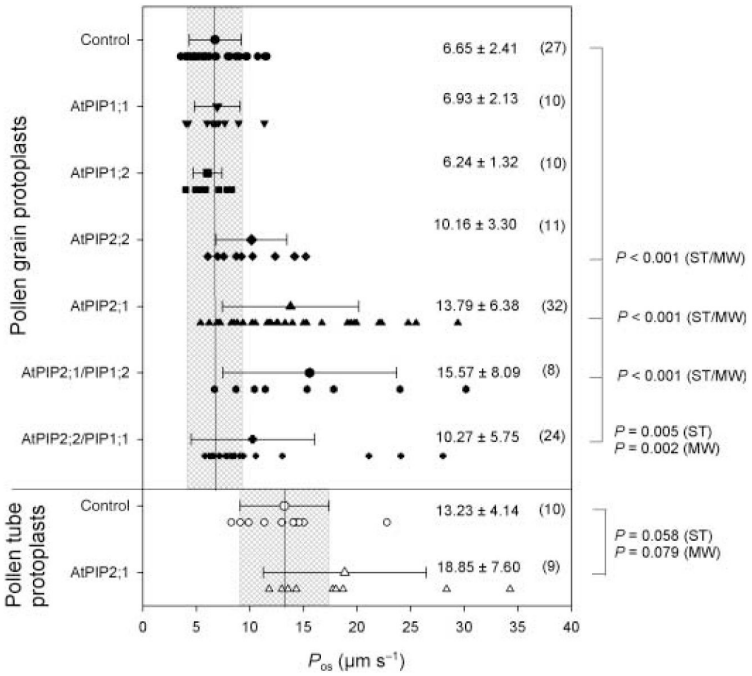

Fig. 3.

Summary of water permeability coefficient (P_{os}) values measured in control and transformed lily (*Lilium longiflorum*) pollen grain protoplasts as well as in control and transformed pollen tube protoplasts. Individual P_{os} values and mean ± SD are presented. Solid lines and grey boxes indicate the mean and the corresponding SD of the respective control P_{os} values. The respective mean ± SD of P_{os} values for each treatment is given in µm s^{-1} and the number in brackets indicates the number of individual protoplasts measured. P_{os} values significantly different from control data are indicated, with significance values (*P*) calculated using Student's *t*-test (ST) and the Mann–Whitney test (MW).

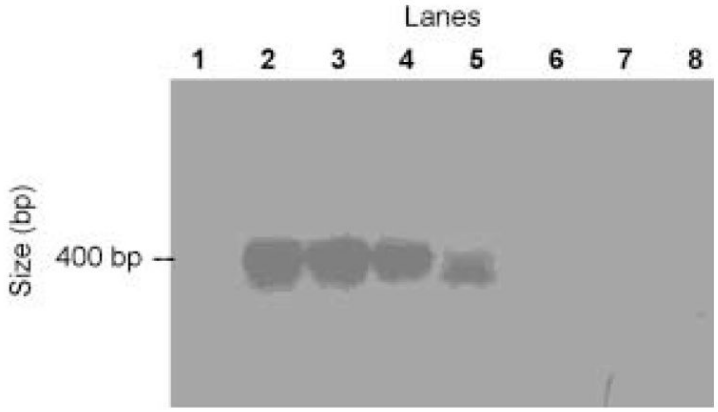

Fig. 4.

Single-cell RT-PCR of *Arabidopsis thaliana* plasma membrane intrinsic protein 1;1 (AtPIP1;1)-transformed and control pollen grain protoplasts from lily (*Lilium longiflorum*). Fluorescent as well as control pollen grain protoplasts were collected and immediately frozen in liquid nitrogen. cDNA was generated by reverse transcription with AtPIP1;1-specific reverse primer, followed by PCR with an AtPIP1;1-specific primer pair. Amplified product was separated by agarose gel electrophoresis, blotted onto a membrane and detected using a digoxigenin-labelled specific AtPIP1;1 probe. Lane 1, water control; lanes 2–5, individual, transformed protoplasts; lanes 6–8, nontransformed protoplasts.

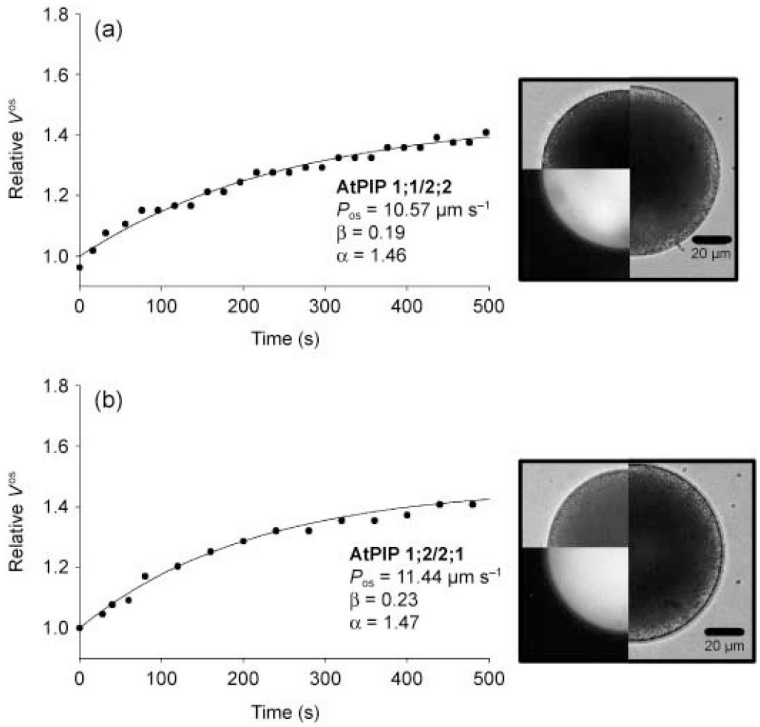

Fig. 5.

Swelling curves of protoplasts obtained from lily (*Lilium longiflorum*) pollen grains co-transformed with *Arabidopsis thaliana* plasma membrane intrinsic protein 1;1 (AtPIP1;1)/AtPIP2;2 (a) and AtPIP1;2/AtPIP2;1 (b). The images show the protoplast at $t = 0$ min as bright field images (upper left quarter) and as fluorescence images (lower left quarter). The right half of the image shows the protoplast at the end of the swelling curve (t_{final}). Bars, 20 μm.

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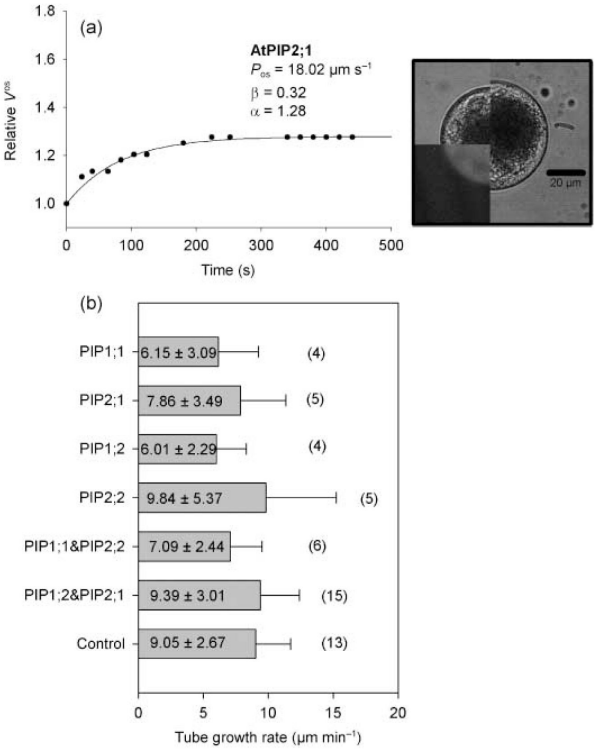

Fig. 6.

Typical swelling curve of a pollen tube protoplast transformed with *Arabidopsis thaliana* plasma membrane intrinsic protein 2;1 (AtPIP2;1) (a) and pollen tube growth rates measured in pollen tubes of control and transformed pollen grains (b). The images show the protoplast at $t = 0$ min as bright field images (upper left quarter) and as fluorescence images (lower left quarter). The right half of the image shows the protoplast at the end of the swelling curve (t_{final}). Numbers in brackets give the number of individual experiments whereas the numbers in the boxes give the mean growth rate ± SD in µm min^{-1}. Bar, 20 µm.

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