PIP1 and PIP2 aquaporins are differentially expressed during tobacco anther and stigma development

Marc Bots1, Richard Feron1, Norbert Uehlein2, Koen Weterings1, Ralf Kaldenhoff2 and Titti Mariani1,*

1 Department of Experimental Botany, Graduate School of Experimental Plant Sciences, Catholic University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands
2 Fachbereich Biologie, TU Darmstadt, Schnittspahnstr. 10, D-64287, Darmstadt, Germany

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Abstract
Several processes during sexual reproduction in higher plants involve the movement of water between cells or tissues, such as occurs during dehiscence of the anther and hydration of the pollen grain after it is deposited on a stigma. To get more insight in these processes, a set of putative aquaporins was cloned and it was found that at least 15 are expressed in reproductive organs, which indicates that the control of water flow is important for reproduction. Functional studies in Xenopus laevis oocytes using two of the cDNAs showed that NtPIP2;1 is an efficient aquaporin, whereas NtPIP1;1 is not. Expression studies on RNA and protein levels showed that PIP1 and PIP2 genes are differently expressed in reproductive organs: PIP1 RNA accumulates in the stigma, and PIP1 and PIP2 RNA can be detected in most tissues of the anther.

Key words: Anther, aquaporin, sexual reproduction, stigma, tobacco.

Introduction
In sexual plant reproduction, control of the movement of water plays an important role in a number of processes that must be completed for fertilization to occur. At flower anthesis, both anthers and pollen grains have undergone dehydration and, in the majority of the angiosperms, pollen contains less than 30% water at anther dehiscence (Goldberg, 1988; Franchi et al., 2002).

Correct timing of anther dehiscence is important in reproduction, as the time of pollen release is crucial for pollination and fertilization to take place. The mechanism of anther dehiscence is complex and involves changes in carbohydrate metabolism and dehydration of the anther and pollen (Table 1; Keijzer, 1987; Bonner and Dickinson, 1990; Clement and Audran, 1995; Pressman et al., 2002). Dehydration may involve evaporation or movement of water following an osmotic gradient via the vascular bundle towards the base of the flower. Although dehydration and the changes in carbohydrate metabolism may prepare the anther for dehiscence, the splitting of the anther is determined by degeneration of the stomium cells (Beals and Goldberg, 1997). Concurrently with anther dehydration, pollen grains also dehydrate. The significance of this process for the functions of the pollen grain is illustrated by the Arabidopsis mutant raring-to-go (Johnson and McCormick, 2001). In these plants, pollen grains hydrate and germinate within the anther when it is exposed to high humidity.

After dehiscence, pollen grains are dispersed and may land on the stigmatic surface of a pistil. If the pistil is receptive, the dehydrated pollen grains rehydrate, mobilizing water from the stigma, before producing a pollen tube. In some species, cross- and self-fertilization is prevented at this level (Dickinson, 1995; Hulskamp et al., 1995).

The water translocated in these processes may move through plasmodesmata in some cases, but this is not always possible. The water that enters the pollen grain during hydration on the stigma, for example, must pass two plasma membranes. Aquaporins are a class of proteins that mediate the passive movement of water and have been found in various cellular membranes (Maurel et al., 2002). The Plasma membrane Intrinsic Protein (PIP) subfamily of aquaporins, which is thought to be plasma membrane localized, can be divided into two subfamilies and has been suggested to function in a number of cellular processes (Tyerman et al., 2002). Aquaporins are ubiquitously
Materials and methods

*These subfamilies may contribute to different extents to the results show that they display distinct expression patterns. To obtain more information about the functions in the tissues of the reproductive organs where they are expressed, to understand if PIP1 and PIP2 family members perform identical functions in plants, and analysis of plants impaired in the expression of PIP1 or PIP2 proteins has shown that they function in various processes, including root water uptake, stress recovery, and CO₂ transport (Martre et al., 2002; Siefritz et al., 2002; Javot et al., 2003; Uehlein et al., 2003). In addition, aquaporins have been implicated in general processes such as cell expansion and osmotic control (Maurel et al., 2002; Reisen et al., 2003).

As the dehydration of anthers and pollen and pollen rehydration on the stigma are likely to involve movement of water across plasma membranes, it may be expected that aquaporins function to facilitate this process. Indeed it was previously shown that PIP1 aquaporins are expressed in the anther and dry-type stigma of *Brassica* sp. (Ruiter et al., 1997; Marin-Olivier et al., 2000; Dixit et al., 2001) and that a PIP2 isoform is expressed in the anther and pistil of *Solanum chacoense* (O’Brien et al., 2002). However, it is unclear if PIP1 and PIP2 family members perform identical functions in the tissues of the reproductive organs where they are expressed. To obtain more information about the possible roles of these proteins in anther and pollen dehydration, pollen hydration, and pollen–stigma interactions in a plant with a wet-type stigma, PIP1 and PIP2 genes in tobacco reproductive organs have been analysed. The results show that they display distinct expression patterns during tobacco anther and stigma development and that these subfamilies may contribute to different extents to the movement of water in these organs.

### Table 1. Events during tobacco anther and stigma development

<table>
<thead>
<tr>
<th>Stage</th>
<th>Events in anther development</th>
<th>Events in stigma development</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3</td>
<td>Tissue differentiation, meiosis, microspores in tetrads</td>
<td>Tissue differentiation, fusion of carpels, first papillae formed near the fusion groove</td>
</tr>
<tr>
<td>4–6</td>
<td>Degradation of circular cell cluster and tapetum, pollen wall formation</td>
<td>Stigma cells dividing, papillae formation continues, start exudate secretion</td>
</tr>
<tr>
<td>7–9</td>
<td>Degradation of connective tissue, anther bilocular, dehydration of anther tissues commences</td>
<td>Stigma cell enlargement</td>
</tr>
<tr>
<td>10–12</td>
<td>Degradation of connective tissue continues, dehydration of anther and pollen completed, rupture of stomium initiates dehiscence</td>
<td>Secretory zone clearly defined</td>
</tr>
<tr>
<td></td>
<td>Large amounts of exudate on stigma surface, stigma ready for pollination</td>
<td></td>
</tr>
</tbody>
</table>

*a* Koltunow et al., 1990.  
*b* Wolters-Arts et al., 1996.

Expressed in plants, and analysis of plants impaired in the expression of PIP1 or PIP2 proteins has shown that they function in various processes, including root water uptake, stress recovery, and CO₂ transport (Martre et al., 2002; Siefritz et al., 2002; Javot et al., 2003; Uehlein et al., 2003). In addition, aquaporins have been implicated in general processes such as cell expansion and osmotic control (Maurel et al., 2002; Reisen et al., 2003).

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### Materials and methods

#### Plant material

*Tobacco* (*Nicotiana tabacum* cv. Petit Havana SR1) plants were grown under standard greenhouse conditions. Flower stages were determined according to Goldberg (1988). Mature pollen grains were obtained from freshly dehisced anthers. Pollen tubes were determined according to Goldberg (1988). Mature pollen grains grown in liquid medium containing 15% sucrose and 0.01% boric acid for 4 h at 28 °C.

#### Nucleic acid isolation

Total RNA was isolated by grinding the tissue in 1 ml extraction buffer (0.1 M TRIS, 50 mM EDTA, 1% (w/v) SDS, 0.1 M NaCl, 1% tri-iso-propanylmaphthalene sulphonic acid sodium salt, 50 mM β-mercaptoethanol, pH 8.0) and 1 ml phenol. After phenol:chloroform extraction and ethanol precipitation, RNA was obtained from the ethanol-precipitated pellet by LiCl precipitation. The integrity of the RNA was assayed by denaturing agarose gel electrophoresis. Plasmid DNA was obtained using standard alkaline-lysis methods.

#### Isolation of aquaporin cDNAs

**RT-PCR**

RT-PCR reactions were performed with the Access RT-PCR kit (Promega), according to the manufacturer’s protocol. The primers that were used in the reactions were NtAQP3: 5’-TGC TTG GCT TTT TGG TGG TAT GAT-3’ and NtAQP4: 5’-AAG ACT CCT AGC GGG GTT GAT GC-3’ (Biela et al., 1999). For the reactions, RNA from stigmas or anthers of stages 1–3, 4–6, 7–9, or 10–12 was pooled. In the RT-PCR, 200 ng RNA was reverse transcribed using an oligo(dT) primer for 45 min at 48 °C, followed by 40 PCR cycles (30 s at 94 °C, 1 min at 60 °C, and 1 min at 68 °C). The DNA-products resulting from the RT-PCR were cloned using the pGEM-T Easy kit (Promega), according to the manufacturer’s protocol.

#### Family transcript profiling

For the isolation of novel aquaporin family members using RTP, cDNA was prepared from RNA of anther, stigma, and root tissue. Aquaporin-like sequences were amplified from the cDNA pools using the gene-specific primer (FSD1: 5’-TGACTGTGATTTCCTGAGGACATATTAAY-CCNGC-3’) and an oligo(dT) primer. The resulting DNA fragments were then digested with MgI or MboI, ligated to adaptors and subsequently amplified with adapter-specific primers (Mse-F: 5’-GACGATGAGTCCTGAC-3’; Mse-R: 5’-TACTCGAGACTCAT-3’; Taq-F 5’-GACGATGAGTCCTGAC-3’; Taq-R 5’-CGGTCAAGACCTCAT-3’; Mbo-F: 5’-ACTCGAGTTCACC-GAAAGTATAGATCCCA-3’; Mbo-R 5’-GATCTGAGAGATCATACTTCCGTGGAGAAAC-3’) and the FSD primer, which was 5’ end-labelled with 32P. The resulting products were separated on a 4.5% polyacrylamide gel and the gel was exposed to Kodak X-omat AR Scientific Imaging Films to identify the aquaporin fragments, which were then isolated from the gel, cloned, and sequenced according to standard methods.

### 5’ and 3’ RACE and isolation of full-length cDNAs

To obtain the 5’ and 3’ ends of the aquaporin cDNA fragments 4.1 and 8.1, rapid amplification of cDNA ends (RACE-PCR) was performed using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc). cDNA was made from 1 μg of polyA+ RNA according to the manufacturer’s protocol. 5’ and 3’ cDNAs were then amplified by PCR (30 cycles 30 s at 94 °C, 3 min at 64 °C, 3 min at 72 °C for the AQP RACE 5 primer or 10 cycles 30 s at 94 °C, 3 min at 70 °C, 3 min at 72 °C followed by 20 cycles 30 s at 94 °C, 3 min at 72 °C followed by 20 cycles 30 s at 94 °C, 3 min at
68 °C, 3 min at 72 °C for the other primers). The primers used in addition to the adapter-specific primers were AQP RACE 5: 5'-TAA TGC AAT GCC TTG GTG CTA TTT GTG GTG C-3' and AQP RACE 6: 5'-ACA CAG CAA ATC CAA TAG GAG GAG GT-3' for clone Aqp 4.1 and AQP RACE 7: 5'-TTG CCC AAT CTT TAG GTG CAA TTT GTG GTG T-3' and AQP RACE 8: 5'-ACA CAG CAA ATC CAA TAG GAG GAG GTG C-3' for clone 8.1.

To generate the full-length clones of NtPIP1;1 and NtPIP2;1, cDNA was made from 1 μg total RNA with an oligo(dT)15 primer and M-MLV reverse transcriptase (Promega). This cDNA was used as a template in a PCR with Pfu Turbo polymerase (Stratagene) with the M-MLV reverse transcriptase (Promega). This cDNA was used as the template for NtPIP1;1 and NtPIP2-1: 5'-CAC TAG TAA AAG GCA AAT GGC AGG AGA A-3'.

BRL) at 16°C and oocyte cell volumes were calculated by measuring the area of each diameter changes were recorded with a microscope video system measured by transfer to 3-fold diluted ND 96 solution. Oocyte incubated for 3 d in ND 96. Water permeability of single oocytes was (or an equivalent volume of water) was injected and the oocytes were incubated for 2 h while either the PIP1 immune serum (1:1000 in blocking buffer) (Biel et al., 1999) or the PIP2 immune serum (1:500 in blocking buffer). The PIP2 immune serum was

**Nucleotide sequence analysis**

Sequence reactions were prepared with the Beckmann Quick Start kit according to the manufacturer’s protocol. Reaction products were sequenced on a Beckmann CEQ 2000 automated sequencer and subsequently with anther cDNA as template for NtPIP2;1. The PCR products were cloned in pGEM-T Easy. The nucleotide sequences of NtPIP1;1 and NtPIP2;1 were determined and submitted to GenBank (accession numbers AF440271 and AF440272, respectively).

**Oocyte assays**

For the oocyte assays, the coding regions of NtPIP1;1 and NtPIP2;1 were amplified by PCR (Fig. 1) and subcloned in pT7Ts (Deen et al., 1994). Sense RNA was transcribed from linearized pT7Ts using T7 polymerase, and the integrity and size of the cRNA were confirmed by denaturing gel electrophoresis. *Xenopus laevis* oocytes of stages V and VI were collected in ND 96 solution (96 mM NaCl, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES-NaOH, pH 7.4) supplemented with penicillin and streptomycin (200 U ml-1) at 16°C. A volume containing approximately 10–50 ng cRNA (or an equivalent volume of water) was injected and the oocytes were incubated for 3 d in ND 96. Water permeability of single oocytes was measured by transfer to 3-fold diluted ND 96 solution. Oocyte diameter changes were recorded with a microscope video system and oocyte cell volumes were calculated by measuring the area of each oocyte (Scion Beta 4). The osmotic permeability coefficient was calculated using the formula P_f=V_0[d(V/V_0)/dt]/[S×(Osm_in–Osm_out)], where V_0 is the initial oocyte volume, S is the initial surface area, and V_t is given as 18 cm² mol⁻¹.

**Northern blot analysis**

10 μg of RNA was separated on 1.3% (w/v) agarose gels containing 0.5% (w/v) formaldehyde in MOPS buffer. The RNA was transferred to a nylon membrane (Nitrans supercharge) by capillary transfer in 10X SSC. After transfer, the RNA was fixed on the membrane by baking at 80°C for 2 h. For DNA probes, 25 ng DNA was 32P-labelled using random primed labelling (Feinberg and Vogelstein, 1983). RNA probes were transcribed from linearized plasmids in the presence of 32P-UTP. Hybridizations with 32P-labelled probes were performed at 65°C for 16 h in hybridization buffer (50% (w/v) formamide, 5X SSC, 1% (w/v) SDS, 10% (w/v) dextran sulphate, and 10 μg ml⁻¹ tRNA for RNA probes; 6X SSC, 0.5% (w/v) SDS, 5X Denhardt’s for DNA probes). After hybridization, filters were washed at 65°C with 2X SSC/0.1% SDS, 1X SSC/0.1% SDS, and 0.2X SSC/0.1% SDS, for 20 min each. The filters were exposed to Kodak X-ray film and the images were digitized and the contrast and brightness were adjusted with Adobe Photoshop. Figures were assembled using Adobe Illustrator.

**Protein isolation and western blot analysis**

Proteins of the desired tissues and stages were extracted in homogenization buffer (330 mM sucrose, 100 mM KCl, 1 mM EDTA, 50 mM TRIS, 0.05% MES, 5 mM DTT, Complete® protease inhibitor cocktail (Roche Mannheim), pH 7.5). The homogenate was centrifuged at 1000 g for 15 min to collect cells and debris, and the supernatant was centrifuged at 10 000 g for 15 min to collect the large cellular organelles. Finally, the microsomal fraction was pelleted by centrifugation at 20 000 g for 75 min. The microsomal pellet was dissolved in membrane buffer (330 mM sucrose, 200 mM DTT, 25 mM TRIS, pH 8.5).

For the western blot, protein samples were prepared in the presence of 50 mM ethanethiol. 15 μg of proteins were separated by 12% SDS–PAGE. The proteins were electroblotted onto a nitrocellulose membrane (Nitran supercharge) by capillary transfer in 10X SSC/0.1% SDS, for 20 min each. The filters were exposed to Kodak X-ray film and the images were digitized and the contrast and brightness were adjusted with Adobe Photoshop. Figures were assembled using Adobe Illustrator.
obtained from Eurogentec and produced by immunization of rabbits with the peptide H2N-QQHGKDYVDPPPAPLC-CONH2. After incubation with the primary antibody, the membranes were rinsed and incubated with the secondary antibody, horseradish-peroxidase conjugated goat anti-rabbit antibody (Pierce) 1:20 000 in blocking buffer for 1 h. All incubations were performed at room temperature. The membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s protocol and exposed to Kodak X-omat AR Scientific Imaging Films. The resulting films were digitized and the contrast and brightness were adjusted with Adobe Photoshop.

In situ hybridization

The in situ hybridization was essentially performed as described in DeBlock and Debrouwer (1993). Briefly, tissue was fixed in 4% paraformaldehyde, 0.25% glutaraldehyde (anthers) or 4% paraformaldehyde, glutaraldehyde 0.75% (stigmas). After embedding the tissue in paraplast (Sigma, St Louis MO), sections of 7 μm were cut and attached to coated object slides (Vectabond). Before hybridization of the probe, the sections were deparafinized and hydrated with successive ethanol/water steps. The probes were transcribed from linearized plasmids using DIG RNA labelling mix (Roche) with T3 or T7 RNA polymerase. Sections were hybridized overnight at 45 °C. After washing in 0.1× SSC for high stringency. Detection was performed by antibody conjugated with alkaline phosphatase. Photography occurred with a Leitz Orthoplan (Leica Microsystems GmbH, Wetzlar, Germany) microscope equipped with a Color Coolsnap digital camera (Roper Scientific, Tucson, AZ) and the MetaVue (Universal Imaging Corporation, West Chester, PA) software.

Results

mRNA of PIP-aquaporin genes is present in anthers and stigmas

Two methods were used to obtain aquaporin sequences from tobacco stigmas and anthers. The first method, RT-PCR, with primers corresponding to conserved aquaporin regions (Biela et al., 1999) resulted in DNA fragments of the expected size of 430 bp. Sequencing of eight individual clones revealed that they corresponded to seven different cDNAs with homology to aquaporins of the PIP1 and PIP2 subfamily. The second method used to isolate aquaporin cDNAs was the novel technique Family Transcript Profiling (FTP). Briefly, this cDNA-AFLP-based technique requires a single degenerate primer complementary to a domain specific for a given gene-family transcript, in combination with an oligo-dT primer. The amplified fragments are then digested, ligated to adaptors, and amplified with adaptor-specific primers. Unlike RT-PCR, where cDNAs of different members of a gene-family are amplified in DNA fragments of identical length, FTP allows the visual identification of individual members of a gene-family expressed in a certain organ. cDNA from anther, stigma, and root was used in the experiments, in combination with an aquaporin primer directed at the first NPA-motif and eight additional aquaporin cDNAs were isolated. Database searches, summarized in Table 2, revealed that all these sequences showed homology to aquaporins of either the PIP or NIP subfamilies.

After performing northern blot analysis using a limited number of cDNA fragments as probes (Table 2), it was decided to isolate full-length cDNAs of fragments Aqp 4.1 and Aqp 8.1 using a two-step RACE-PCR (see Materials and methods). The resulting clones were named NtPIP1;1, corresponding to Aqp 4.1, and NtPIP2;1, corresponding to Aqp 8.1. The NtPIP1;1 cDNA contained an open reading frame encoding a protein of 286 amino acids, 97% homologous to a PIP1 family member from Nicotiana tabacum.

Table 2. Aquaporin fragments isolated from tobacco anther and stigma

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Source</th>
<th>Method</th>
<th>Closest homologue</th>
<th>Identitya</th>
<th>Expressionb</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Anther</td>
<td>RT-PCR</td>
<td>Ntasp1 (a01416)</td>
<td>98%</td>
<td>nd</td>
<td>CK720584</td>
</tr>
<tr>
<td>6.1</td>
<td>Anther</td>
<td>RT-PCR</td>
<td>Scpi2a (a229301)</td>
<td>95%</td>
<td>nd</td>
<td>CK720585</td>
</tr>
<tr>
<td>7.1</td>
<td>Anther</td>
<td>RT-PCR</td>
<td>B. Oleracea pip2</td>
<td>92%</td>
<td>nd</td>
<td>CK720586</td>
</tr>
<tr>
<td>8.1</td>
<td>Anther</td>
<td>RT-PCR</td>
<td>Scpi2a (a229301)</td>
<td>94%</td>
<td>A</td>
<td>CK720587</td>
</tr>
<tr>
<td>P2</td>
<td>Anther</td>
<td>FTP</td>
<td>N. Alata nip</td>
<td>91%</td>
<td>A</td>
<td>CK720588</td>
</tr>
<tr>
<td>P16</td>
<td>Anther</td>
<td>FTP</td>
<td>N. Alata nip</td>
<td>70%</td>
<td>A</td>
<td>CK720590</td>
</tr>
<tr>
<td>P17</td>
<td>Anther</td>
<td>FTP</td>
<td>Ntasp1 (a01416)</td>
<td>93%</td>
<td>A, S, R</td>
<td>CK720591</td>
</tr>
<tr>
<td>P14</td>
<td>Anther</td>
<td>FTP</td>
<td>Scpi2a (a229301)</td>
<td>95%</td>
<td>A, S</td>
<td>CK720589</td>
</tr>
<tr>
<td>1.1</td>
<td>Stigma</td>
<td>RT-PCR</td>
<td>Tramp (x73847)</td>
<td>97%</td>
<td>A, S</td>
<td>CK720592</td>
</tr>
<tr>
<td>2.1</td>
<td>Stigma</td>
<td>RT-PCR</td>
<td>N. Excelsior pip1</td>
<td>98%</td>
<td>nd</td>
<td>CK720593</td>
</tr>
<tr>
<td>3.1</td>
<td>Stigma</td>
<td>RT-PCR</td>
<td>Scpi2a (a229301)</td>
<td>95%</td>
<td>nd</td>
<td>CK720594</td>
</tr>
<tr>
<td>4.1</td>
<td>Stigma</td>
<td>RT-PCR</td>
<td>Tramp (x73847)</td>
<td>99%</td>
<td>S, R</td>
<td>CK720595</td>
</tr>
<tr>
<td>P3</td>
<td>Stigma</td>
<td>FTP</td>
<td>N. Excelsior pip1</td>
<td>98%</td>
<td>A, S, R</td>
<td>CK720596</td>
</tr>
<tr>
<td>P8</td>
<td>Stigma</td>
<td>FTP</td>
<td>N. tabacum pip1</td>
<td>97%</td>
<td>S</td>
<td>CK720597</td>
</tr>
<tr>
<td>P15</td>
<td>Stigma</td>
<td>FTP</td>
<td>N. Excelsior pip1</td>
<td>86%</td>
<td>A, S, R</td>
<td>CK720598</td>
</tr>
<tr>
<td>P5</td>
<td>Root</td>
<td>FTP</td>
<td>Pyrus communis pip2</td>
<td>89%</td>
<td>S, R</td>
<td>CK720599</td>
</tr>
</tbody>
</table>

a BLASTX.

b Determined by northern blot with 10 μg RNA (ND: not determined; A, anther; S, stigma; R, root; other tissues not tested).

c Siefritz et al., 2002.

d O’Brien et al., 2002.

e Fray et al., 1994.
a PIP2 family member from Solanum chacoense expressed in anthers and pistils (O’Brien et al., 2002).

NtPIP2;1 encodes an active water channel protein

To test the water transport activity of NtPIP1;1 and NtPIP2;1, capped sense cRNA was transcribed from the full-length cDNA clones of both genes and equal amounts of cRNA was injected in Xenopus laevis oocytes. After a 3 d incubation to allow cRNA translation and targeting of the encoded proteins to the oocyte membrane, the oocytes were transferred to a hypo-osmotic medium and the increase in area was measured in time and used to calculate the increase in volume (Fig. 1a). This change in volume was used to calculate the relative water permeability of the oocytes ($P_f$). The results of the experiments are presented in Fig. 1. Control oocytes that were injected with water showed a $P_f$ of 17±13 cm s$^{-1}$×10$^{-4}$. The $P_f$ of oocytes injected with NtPIP1;1 cRNA was slightly above control values but not significantly different (27±6 cm s$^{-1}$×10$^{-4}$), whereas the $P_f$ of oocytes injected with NtPIP2;1 cRNA was 8-fold higher: 137±50 cm s$^{-1}$×10$^{-4}$ (Fig. 1b). These data showed that NtPIP2;1 functions very efficiently as a water channel when expressed in oocytes, and that NtPIP1;1 has negligible water-transport capacity.

Expression patterns of NtPIP1;1 and NtPIP2;1

To analyse the accumulation pattern of NtPIP1;1 and NtPIP2;1 individually, a search was made for regions in their full-length cDNAs that shared the lowest homology to their closest family members and these regions were used as probes. Sequence alignments of the NtPIP1;1 and NtPIP2;1 cDNAs with homologues of the PIP1 and PIP2 families revealed that the 3′ UTR was most suited for these purposes. For instance, the 3′ UTR of NtPIP1;1 was only 60% homologous to the 3′ UTR of its closest relative, compared with 95% for the coding region (data not shown).

Figure 2a shows that the NtPIP1;1 probe hybridized to RNA from anthers, pistils, and roots. The highest hybridization level was detected with RNA of roots. The NtPIP2;1 probe hybridized to pistil and stem RNA and to a lower level to anther and leaf RNA. Both probes did not hybridize to seed, pollen, and pollen tube RNA.

To determine whether NtPIP1;1 and NtPIP2;1 RNA in pollen and pollen tubes was present below the detection limit of the northern blot, RT-PCR was performed with pollen and pollen tube RNA in combination with NtPIP1;1 and NtPIP2;1 3′ UTR specific primers. The results showed that fragments of the expected size were amplified from RNA of both samples (Fig. 2b), indicating that both NtPIP1;1 and NtPIP2;1 RNAs accumulate in pollen and pollen tubes, albeit at a low level. Control reactions with RNase added to the RT reaction did not yield any products.

Northern blots were used to determine the accumulation patterns of NtPIP1;1 and NtPIP2;1 during stigma and anther development. Figure 2c shows that the 3′ UTR probe of NtPIP1;1 hybridized at very low level to anther RNA of flowers at all stages. In the stigma, RNA was detected at flower stages 6–10, and increased strongly at stages 10–12. The NtPIP2;1 probe hybridized at high level to RNA of anthers at stages 1–6 and 10–12. Interestingly, at flower stages 7–9 the hybridization level was considerably lower. No signal was obtained with RNA of stigmas at stages 6–12.

PIP proteins are present in anthers and stigmas

Western blotting was used to analyse the presence of PIP1 and PIP2 proteins in anthers and stigmas. Figure 3a shows the result of a western blot analysis using an affinity purified antibody raised against an N-terminal peptide of NtPIP1;1. The results of this western blot showed that, in the microsomal fraction of stigmas from stages 6–10, no protein could be detected, whereas in the microsomal...
fraction of stigmas from stages 10–12 a single protein was detected of approximately 34 kDa. A protein with the same molecular weight was detected in developing anthers; the expression level of this protein declined during anther development. NtPIP1:1 mRNA only accumulates to very low levels in developing anthers, and therefore it is not likely that all the protein detected in anther samples is NtPIP1:1 itself. Database searches for the peptide sequence that was used to raise the antibody revealed that closely related peptides are present in other PIP1 aquaporins, and therefore the protein that is detected in anthers is probably another member of the PIP1 homologue subfamily.

For the analysis of NtPIP2;1 protein expression during anther and stigma development, an antibody was raised in rabbits against an N-terminal peptide encoded by the cDNA of NtPIP2;1, and this antibody was used for a western blot containing microsomal protein fractions of developing anthers. Figure 3b shows that a protein with an apparent molecular weight of 36 kDa is detected at all anther stages, but expression appears lower in stages 8–10 of anther development. In addition, a protein with a molecular weight of approximately 32 kDa is detected from stages 7 onwards, increasing until stage 12 of anther development. By contrast, no PIP2 proteins were detected in stigmas (data not shown).

**In situ hybridization of PIP aquaporins in reproductive organs**

To obtain information about the spatial localization of PIP aquaporin transcripts in tobacco stigmas, *in situ* hybridization was performed. Because the PIP1 and PIP2 aquaporin subfamilies share considerable homology, the specificity of the candidate probes was tested on a Southern blot containing DNA fragments of different members of the PIP1 and PIP2 subfamilies. The results showed that, at low stringencies, cross-hybridization occurred, whereas at higher stringency the probes only hybridized to their corresponding coding sequences (data not shown). Therefore it was decided to use these probes under highly stringent conditions in the *in situ* experiments.

As PIP1 expression in tobacco stigmas increases during the later stages of development, stigmas of almost mature flowers (stage 11) were used. Figure 4 shows that compared with the control (sense probe), a signal is present in the neck-cells of the stigma and transmitting tissue of the style, and that a weaker signal is present in the cells of the cortex.

Similarly, *in situ* hybridizations were performed using the PIP1 and PIP2 probes on sections of tobacco anthers. Anthers of stage 2 were used for the *in situ* hybridizations because, at this stage, all different tissues of the anther are still present (Koltunow et al., 1990), and because PIP1 and PIP2 are both expressed at this stage (Fig. 2). The results of these *in situ* hybridizations are shown in Fig. 5. PIP1 and PIP2 transcripts were detected in the cells of the connective tissue, the anther wall, and the vascular bundle. In the cells of the circular cell cluster (CCC), the PIP2 probe gave a very weak signal, whereas the signal with the PIP1 probe was stronger. PIP1 or PIP2 transcripts were not detected in the cells of the epidermis. The tapetum and the microspores stained equally dark in all sections after hybridization with both the sense and the antisense probes, thereby making it difficult to ascertain the presence or absence of PIP transcripts. Together, PIP1 and PIP2 transcripts are present in most of the tissues of young anthers.

**Discussion**

The control of movement of water plays an important role in plant development and reproduction. In anthers, correctly timed dehydration is important for pollen development and anther dehiscence, and on the stigma hydrated pollen...
must hydrate with stigmatic water in order to produce a pollen tube. Previously it was shown that functional aquaporins are expressed in floral organs of *Brassica* and *Solanum* (Ruiter et al., 1997; Marin-Olivier et al., 2000; Dixit et al., 2001; O’Brien et al., 2002). To get more insight into the role of aquaporins in reproductive processes in species with wet-type stigmas, PIP1 and PIP2 aquaporins of tobacco anthers and stigmas have been characterized.

Using RT-PCR and the novel technique FTP, 15 different fragments were obtained that shared homology with aquaporins of the PIP and NIP subfamilies of aquaporins. This high number indicates the presence of a large aquaporin gene family in tobacco, as found in *Arabidopsis* (Quigley et al., 2002). There are at least three possible reasons for the presence in the genome of such a large aquaporin gene family: (i) redundancy may guarantee an optimal cellular water balance in plants, (ii) the proteins are functionally different with respect to their transport properties, or (iii) the proteins have identical functions but are temporally and spatially differently expressed. In the latter case, the regulation of expression of functionally identical aquaporins could be integrated in the changing expression patterns that accompany specific developmental stages.

The possibility that related aquaporins perform different functions is suggested by the higher water permeability of oocytes expressing NtPIP2;1 versus that of oocytes expressing NtPIP1;1 (Fig. 1). The level of NtPIP1;1 and NtPIP2;1 proteins in the oocyte plasma membrane were not analysed, and therefore it cannot be excluded that inefficient translation of NtPIP1;1 mRNA is the major cause for the difference in water permeability. However, similar results have been found for PIP1 and PIP2 proteins of other species (Tyerman et al., 2002), indicating that the low water permeability of PIP1 proteins expressed in oocytes may be inherent to most PIP1 family members. Recently, it has been shown that simultaneous expression of inactive PIP1 and active PIP2 proteins in oocytes results in higher water permeability depending on the amount of PIP1 proteins, indicating that the formation of PIP1 and PIP2 heterotetramers may play a role in plant cells (Fetter et al., 2004).

Analysis of the spatial and temporal expression patterns of NtPIP1;1 and NtPIP2;1 transcripts and PIP1 and PIP2 proteins indicates that they have overlapping, but not identical patterns (Figs 2–5). This could indicate that PIP1 and PIP2 aquaporins contribute to a different extent to the regulation of water movement in reproductive development. Both PIP subfamilies, for example, might function in the expanding cells, the connective tissue, and the cell wall in young anthers, but only PIP1 proteins are present in the cells of the circular cell cluster (Fig. 5). By contrast, mainly PIP2 and not PIP1 proteins accumulate at late stages of anther development and, therefore, may play a role during anther dehydration and dehiscence. These data indicate that PIP1 and PIP2 proteins are not fully functionally redundant during anther and stigma development.

The results of western blots with the PIP2 antibody show that during anther development at least two PIP2 isoforms are expressed (Fig. 3b). Detection of multiple aquaporins by a single antibody is not unexpected, taking into account the high homology within a subfamily of aquaporins. The expression of the PIP2 isoform with the larger size (apparent mass 36 kDa; PIP2a) is strong early in development, then decreases, and increases again in the last stages.
of anther development. The smaller isoform (apparent mass 32 kDa; PIP2b) is not expressed until stage 7 of anther development, and then expression increases towards anther maturity. As anthers enlarge until approximately stage 6, and start dehydrating around stage 8, these results suggest that only PIP2a may contribute to the anther growth that takes place between anther stages 2 and 5. In addition, PIP2a may have a role in anther and pollen dehydration shortly before dehiscence. PIP2b, by contrast, may only function in dehydration of the anther and pollen before dehiscence.

The presence of aquaporins in the stigma suggests a role in pollen hydration, but these results and data from Brassica show that PIP1 aquaporins are not expressed in the stigmatic papillae (Marin-Olivier et al., 2000), making such a function less likely. However, during tobacco stigma development, PIP1 transcripts were expressed with increasing strength in the last stages before pollination would take place. Based on the observed localization, the actual function of these proteins may be in another aspect of fertilization than pollen hydration. One possibility would be that PIP1 proteins function in pollen tube elongation in the stigma–style transition. Alternatively, the PIP1 proteins in the neck cells could be present to ensure the movement of water into the transmitting tissue, the site of pollen tube elongation within the style.

Together, it is shown that PIP1 and PIP2 proteins are differentially expressed in the anther and stigma of tobacco flowers. Together with the observation that PIP1 and PIP2 proteins have different water permeability in oocytes this could indicate that these related aquaporin family members do not act redundantly, but have distinct functions in changing genetic programmes. However, it is unclear if these distinct roles of PIP1 and PIP2 proteins are conserved between plant species, as in Brassica anthers PIP1 proteins are expressed in a similar fashion as PIP2 proteins in tobacco anthers. It cannot be excluded that other PIPs and other proteins than aquaporins also play an important role in the movement of water during reproduction. Detailed analysis of the reproductive processes in plants where PIP expression is suppressed may contribute to understanding whether aquaporins alone are sufficient for the regulation of water movement in reproductive organs.

References


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