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ORIGINAL ARTICLE

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Characterization of a fertilization-induced and developmentally regulated plasma-membrane aquaporin expressed in reproductive tissues, in the wild potato *Solanum chacoense* Bitt.

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Abstract Fertilization triggers a unique and complex developmental program leading to embryogenesis and seed set. Growth and differentiation are accompanied by rapid metabolic changes as well as massive cellular reorganization inside the fertilized ovule. Using differential display to isolate genes involved in pollen–pistil interactions and early fertilization events, we isolated from *Solanum chacoense* Bitt. a fertilization-induced plasma-membrane intrinsic protein of the PIP2 family that is predominantly expressed in pistil and anthers tissues. Major intrinsic proteins (MIPs) consist of a large family of highly conserved membrane-spanning proteins that are mainly represented in plants by the aquaporins. Aquaporins, mainly of the PIP and TIP type, have been shown in many species to serve as water channels. In *S. chacoense* the ScPIP2a mRNA is developmentally regulated during anthesis, with mRNA levels gradually decreasing as the pistil reaches maturity. In flowers, strongest expression was observed in elongating styles, in stamens, and transiently in ovules following fertilization. In styles, maximal expression levels correlated with phases of rapid style elongation and with the formation of epidermal papillae. ScPIP2a mRNA was also strongly expressed in developing fruit, consistent with a role in cell expansion during maturation and development.

Keywords Differential display · Fertilization-induced gene · Major intrinsic protein · Plasma-membrane intrinsic protein · *Solanum* (aquaporin) · Stylar cortex papillae

The GenBank accession number for the ScPIP2a aquaporin cDNA is AF290201

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Abbreviations DD: differential display · MIP: major intrinsic protein · PIP: plasma-membrane intrinsic protein · TIP: tonoplast intrinsic protein

Introduction

The characterization in recent years of numerous water-transporting molecular channels in plants, collectively known as aquaporins, has led to the emergence of a dynamic picture of rapid and regulated water transport across biological membranes. Although transmembrane water transport can occur by diffusion through the lipid bilayer, the presence of proteinaceous water channels enables fast and controlled translocation of large volumes of water across membranes. The plant aquaporins are now classified into four major subfamilies (Johanson et al. 2001). Two major groups or subfamilies are named after their location in the cell: the tonoplast intrinsic proteins or TIPs, localized to the vacuolar membrane and, the plasma-membrane intrinsic proteins or PIPs, localized to the plasma membrane (Maurel 1997; Johansson et al. 2000). A third subfamily comprises the NOD-26-like MIPs (NIPs) and, recently, a fourth subfamily, the small basic intrinsic proteins (SIPs), was characterized (Johanson et al. 2001).

The recent sequence completion of the *Arabidopsis* genome has enabled the identification of more than 600 predicted membrane transport systems. Of these transporters, 15% are channel proteins and half of these (approx. 40) could be classified as putative aquaporins, based on sequence identity (The Arabidopsis Genome Initiative 2000). In comparison with other fully sequenced organisms, *Arabidodpsis* has 4- to 10-fold more major intrinsic protein (MIP) water channels, underscoring their importance in plant water transport and homeostasis (The Arabidopsis Genome Initiative 2000; Zeuthen 2001). Although most aquaporins have been assigned through sequence similarities, water transport activity has been clearly demonstrated for more than 20 PIPs and TIPs (reviewed in Johansson et al. (2000).

Based on high sequence conservation, it is assumed that most putative aquaporins will turn out to be functional water or small-solute channels.

The large number of aquaporins within an organism is also paralleled by a tight tissue- or development-specific expression pattern for numerous plasma-membrane (PIP) and tonoplast (TIP) aquaporins. Aquaporins of the TIP family in maize are expressed in zones of cell division and elongation (Chaumont et al. 1998), and in epidermal and conducting tissues (Barrieu et al. 1998). Expression of a γ-TIP aquaporin in *Arabidopsis*, mainly in the vascular bundles of roots and leaves, has been correlated with cell enlargement (Ludevid et al. 1992). A γ-TIP aquaporin from tulip has also been shown to be restricted to the vascular tissue, and was linked to the stretching of the stalk cells during rapid stalk elongation (Balk and de Boer 1999). In reproductive tissues, a *Brassica* aquaporin (PIP1 family) linked to a self-incompatibility modifier locus (Fukai et al. 2001) is strongly expressed in the stigma epidermis, where pollen grains hydrate (Dixit et al. 2001). External stimuli and phytohormones have also been shown to influence aquaporin expression. Plasma-membrane aquaporins can be regulated by light (Kaldenhoff et al. 1995), phytohormones (Phillips and Huttly 1994; Pih et al. 1999; Morillon et al. 2001; Suga et al. 2001), drought (Yamaguchi-Shinozaki et al. 1992) and salt stresses (Pih et al. 1999).

In this study, we report the isolation of a fertilization-induced and developmentally regulated PIP2-type aquaporin, predominantly expressed in floral tissues in the wild potato species *Solanum chacoense*.

Materials and methods

Plant material

The diploid ($2n = 2x = 24$) *Solanum chacoense* Bitt. self-incompatible genotypes used include line PI 458314 (which carries the S_{11} and S_{12} self-incompatibility alleles) and line PI 230582 (which carries the S_{13} and S_{14} alleles), and were originally obtained from the Potato Introduction Station (Sturgeon Bay, Wis., USA). Plants were grown in greenhouses with 14–16 h of light per day.

Differential display conditions and isolation of full-length cDNA

For the differential display procedure the RNAimage protocol (GenHunter Corporation, Nashville, Tenn., USA) was followed, with modifications described herein. Reverse transcription (RT)-PCR was done in the presence of 0.2 µl of α-[^{32}P]dCTP. Two microliters of loading dye was added to 3.5 µl of the PCR product, heated to 80 °C in a thermocycler (PTC-200; MJ Research) with a heated lid and run on a 0.4-mm, 6% denaturing polyacrylamide gel in 1× TBE (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA). DNA migration was stopped when the xylene cyanol dye ran out of the gel. Afterwards, the gel was dried directly on the glass plate (before any urea-removal treatment) and washed gently with distilled water until no urea was left, and then re-dried. Displayed bands were detected with Fujifilm RX film (Groupe Christie, St-Eustache, Qc., Canada) after an overnight exposure at room temperature. Differentially displayed bands were briefly rehydrated from the glass plate with 50 µl of water prior to careful excision of the gel slice with a scalpel blade. The gel slice was put in a microfuge tube with 100 µl of water and then heated at 70 °C for 1 h. To further facilitate the diffusion of the DNA, the tubes were left at room temperature overnight (Konecny and Redinbaugh 1997). After the diffusion step, the tubes were centrifuged at 10,000 *g* for 1 min to pellet the gel slice, and the DNA in the supernatant was precipitated with 1/10 vol. of 3 M sodium acetate (pH 5.2), 5 µl of glycogen (10 mg/ml) and 450 µl of 100% ethanol. After centrifugation, the pellet was dissolved in 12 µl of water and used for secondary PCR amplification. PCR amplification was done as described by the manufacturer (GenHunter Corporation) with 4 µl of DNA. A full-length clone was isolated with the differential display (DD) fragment as a probe from a pollinated-pistil cDNA library and sequenced on both strands on an ABI Prism 310 (Applied Biosystems) automated sequencer.

Isolation and gel blot analysis of RNA and DNA

Total RNA was isolated as described by Jones et al. (1985). RNA concentration was determined by measuring its absorbance at 260 nm and verified (adjusted if necessary) by agarose gel electrophoresis and ethidium bromide staining. To confirm equal loading of total RNA on RNA gel blots, a 1-kb fragment of the *S. chacoense* 18S RNA was PCR-amplified and used as a probe. Genomic DNA was isolated via a modified CTAB extraction method (Reiter et al. 1992). DNA gel blot analysis, including restriction, electrophoresis, and capillary transfer to a positively charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Baie D'Urfé, Qc., Canada) was done as described by Sambrook et al. (1989). The membrane was hybridized under high-stringency conditions at 65 °C as described by Church and Gilbert (1984) for 16–24 h and, following hybridization, the membrane was washed at room temperature, twice with 2× SSC/0.1% SDS for 30 min (first at room temperature, then at 35 °C), twice with 1× SSC/0.1% SDS for 30 min (first at 45 °C, then at 55 °C), and twice with 0.1× SSC/0.1% SDS at 55 °C for 10 min (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA gel blot analyses were performed as described by Sambrook et al. (1989), following the formaldehyde denaturing protocol. RNAs were capillary-transferred to Hybond N+ nylon membranes and cross-linked (120 mJ/cm^2) with a Hoefer UVC 500 UV cross-linker. The membranes were hybridized under high-stringency conditions at 45 °C in 50% deionized formamide, 5× Denhardt's solution, 0.5% SDS, 200 µg/ml denatured salmon sperm DNA and 6× SSC for 16–24 h. Following hybridization, the membranes were washed as described previously for the DNA gel blot analyses. Probes for DNA gel blot analysis were synthesized from random-labeled isolated DNA inserts (Roche Diagnostic, Laval, Qc., Canada) with α-[^{32}P]dCTP (ICN Biochemicals, Irvine, Calif., USA). For RNA gel blot analyses, probes were made with α-[^{32}P]dATP with the Strip-EZ DNA labeling kit (Ambion, Austin, Tex., USA). The membranes were autoradiographed at –85 °C with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Ont., Canada).

Scanning electron microscopy

Styles were fixed in 4% glutaraldehyde for 4 h at room temperature in 0.1 M phosphate buffer ($NaHPO_4$, pH 7.0). After two rinsing steps in 0.1 M phosphate buffer, the styles were dehydrated in an increasing ethanol series (from 30% to 100%) and critical-point-dried with CO_2, coated with gold–palladium, and viewed in a JEOL JSM-35 SEM.

Phytohormone treatments

Flowers were sprayed with aqueous solutions (in 0.05% Tween) of 50 µM abscisic acid (ABA), 50 µM jasmonic acid (mixed isomers), 50 µM 22(S), 23 (S)-homo-brassinolide, 5 mM salicylic acid, a 1 mg/ml emulsion (3.28 mM) of arachidonic acid, or 0.05% Tween

as a control once per day on two consecutive days. For treatment with volatiles, methyl jasmonate or trans-2-hexenal were first diluted to 0.1 M in ice-cold methanol and 100 µl of the compound (equivalent to 10 µl/l of air space) was added to a piece of Whatman paper suspended over the plant in an airtight container as described by Bate and Rothstein (1998). Treatments were repeated on two consecutive days. As a control, 100 µl of methanol was used in the same way. All phytohormones and elicitors were purchased from Sigma/Aldrich (Oakville, Ont., Canada).

Results

Isolation of the ScPIP2a cDNA and sequence analysis

We used differential display (Liang and Pardee 1992) to characterize genes involved in pollen–pistil interactions and early fertilization events. In *Solanum chacoense*, a self-incompatible wild tuber-bearing potato, pollen tubes reach the ovules around 36 h after pollination and fertilization of most of the ovules has occurred by 42 h after pollination. Reverse transcription of total mRNA from four different time points after pollination (from 0 to 96 h) was used to produce first-strand cDNAs. This substantially decreased the number of false-positives and gave a good prediction of the expression pattern as determined afterward by RNA gel blot analysis. Using the RNA image primer pair H-AP9 and HT11-A, two displayed bands showed a strong increase (U26 and U27) and another two bands displayed a marked reduction (D6 and D7) after pollination and/or fertilization, and were isolated from the gel. After isolation and re-amplification of the selected displayed bands from the gel, the DD fragments were used to probe RNA gel blots containing unpollinated-pistil mRNAs (control) and pollinated-pistil mRNAs (1, 2 and 4 days after pollination) to ensure that the DD fragments isolated corresponded to genes truly responding to pollination or fertilization (data not shown). Here we present data corresponding to clone U26 (up-regulated 26). The U26 DD fragment was cloned and sequenced. A full-length clone corresponding to U26 was isolated from a 48-h pollinated-pistil library and named ScPIP2a (for *Solanum chacoense* PIP2a cDNA).

The ScPIP2a clone contains a cDNA of 1,113 bp (excluding the poly-A tail) with a short 5′ untranslated leader of 60 nucleotides (nt) and a 3′-untranslated region (UTR) of 201 nt (GenBank accession number AF290201). The size of the ScPIP2a cDNA corresponds to the size of the mRNA, as determined by RNA gel blot analysis (1.14 kb, see Fig. 3), suggesting that the ScPIP2a cDNA is full-length or near full-length. Furthermore, an in-frame stop codon (position 13–15) is found in the 5′-UTR, suggesting that the first ATG encountered (position 61–63) is the translation start site. The ScPIP2a cDNA is predicted to encode a 283-amino-acid polypeptide of 30.3 kDa with a predicted isoelectric point (pI) of 8.9. Prediction servers (TM Pred, TMHMM) all strongly supported the presence of six transmembrane helices, suggesting that the protein is membrane-localized. Homology searches in DNA and protein databases revealed strong similarities to plant aquaporins, a large family of water-channel proteins (Johansson et al. 2000). A ClustalX alignment followed by a phylogenetic analysis (PAUP 4.0b8) grouped ScPIP2a with the PIP2 subfamily (data not shown). ScPIP2a has all of the distinctive features of PIP2, as deduced from sequence similarities in the N- and C-terminal domains as well as in the B and E loops (Schäffner 1998). A protein alignment with the aquaporins closest to the ScPIP2a deduced protein is shown in Fig. 1. Among the MIPs with known expression profiles, the ScPIP2a deduced protein shares strongest sequence similarities to the *Arabidopsis thaliana* SIMIP and AtPIP3 (now AtPIP2;7) proteins (84% amino acid identity, 90% similarity; Weig et al. 1997; Pih et al. 1999), and to the spinach PM28A aquaporin (80% identity, 86% similarity; Johansson et al. 1996). Other MIP sequences closely related to ScPIP2a include the *Brassica oleracea* PIP3 gene, a PIP2-type aquaporin expressed in seed during development and germination (84% amino acid identity, 90% similarity, accession number AF314656), the PIP2-2 aquaporin from a *Vitis* hybrid (83% identity, 90% similarity, accession number AF141900), and the mipE protein from the ice plant *Mesembryanthemum crystallinum* (82% identity, 89% similarity; Kirch et al. 2000). All of the above-mentioned MIPs can be grouped in the PIP2 family through specific signature sequence identities (Schäffner 1998).

Gene copy number of the ScPIP2a aquaporin

A Southern blot of *S. chacoense* genomic DNA was probed with the complete ScPIP2a cDNA insert (Fig. 2). Two strongly hybridizing fragments could be detected from the *Hin*dIII (8.0 and 1.7 kb), *Eco*RV (23 and 17.5 kb), and *Eco*RI (7.9 and 5.7 kb) restriction enzymes. Since there is one *Eco*RI and one *Hin*dIII site in the cDNA sequence (positions 190 and 875, respectively), this suggests that ScPIP2a is a single-copy gene in *S. chacoense*. Weaker-hybridizing fragments could also be detected with *Eco*RI and *Hin*dIII, and might correspond to other related aquaporin sequences.

ScPIP2a mRNA expression in mature tissues

Tissue-specific expression of ScPIP2a was determined using total RNA extracted from different tissues of *S. chacoense* and the complete ScPIP2a insert as a probe. Although MIPs inside a given family can have highly similar sequences, little cross-hybridization could be detected in the DNA gel blot analysis (Fig. 2). Furthermore, when only the 3′-UTR was used as a probe (the U26 fragment obtained from the differential display analysis), the same RNA hybridization patterns were observed (data not shown). This strongly suggests that the RNA hybridization data correspond to a single

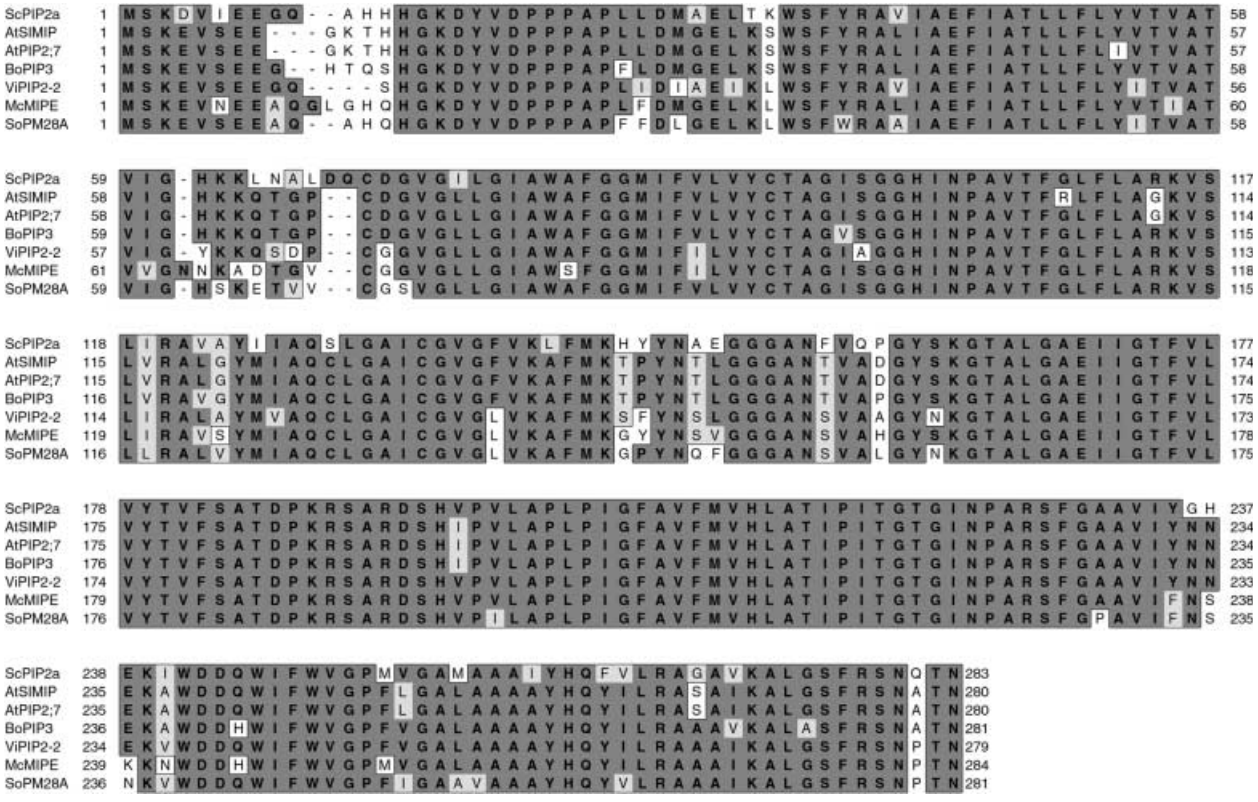

Fig. 1 Alignment of the deduced protein sequence of *Solanum chacoense* PIP2a (ScPIP2a) with those of closely related aquaporins. The deduced amino acid sequence of ScPIP2a was aligned with those of the following PIP2-type aquaporins: AtSIMIP (GenBank accession number AF003728), AtPIP2;7 (GenBank accession number U78297, formerly known as AtPIP3), MipE (GenBank accession numberU73467), PIP2-2 GenBank accession number AF141900), and PM28A (GenBank accession number L77969)

RNA species. RNA gel blot analyses indicated that ScPIP2a is predominantly expressed in pistil and anther tissues, and to much weaker levels (≈9-fold less, as determined by densitometric scans) in mature leaves and petals (Fig. 3a). No hybridization signal could be detected in pollen or in pollen tubes grown in vitro (Fig. 3a). High expression levels, comparable to levels observed in anthers, occurred in pollinated pistils from 48 to 96 h post-pollination, where there was a 3-fold increase in ScPIP2a mRNA abundance compared with unfertilized pistil tissues. Since pollen tubes reach the embryo sac about 36 h after pollination, and fertilization occurs between 36 and 48 h after pollination in *Solanum* spp. (Clarke 1940; Williams 1955; and our unpublished results), the increase in ScPIP2a mRNA expression is thus most probably a fertilization-dependant event, and not only triggered by pollen tube growth in the style as observed before for other genes responding to pollination and fertilization (Lantin et al. 1999). To ascertain that the ScPIP2a mRNA increase was not due to a late response to pollination, an incompatible self-pollination was performed. In *S. chacoense*, a gametophytic self-incompatible species, self

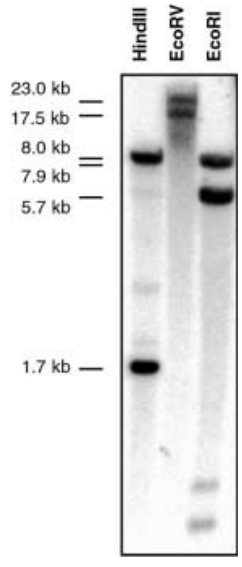

Fig. 2 DNA gel blot analysis of the ScPIP2a gene. Genomic DNA (10 μg) isolated from *S. chacoense* leaves was digested with *Eco*RI, *Eco*RV and *Hin*dIII restriction enzymes and probed with the 1.1-kb complete ScPIP2 cDNA insert. Molecular weights of the fragments as determined from the DNA MW ladder appear on the *left*

pollen tubes are arrested in the top half of the style and never reach the ovary (Matton et al. 1999). No ScPIP2a mRNA increase could be detected after a self-incompatible pollination (data not shown), confirming that the ScPIP2a mRNA increase is triggered by fertilization. Since fertilization triggered a substantial increase in ScPIP2a mRNA abundance in pistil tissues, fertilized

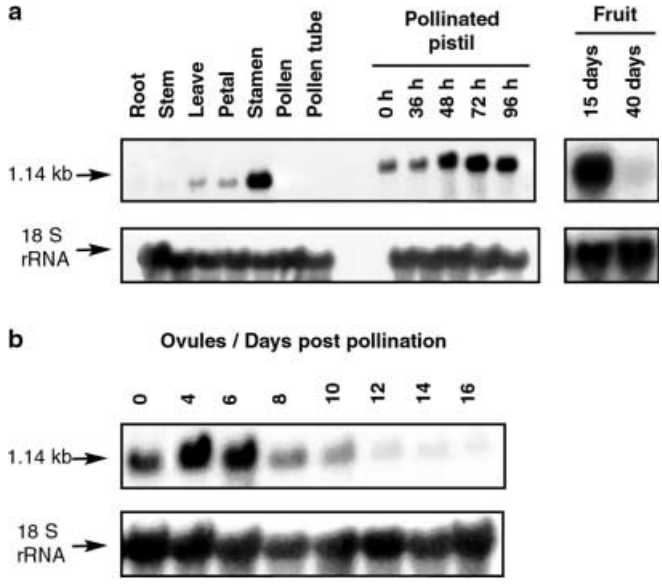

Fig. 3a, b RNA expression analysis of ScPIP2a transcript levels in tissues of *S. chacoense*. **a** RNA gel blot analysis of ScPIP2a mRNA accumulation in mature tissues, pistils at different times after pollination, and in developing fruits. Ten μg of total RNA from the various tissues was probed with the 1.1-kb complete (*Eco*RI/*Xho*I) ScPIP2a cDNA insert. **b** RNA gel blot analysis of ScPIP2a mRNA accumulation in isolated ovules at different times after pollination. Same conditions as in **a**

ovules were also tested for ScPIP2a mRNA accumulation. As can be seen in Fig. 3b, a transient increase could be observed in ovules, similar to that previously observed in whole pistils. Similarly, ScPIP2a mRNA abundance increased transiently in young developing fruits (Fig. 3a). Mature fruits that had reached their maximal size, did not express detectable levels of ScPIP2a mRNA (Fig. 3a).

Developmental expression of ScPIP2a mRNA in pistil tissues

Aquaporin expression has often been linked to tissue growth, being detected in zones of cell division and elongation (Chaumont et al. 1998). In order to determine if ScPIP2a is developmentally regulated during pistil development, an RNA gel blot analysis was conducted on styles and ovaries from 3 days before anthesis to 2 days after anthesis. As can be seen in Fig. 4, ScPIP2a mRNA levels are higher in immature style and ovary tissues than in mature tissues at anthesis or later. Thus, unless fertilized, ScPIP2a mRNA levels decline in pistils (compare Fig. 4a, left, with Fig. 3a, b). When upper and lower halves of styles were collected separately 2 days before anthesis, ScPIP2a mRNA abundance was higher in the lower part of the style (Fig. 4a, right). In almost mature styles, 1 day before anthesis, only a slight difference between lower and upper style could still be observed (Fig. 4a, right). The elevated levels of ScPIP2a mRNA in young styles combined with a stronger expression level in the basal part of the style could be explained if most of the style elongation was produced a few days before anthesis and/or if specialized tissue was made during style maturation. To determine if there was any correlation between ScPIP2a mRNA expression levels and the daily growth rate, style length was measured from 4 days before anthesis until the day of anthesis. Figure 4b shows that maximum gain in length, expressed as the percentage length increase per day, was observed in young styles with a 65% gain of total length from –4 to –3 days before anthesis, consistent with the highest ScPIP2a aquaporin mRNA expression detected in young styles (Fig. 4a).

Styles were examined by scanning electron microscopy for the presence of external specialized structures or appendages from 4 days before anthesis to anthesis. The major difference observed was the presence of numerous papillae in the median and lower regions of the style (Fig. 5a). Papillae were never observed in the upper style (Fig. 5), where ScPIP2a mRNA levels were always lower (Fig. 4a). As the style matured, the papillae started to appear in the median and basal region of the style (Fig. 5a). Apart from style elongation and papilla formation, radial expansion of the style could also be observed (compare from –4 to 0 days before anthesis, same magnification for all figures, Fig. 5a). As the style reached maturity, a thick deposit of cuticular waxes could also be observed under higher magnification, on all cells of the upper and median region, including the papilla cells (Fig. 5b).

Hormonal and stress-related responses of ScPIP2a in pistil tissues

Many aquaporins have been shown to be transcriptionally regulated by various stimuli, including salt and drought stresses, phytohormone treatments, as well as blue light (Kaldenhoff et al. 1993; Phillips and Huttly 1994; Yamada et al. 1995; Morillon et al. 2001). Various treatments were attempted to determine if ScPIP2a transcription was influenced by phytohormone or stress treatments. Since ScPIP2a is mostly expressed in flower tissues, and because of the difficulty of delivering the chemicals inside the target tissues, flower spraying was chosen to determine the effect of various phytohormones on ScPIP2a transcription. In stylar tissues, salicylic acid, a potent inducer of defense response genes (Durner et al. 1997) induced a slight (2-fold) increase in ScPIP2a mRNA levels (data not shown). Trans-2-hexenal, a C_6 volatile produced from damaged or wounded tissue (Bate and Rothstein 1998) also induced a 2-fold increase in ScPIP2a stylar mRNA levels (data not shown). In ovaries, no externally applied phytohormone or elicitor treatments (see *Materials and methods*) had any appreciable effect on ScPIP2a mRNA levels (data not shown).

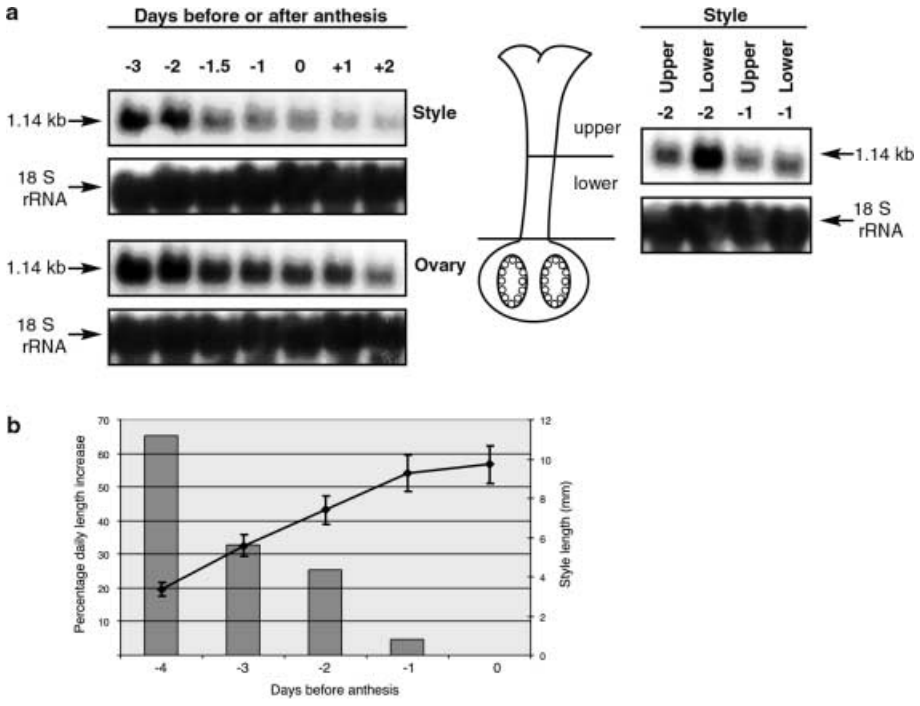

Fig. 4 **a** Developmental expression pattern of ScPIP2a mRNA levels in unpollinated pistil tissues. *Left panel* ScPIP2a mRNA levels in styles and ovaries were analyzed by RNA gel blot analysis. Ten-μg samples of total RNA from styles and ovaries of pistils 3 days before anthesis to 2 days after anthesis were probed with the 1.1-kb ScPIP2a cDNA insert. *Right panel* RNA gel blot analysis of ScPIP2a mRNA levels in the upper and lower halves of styles from flowers 2 days (*–2*) and 1 day (*–1*) before anthesis. **b** Style length and daily growth rate of styles during pistil development. Style length (*graph*; means ± SD) was measured on 10 individual styles per day from 4 days before anthesis to the day of anthesis. The percentage daily length increase (*columns*; mean values) was calculated as: (L day_n–L day_{n-1}/L day_{n-1})×100where L is the measured length on any particular day

Discussion

The isolation in recent years of numerous proteinaceous water channels, the aquaporins, has changed the picture of how large volumes of water can cross biological membranes in a fast and controlled way, in order to adjust the osmotic balance either needed for cell enlargement and development, or influenced by environmental factors. Furthermore, the large numbers of aquaporins present in plants is also paralleled by a tight tissue-, developmental-, or stimulus-specific expression pattern. Using an mRNA differential display strategy, numerous candidate genes, either up- or down-regulated following fertilization, were isolated from pistil tissues in the self-incompatible potato *Solanum chacoense*. In this paper, we report the isolation and characterization of a new developmentally regulated PIP2-family aquaporin, mainly expressed in reproductive tissues, and transcriptionally regulated during fertilization.

Comparison of the expression patterns of the aquaporins most closely related to ScPIP2a reveals that they are also preferentially expressed in floral organs, and might thus represent a special class of plant aquaporins. The transcript levels of the *Arabidopsis* SIMIP aquaporin are increased by salt-stress and ABA treatment (Pih et al. 1999). The SIMIP aquaporin is also expressed in a tissue-specific manner, highest transcript levels being found in flowers, and very low levels in leaves and roots. Similarly, the *Arabidopsis* PIP3 aquaporin (now renamed AtPIP2;7) has been shown to be a functional aquaporin in a *Xenopus laevis* oocyte assay, and its mRNA abundance in whole plants is highest in floral organs, as well as being strongly expressed in tissue culture cells (Weig et al. 1997). The *Brassica oleracea* PIP3 gene is expressed in seeds during development and germination (GenBank accession number AF314656). The floral expression pattern has not been determined for the *Mesembryanthenum* MipE (Kirch et al. 2000), the spinach PM28A (Johansson et al. 1996), or the *Vitis* PIP2-2 aquaporins (only sequence data available). Other MIPs also have floral-specific, or floral-predominant expression, like the PIP1-family mipA and mipB genes in *B. oleracea*, that show strongest expression in stamens, sepals, petals, and carpels (Ruiter et al. 1997). This reinforces the view that the large number of aquaporins within an organism is paralleled by a tight tissue- or development-specific expression pattern.

As mentioned in the *Introduction*, numerous reports have linked aquaporin expression with zones of cell division, elongation and expansion in various tissues (Ludevid et al. 1992; Chaumont et al. 1998; Balk and de Boer 1999). Our ScPIP2a mRNA expression results, mainly with style elongation, papilla formation on stylar cortical cells, and early fruit growth, would support such a function for the ScPIP2a aquaporin in cell elongation and expansion. By comparing transmitting-tissue cell

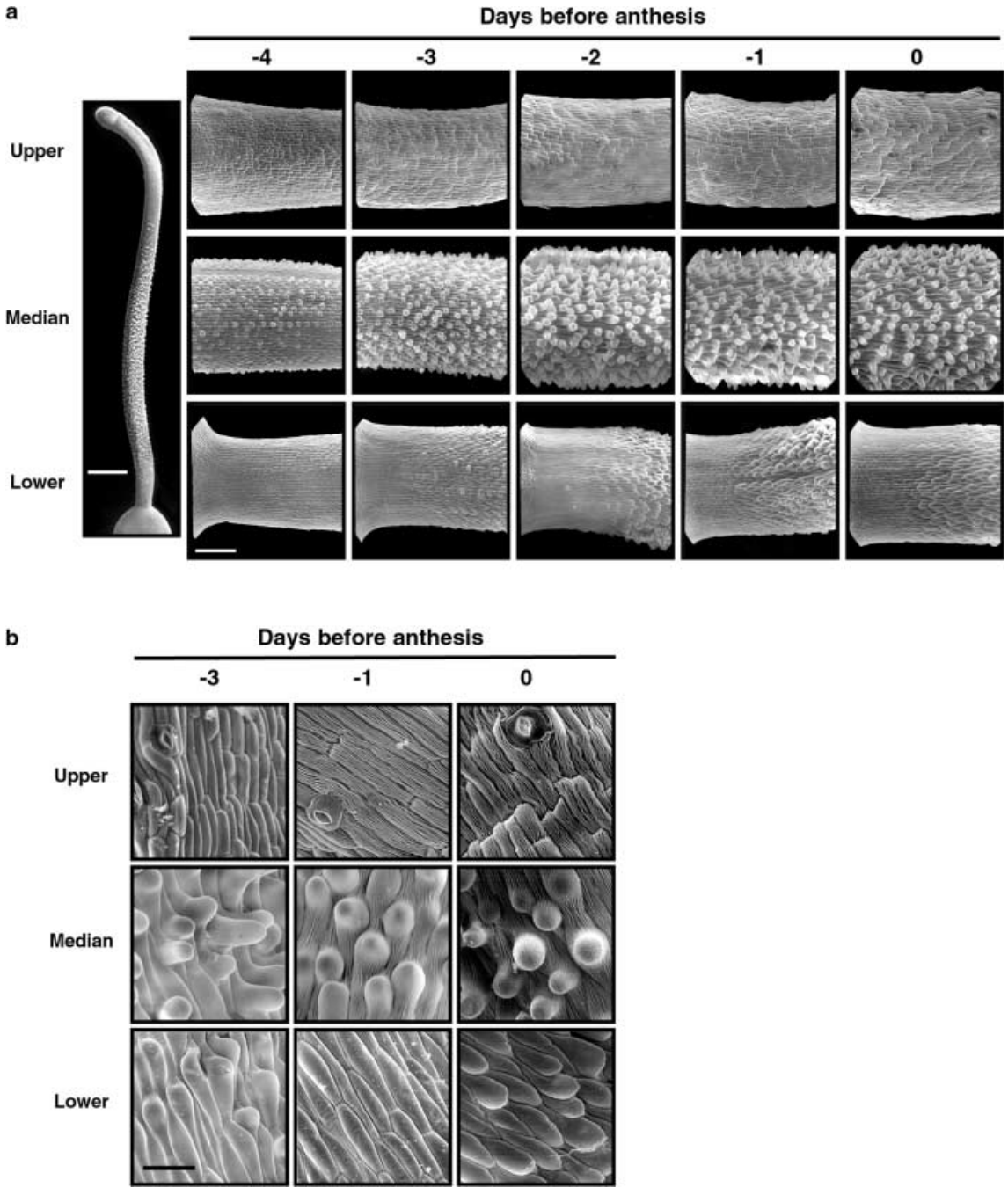

Fig. 5 Scanning electron microscopy of a whole *S. chacoense* style at anthesis (**a**) and sections of styles at low (**a**) and high magnification (**b**) from 4 days before anthesis to the day of anthesis. Development of numerous papillae can be observed mainly on the median and lower sections of the style. Bars = 1 mm (**a**, whole pistil), 100 μm (**a**, ×200 sections), 50 μm (**b**, ×500 sections)

length and overall style length during the major growth period of the style, Linskens (1974) noted that there was a linear relation between these two, and that style growth was characterized by cell elongation with a constant cell number. Furthermore, at an early maturation stage, when the style length of petunia is around one-sixth of its total length, (less than 5 mm in a total length of 29 mm at anthesis), cell divisions were only observed at the base of the style (Linskens 1974). A kinematic analysis of gynoecial growth was also described for *Lillium longiflorum* by Crone and Lord (1991). Utilizing surface-marking techniques, the authors measured the local relative growth rate (LRGR) along the style during gynoecial growth. A triphasic pattern of growth was observed for all floral organs. From late phase I, after the start of stylar intercalation from stigma and ovary, till the end of phase II (when total style length reaches 77% of its final length) the lower style region was determined to be the predominant site of growth, having the highest LRGR (Crone and Lord 1991).

By comparison, the period during which ScPIP2a expression was determined in the style would correspond to the second phase (phase II) in lily. This again correlates well with the observed results that ScPIP2a aquaporin expression is strongest in young developing tissues and peak expression is detected in the style's basal region (Fig. 4).

Another striking developmental feature is the appearance, during style elongation, of papillae on the style's epidermis (Fig. 5). These papillae are most similar to those observed on petals, and are projections of the outer periclinal cell wall (Jeffree 1986). Unlike the stigmatic papillae, these stylar papillae are most probably not collecting hairs, and have probably no function in retaining pollen grains. They do not secrete any mucilaginous substances and are thickly covered with epicuticular waxes. In *S. chacoense*, the style grows almost twice as long as the anthers, and most of the papillae would be below the anther opercula where the pollen is shed. Another possible role for the papillae was proposed by Haberlandt, who suggested that the presence of papillose epidermal cells was to serve as lenses, concentrating the limited light that can reach the leaves below the canopy of thick forests (Haberlandt 1924). The papillate structure would be more efficient at collecting light arriving from a wide range of angles than a flat surface (Jeffree 1986). Although the style is not thought to be a photosynthetic organ, it possesses well-developed chloroplasts, and is indeed, photosynthetically active (Jansen et al. 1992). In fact, a 20% increase in photosynthetic flux could be measured in styles following pollination. Although a role for these stylar papillae is far from obvious, the increased turgor pressure needed for the outer periclinal cell wall to become a papilla has to rely on increased water flow, and expression of the ScPIP2a aquaporin described here could be involved in such cell-expansion event, as well as participating in style elongation.

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