

Specific plasma membrane aquaporins of the PIP1 subfamily are expressed in sieve elements and guard cells

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Background information. Transmembrane water flow is aided by water-specific channel proteins, aquaporins. Plant genomes code for approx. 35 expressed and functional aquaporin isoforms. Plant aquaporins fall into four different subfamilies of which the PIPs (plasma membrane intrinsic proteins) constitute the largest and evolutionarily most conserved subfamily with 13 members in *Arabidopsis* and maize. Furthermore, the PIPs can be divided into two phylogenetic groups, PIP1 and PIP2, of which the PIP1 isoforms are most tightly conserved, sharing > 90% amino acid sequence identity. As the nomenclature implies, the majority of PIPs have been shown to be localized at the plasma membrane. Recently, two highly abundant plasma membrane aquaporins, SoPIP2;1 and SoPIP1;2, have been purified and structurally characterized.

Results. We report the cloning of a cDNA encoding SoPIP1;2 and show that there are at least five additional sequences homologous with SoPIP2;1 and SoPIP1;2 in the spinach genome. To understand their role *in planta*, we have investigated the cellular localization of the aquaporin homologues SoPIP1;2 and SoPIP1;1. By Western- and Northern-blot analyses and by immunocytochemical detection at the light and electron microscopic levels, we show that SoPIP1;2 is highly expressed in phloem sieve elements of leaves, roots and petioles and that SoPIP1;1 is present in stomatal guard cells.

Conclusions. Localization of the two abundant aquaporin isoforms suggests roles for specific PIPs of the PIP1 subgroup in phloem loading, transport and unloading, and in stomatal movements.

Introduction

Short distance water transport occurs by three distinct pathways: symplastic, through plasmodesmata; apoplastic, through cell walls; and across membranes (Steudle and Frensch, 1996). In plants, transmembrane water flow is crucial, for example, for changes in stomatal aperture, for rapid elongation of cells

(pollen tubes and root tip) and for diurnal regulation of leaf movements (due to turgor changes in suspensor cells of pulvini). Water channel proteins, or aquaporins, are believed to play a major role in these processes. Their function and regulation have been extensively documented since the first report of γ TIP, a tonoplast aquaporin of *Arabidopsis thaliana* showing water transport activity in *Xenopus laevis* oocytes (Maurel et al., 1993; for reviews, see Johansson et al., 2000 and Maurel et al., 2002).

Aquaporins are members of the MIP (major intrinsic protein) family and have been found in bacteria, fungi, plants and animals. These 26–34 kDa proteins have six transmembrane helices, with both N- and C-termini on the cytosolic side of the membrane. They form tetrameric complexes of which

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Abbreviations used: ABA, abscisic acid; MIP, major intrinsic protein; OTG, *N*-octylthioglucoside; PEG, poly(ethylene glycol); PIP, plasma membrane intrinsic protein; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; UTR, untranslated region.

each subunit is a functional water channel. Highly conserved amino acid residues are grouped into two signature sequences, Asn-Pro-Ala or NPA boxes, in the second intracellular and in the fifth extracellular loops (LB and LE). In addition to water, some MIPs have been reported to transport solutes including glycerol (aquaglyceroporins), urea, boric acid, ammonia, carbon dioxide and ions.

Among the members of the plant MIP family, the PIPs (plasma membrane intrinsic proteins) form the most highly conserved subfamily. There are 13 expressed PIPs in *Arabidopsis* and maize (*Zea mays*), which can be further divided into two groups, PIP1 and PIP2 (Chaumont et al., 2001; Johanson et al., 2001). Divergence into PIP1 and PIP2 groups predates the emergence of vascular plants, as shown by the presence of these two classes of aquaporins in the moss *Physcomitrella patens* (Borstlap, 2002).

At the sequence level, PIP1 group members have a longer N-terminal region and a shorter C-terminal region when compared with PIP2 group members. Functional differences between members of the PIP1 and PIP2 subclasses have also been observed. Generally, PIP1 proteins exhibit no or very low water channel activity compared with PIP2 proteins, when expressed in the *X. laevis* oocyte heterologous system (Chaumont et al., 2000). The PIP2 isoforms that have been functionally tested are specific for water, whereas PIP1 proteins have been reported to facilitate the transport of other solutes such as glycerol (Biela et al., 1999; Moshelion et al., 2002), boric acid (Dordas et al., 2000), urea (Gaspar et al., 2003) and, recently, carbon dioxide (Uehlein et al., 2003). However, studies of the down-regulation and overexpression of PIP1 isoforms in *Arabidopsis* and tobacco (*Nicotiana tabacum*) indicate a major role of these proteins in transmembrane water transport in plants (Kaldenhoff et al., 1998; Martre et al., 2002; Siefritz et al., 2002; Aharon et al., 2003). By studying the distribution and regulation of single PIP1 isoforms, we may obtain new insight regarding their respective roles in plants.

In spinach (*Spinacia oleracea*), one PIP1 isoform, PM28B, and one PIP2 homologue, PM28A, have previously been identified and characterized (Johansson et al., 1996, 1998). Fotiadis et al. (2001) reported the purification of PM28 from spinach leaf plasma membranes and showed that this protein fraction mainly contains two isoforms: the previously charac-

terized PIP2 isoform, PM28A, and a newly identified PIP1 homologue, PM28C. Structural analyses of two-dimensional crystals revealed that PM28A and PM28C crystallize separately in different patches when co-crystallized and the PM28A and PM28C homotetramers differ in their size and shape (Fotiadis et al., 2001).

In the present paper, we report the cloning of a cDNA encoding PM28C, the most abundant PIP isoform in spinach leaf plasma membrane. Analyses of mRNAs show that the two spinach PIP1 isoforms, PM28B and PM28C, renamed SoPIP1;1 and SoPIP1;2 according to the nomenclature proposed by Johanson et al. (2001), are differentially expressed. We report evidence for the presence of at least seven PIP genes in spinach. Immunogold labelling of spinach leaves, petioles and roots using peptide-specific antisera shows that SoPIP1;2 is abundant in the plasma membrane of sieve elements and SoPIP1;1 in guard cells. These results are consistent with a role for PIP1 homologues in phloem loading and unloading and in control of stomatal aperture size. Possible modes of regulation of the water channel activity of PIP1s are discussed.

Results

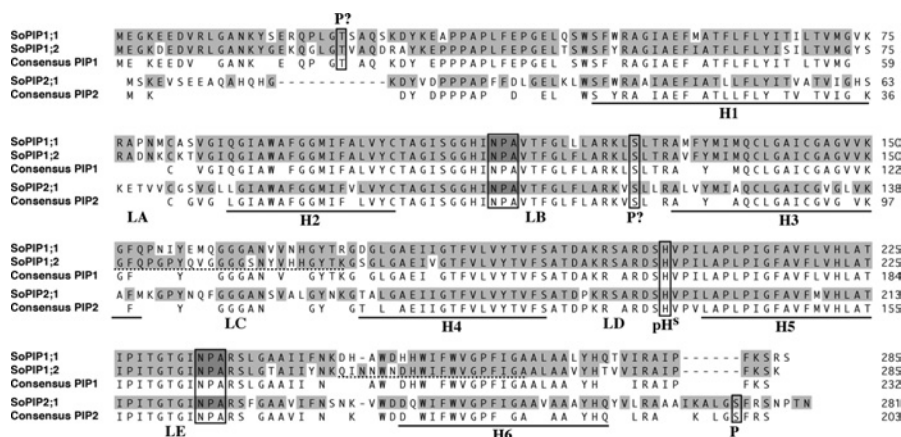
Cloning of SoPIP1;2 and sequence analysis

The cDNAs encoding PM28A and PM28B, two plasma membrane aquaporin homologues from spinach, have previously been cloned using PCR and degenerate oligonucleotides based on partial amino acid sequences from a 28 kDa SDS/PAGE band (Johansson et al., 1996). The cDNA encoding PM28C was cloned using degenerate oligonucleotides corresponding to internal peptide sequences obtained by MS (Fotiadis et al., 2001). Recently, a new nomenclature for plant MIP genes and proteins has been adopted (Johanson et al., 2001) and we subsequently refer to PM28A as SoPIP2;1, PM28B as SoPIP1;1 and PM28C as SoPIP1;2.

The 1248 bp cDNA encoding SoPIP1;2 (GenBank[®] accession no. AY372191) comprises a 45 bp 5'-UTR (untranslated region), 858 bp coding sequence and 345 bp 3'-UTR. The coding sequence shares a sequence identity of 70 and 60% with *SoPIP1;1* (GenBank[®] accession number AJ249384) and *SoPIP2;1* (GenBank[®] accession no. L77969) respectively. In contrast, the 3'-UTR of the *SoPIP1;2* cDNA shares only 30 and 35% identities with the

Figure 1 | Alignment of the deduced amino acid sequences of the spinach plasma membrane MIP isoforms SoPIP1;1 (CAB56217), SoPIP1;2 (AAR23268) and SoPIP2;1 (AAA99274)

Identical amino acids occurring in at least two of the sequences are shown on a grey background. The internal peptide sequences of SoPIP1;2 obtained by Fotiadis et al. (2001) are underlined with a dotted line. The consensus sequences for PIP1 isoforms include amino acids conserved in at least 80% of 58 aligned sequences and for PIP2 isoforms, 80% of 55 aligned sequences, from 28 plant species. The six transmembrane α -helices H1–H6, based on alignment with bovine AQP1, for which the structure has been solved (Sui et al., 2001), are underlined. The connecting loops are labelled LA–LE. Highly conserved amino acids believed to be important for the water channel activity of the proteins are boxed, including the canonical NPA boxes in loops B and E. Conserved phosphorylation sites are marked with the letter P. The question mark indicates that the phosphorylation on this residue has not been demonstrated. The conserved histidine residue involved in the modulation of the water channel activity by pH sensing is indicated by pH^s (Tournaire-Roux et al., 2003).



corresponding regions of *SoPIP2;1* and *SoPIP1;1* respectively.

The deduced protein sequence of *SoPIP1;2* is 286 amino acids long and contains characteristic features of MIPs: six hydrophobic, membrane-spanning α -helices (predicted by hydropathy analyses) and NPA motifs in loops B and E (designated as LB and LE in Figure 1). Consensus sequences for PIP1 and PIP2 homologues illustrate a high degree of conservation in the PIP subfamily. The amino acid sequence of *SoPIP1;2* shares 67 and 84% identities with those of *SoPIP2;1* and *SoPIP1;1* respectively. *SoPIP1;2* is similar to other members of the PIP1 group: in particular it has a longer N-terminal end and a shorter C-terminal region compared with PIP2 group members. Phylogenetic analyses confirmed that it indeed belongs to this subfamily (results not shown).

Steady-state mRNA levels of *SoPIP1;1* and *SoPIP1;2*

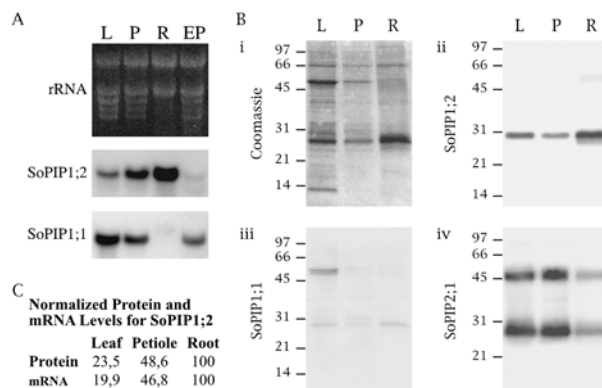
Northern-blot analyses were performed using RNAs extracted from leaves, petioles, roots and epidermal

peels. To minimize cross-hybridization, probes were chosen in the 3'-UTR regions of the *SoPIP1;1* and *SoPIP1;2* cDNA clones. The signal corresponding to the *SoPIP1;2* transcript was very strong and rapidly saturated: film exposure times were kept short (30 s to 1 min), whereas Northern blots with *SoPIP1;1* probes required longer exposure times (5–10 min) to obtain signals (Figure 2A). Since the riboprobes used in these experiments had similar length and labelling density, these differences in signal intensities indicate that the *SoPIP1;2* transcript was much more abundant than the *SoPIP1;1* transcript. *SoPIP1;1* was expressed in leaves, petioles and epidermal peels, with the highest level in leaves, but no signal could be detected in roots. The *SoPIP1;2* transcript was observed in leaves, petioles and roots, with the highest abundance in roots and barely detectable levels among epidermal peel RNAs (Figure 2A). The signal intensities corresponding to *SoPIP1;2* mRNAs were recorded with a densitometer. The relative mRNA amounts are shown in Figure 2(C).

The expression of certain members of the PIP family follows diurnal and circadian rhythms (Moshelion

Figure 2 | Transcript and protein levels of spinach PIPs in different organs

(A) Northern blots performed on total RNA extracted from leaves (L), petioles (P), roots (R) and epidermal peels (EP). The top panel shows the ethidium bromide-stained RNA-denaturing gel; in the middle panel, the corresponding nylon membrane was hybridized with an *SoPIP1;2*-specific probe; in the lower panel, an *SoPIP1;1*-specific probe was used. (B) Plasma membranes were purified from leaves (L), petioles and midribs (P) and roots (R). Proteins associated with the plasma membrane were solubilized with 3% OTG and separated by SDS/PAGE. (i) Coomassie-stained gel. (ii), (iii) and (iv) Immunoblots using peptide antibodies that recognize *SoPIP1;2* (loop C), *SoPIP1;1* (loop C) and *SoPIP2;1* (C terminus) respectively. (C) The percentages of *SoPIP1;2* protein and *SoPIP1;2* transcript in different tissues are expressed relative to roots (100%), and deduced from the intensities of the corresponding bands in (B, ii) and (A). Band intensities were quantified using ImageQuant software (Molecular Dynamics). The intensities of the different lanes in (B, i) were measured in the same way and were used to correct the percentages given in (C) for uneven loading of the protein gel (B, i).



et al., 2002), whereas others are regulated by light or by abiotic stresses (reviewed in Maurel et al., 2002). Populations of mRNAs were prepared from leaves and roots collected at different time points during a 24 h period. Northern-blot analyses did not reveal any significant variation of the mRNA levels for any of the three PIP isoforms (results not shown). Spinach plants were exposed to salt, drought and osmotic stress and to treatment with ABA (abscisic acid). There were no changes in the three PIP mRNA levels after 1–2 h of treatment with 250 mM NaCl, drought, 10% (w/v) PEG [poly(ethylene glycol)] or 100 μ M ABA (results not shown).

Generation of isoform-specific antibodies

Polyclonal antibodies that recognize peptides in the N-terminal region of PIP1s and in the C-terminal region of PIP2s respectively have been used in several studies to discriminate between proteins of these two groups. However, due to high sequence similarities, such antibodies recognize several isoforms within a group. In PIPs, the extracellular loop C is one of the most variable domains (Figure 1). Kirch et al. (2000) reported that three antibodies directed against peptides in the loop C of two PIP1s and one PIP2 member of *Mesembryanthemum crystallinum* specifically recognize these isoforms.

To assess the degree of conservation of loop C in spinach, DNA fragments corresponding to the region 137–182 in *SoPIP1;2* amino acid sequence were amplified by PCR from genomic DNA using degenerate primers. The fragments were subcloned into pBluescript and the transformants were sequenced. Four additional loop C sequences could be deduced, which belong to one putative PIP1 member and three putative PIP2 proteins. An alignment of these sequences with those of *SoPIP1;1*, *SoPIP1;2* and *SoPIP2;1* (Figure 3) shows that loop C is also variable in spinach. Peptides in loop C were therefore used to produce *SoPIP1;1*- and *SoPIP1;2*-specific antibodies (Figure 3). Within these, eight out of 21 amino acids are unique for *SoPIP1;1* and seven out of 21 for *SoPIP1;2*. On dot-blot, *SoPIP1;1*- and *SoPIP1;2*-antibodies did not bind to the *SoPIP1;2*- and *SoPIP1;1*-peptides respectively (results not shown). By analogy with *Arabidopsis* and maize, the number of spinach PIPs may amount to approx. 13. Despite the variability of PIP sequences in the loop C region, the possibility that these antibodies cross-react with unidentified spinach PIPs cannot be excluded. However, our results and the results of Kirch et al. (2000) suggest that they are specific.

Organ distribution of *SoPIP1;1* and *SoPIP1;2*

Plasma membranes from leaves, petioles including midribs and roots were isolated. Equal amounts of membrane proteins were solubilized with 3% (w/v) OTG (*N*-octylthioglucoside) and analysed by Western blotting. Using this procedure, at least 80% of the PIPs were solubilized, as indicated by Western blotting on the pellet and supernatant protein fractions (results not shown).

Figure 3 | Alignment of the amino acid sequences of the loop C region of spinach PIPs

The sequences corresponding to the region between the amino acids 137 and 182 (numbering refers to the sequence of SoPIP1;2) were aligned for seven spinach PIPs. For the isoforms marked with an asterisk, amino acid sequences were deduced from the nucleotide sequences of fragments amplified by PCR using genomic DNA and degenerate primers. Residues occurring in at least four of the sequences are boxed. Identical or similar amino acids are shown in bold. The peptides used to produce antibodies against SoPIP1;1 and SoPIP1;2 are indicated by a black bar above the peptides. The amino acids that are unique for SoPIP1;1 or SoPIP1;2 are shown on a grey background.

SoPIP1;1	M	Q	C	L	G	A	I	C	G	A	G	V	V	K	G	F	Q	P	N	I	Y	E	M	Q	G	G	G	A	N	V	V	N	H	G	Y	T	R	G	D	G	L	G	A	E	I	I
SoPIP1;2	M	Q	C	L	G	A	I	C	G	A	G	V	V	K	G	F	Q	P	G	P	Y	Q	V	G	G	G	S	N	Y	V	H	H	G	Y	T	K	G	S	G	L	G	A	E	I	V	
SoPIP1;3*	M	Q	C	L	G	A	I	C	G	A	G	V	V	K	G	F	Q	P	T	P	Y	A	L	L	G	G	G	A	N	V	V	A	E	G	Y	T	K	G	A	G	L	G	A	E	I	V
SoPIP2;1	A	Q	C	L	G	A	I	C	G	V	G	L	V	K	A	F	M	K	G	P	Y	N	Q	F	G	G	G	A	N	S	V	A	L	G	Y	N	K	G	T	A	L	G	A	E	I	I
SoPIP2;2*	M	Q	C	L	G	A	I	C	G	V	G	L	V	K	A	F	Q	S	S	Y	Y	V	R	Y	G	G	G	A	N	V	M	A	H	G	Y	N	K	G	T	G	L	G	A	E	I	I
SoPIP2;3*	A	Q	C	L	G	A	I	C	G	V	A	L	V	K	A	F	H	G	V	Y	Y	D	K	Y	G	G	G	A	N	K	V	S	P	G	Y	S	S	G	T	A	L	A	A	E	I	I
SoPIP2;4*	A	Q	C	L	G	A	I	C	G	V	G	L	V	K	A	F	Q	S	A	Y	Y	H	E	Y	G	G	G	A	N	T	L	S	Q	G	Y	S	K	G	T	G	L	A	A	E	I	V

Antibodies raised against the SoPIP1;2 loop C peptide recognize a major band at 29 kDa [Figure 2B(ii)]. The signal was more intense for the root protein fraction where two additional bands at 27 and 59 kDa could be detected. The band at 59 kDa probably represents the dimeric form of SoPIP1;2. The faint band at 27 kDa may be the result of either post-translationally modified SoPIP1;2 or another isoform recognized by the antibodies. The intensity of the band at 29 kDa in the three different organs [Figure 2B(ii)] was quantified by densitometry. The values were corrected for uneven loading of the gel due to differential solubilization of membrane proteins by OTG [Figure 2B(i)] and relative amounts of the SoPIP1;2 protein were calculated (Figure 2C). The relative proportions of *SoPIP1;2* mRNA and SoPIP1;2 protein obtained from Northern- and Western-blot analyses are strikingly similar after correction for uneven loading of the protein gel (Figure 2C). *SoPIP1;2* mRNA and protein are most abundant in roots; in petioles, they are approximately half and in leaves one-fifth as abundant as in roots.

Western-blot results using antibodies to SoPIP1;1 were consistent with Northern-blot results, showing that the *SoPIP1;1* gene is expressed at low levels and mainly in leaves [Figure 2B(iii)]. A double band at 56–59 kDa in the leaf fraction was immunostained in the Western blot and was also detectable in the petiole fraction. Aquaporins are known to exist as homotetramers in native membranes and these interactions often resist SDS/PAGE separation (Karlsson et al., 2003). Therefore the band at 56–59 kDa may correspond to SoPIP1;1 dimers. The faintness of the bands in this Western-blot analysis suggests that SoPIP1;1 is scarce in the organs examined. In the

28 kDa region, faint bands were present for all organs, including roots. In immunocytochemistry experiments, SoPIP1;1 antibodies seem to cross-react with SoPIP1;2 (see below). This may explain the weak bands at 28 kDa in the root plasma membrane fraction. Unlike SoPIP1;2, SoPIP1;1 is not, or little, resolved on SDS/PAGE as a monomer for leaf plasma membranes, indicating that there may be physicochemical and/or structural differences between the two PIP1 homologues.

Figure 2B(iv) shows a Western blot using antibodies directed against a peptide in the C-terminal region of SoPIP2;1 (Johansson et al., 1998, Fotiadis et al., 2001). These antibodies do not cross-react with PIP1 proteins. The signals for the monomeric (at 28 kDa) and dimeric (at 55 kDa) forms are equally intense and SoPIP2;1 is most abundant in petiole plasma membranes and least abundant in roots.

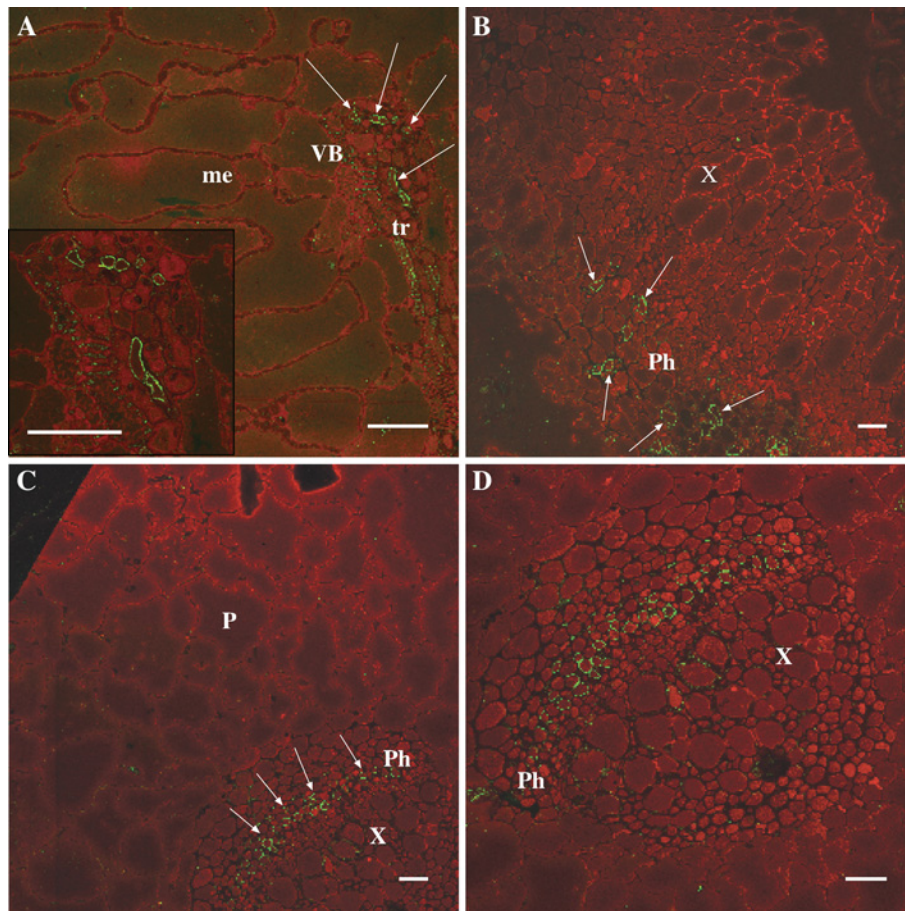
SoPIP1;1 is specifically expressed in guard cells and SoPIP1;2 in enucleate sieve elements

Antibodies raised against SoPIP1;1, SoPIP1;2 and SoPIP2;1 were used for immunolocalization in fully expanded and young leaves, petioles, and mature and immature roots. For light microscopy, immunogold-labelled sections of resin-embedded tissues were silver-enhanced and imaged by laser scanning confocal microscopy. Despite strong signals in immunoblots [Figure 2B(iv)], no specific labelling was observed with the SoPIP2;1 antibodies (results not shown). However, both SoPIP1;1 and SoPIP1;2 epitopes were localized in specific cell types.

SoPIP1;2 epitopes were present in the phloem of leaves (Figure 4A), roots (Figure 4B) and petioles

Figure 4 | SoPIP1;2 is present in phloem cells in leaves, petioles and roots

Immunogold labelling was performed on cross-sections of petioles, roots and leaves using polyclonal antibodies directed against SoPIP1;2. **(A)** Leaf cross-section. SoPIP1;2 epitopes are localized in the phloem, as indicated by the arrows. Some non-specific labelling is observed in xylem tracheary elements (tr). The inset shows the vascular bundle (VB) at higher magnification. **(B)** Root cross-section. Only phloem cells are labelled with the SoPIP1;2 antibodies (arrows). **(C)** Petiole cross-section. Arrows indicate immunolabelled phloem cells. **(D)** Higher magnification of the vascular bundle shown in **(C)**. me, palisade mesophyll cell; Ph, phloem; P, parenchyma; X, xylem. Scale bars, 30 μm .



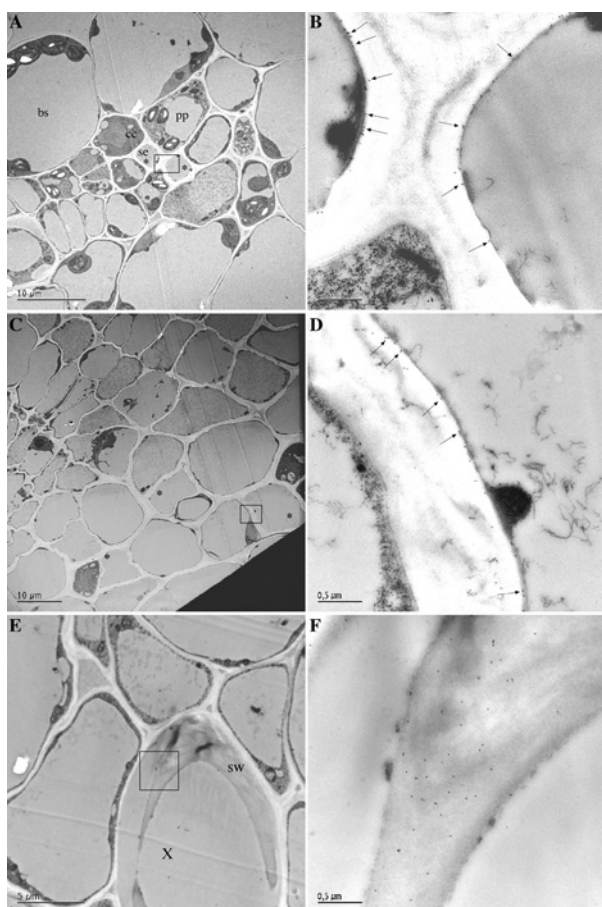
(Figures 4C and 4D). Weak staining of tracheary elements in leaves (Figure 4A) and of the secondary wall thickenings of xylem cells in petioles (Figure 4D) were also observed. However, the immunolabelling of xylem vessels diminished when the primary antibody concentration was decreased, whereas labelling in phloem cells remained intense, indicating that xylem labelling was an artefact. No immunolabelling was detected in other cell types.

Ultrathin sections of equivalent samples were immunogold-labelled and imaged by transmission electron microscopy. The labelled phloem cells observed display features typical for enucleate sieve el-

ements: a low electron density, a thin layer of cytoplasm and the presence of protein bodies (Figures 5A and 5C). The SoPIP1;2 epitopes are present only at the plasma membrane of these cells (Figures 5B and 5D). Sieve elements of both mature and immature tissues were equally labelled. In rare cases, we observed labelling in developing sieve elements. No signal was seen in other cell types with the SoPIP1;2-antibodies, including companion cells. Some immunogold-labelling of the secondary wall thickenings of xylem vessels was observed both with the antiserum and with the preimmune serum (Figures 5E and 5F). This is probably caused by

Figure 5 | Localization of SoPIP1;2 in vascular tissues of leaves and roots by immunoelectron microscopy

Immunogold labelling with SoPIP1;2 antibodies (dilution 1:1000, v/v) was performed on ultrathin cross-sections of leaves and roots (A–D). (A) Minor vein of leaf. (B) Higher magnification of the region delimited by a rectangle in (A), showing two sieve elements; arrows indicate the gold colloids coupled to the secondary antibody. (C) Root cross-sections. (D) Higher magnification of the region corresponding to the rectangle in (C), showing the plasma membrane of a sieve element labelled with colloidal gold. The plasma membrane of the neighbouring cell is not labelled. As a control, the preimmune serum was used in immunogold labelling on sections of petioles (E, F). (E) Part of a vascular bundle of petiole. (F) Higher magnification of the region delimited by a rectangle in (E). The labelled cells, indicated by asterisks, are sieve elements. bs, bundle sheath; cc, companion cells; pp, phloem parenchyma; se, sieve elements; X, xylem vessel; sw, secondary wall.



non-specific binding of antibodies to the secondary cell wall, also observed by light microscopy (see above).

Using the antibodies raised to the SoPIP1;1 peptide, not only guard cells in the leaf epidermis were abundantly labelled (Figure 6), but also phloem cells and xylem vessels in all organs (leaves, Figure 6A; petioles and roots, results not shown). It is possible that SoPIP1;1-antibodies may cross-react with SoPIP1;2 epitopes in phloem cells, although dot-blots suggest no cross-reactivity. Some labelling was also observed in mesophyll cells. However, imaging of the immunogold-labelled ultrathin sections by electron microscopy showed that the gold colloids were not localized in a particular membrane in mesophyll cells but rather reflected unspecific binding of the antibodies to the section. It is worth noting that, in many cases, the epitopes in guard cells are not evenly distributed around the cells, but rather concentrated toward the stomatal opening (Figures 6A, 6B and 6D). In some cases, epitopes were observed on the outer side of the stomata (Figure 6C). Using transmission electron microscopy, SoPIP1;1 antibodies were seen to specifically label guard cells (Figures 7B and 7D), whereas preimmune serum did not (Figures 7A and 7C).

We conclude that SoPIP1;1 is expressed in stomatal guard cells and is concentrated in different zones around the guard cells in different stomata. SoPIP1;2, the most abundant PIP in spinach leaves, is localized in phloem sieve elements in source and sink tissues.

Discussion

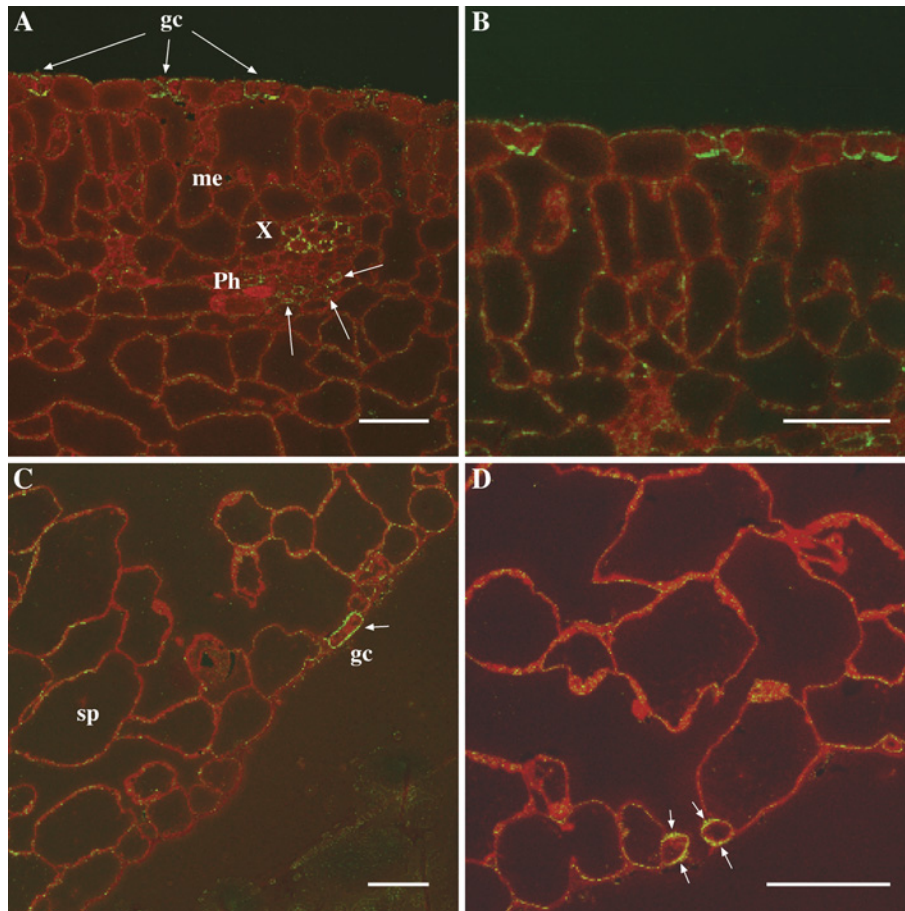
In the present paper, we report the cloning of a 1248 bp cDNA encoding SoPIP1;2/PM28C. Fotiadis et al. (2001) have previously shown that SoPIP1;2 is the most abundant protein in a 28 kDa protein fraction (PM28), purified from spinach leaf plasma membranes. Although the cDNA for SoPIP1;1/PM28B was cloned from a leaf cDNA library, the protein could not be detected by Fotiadis et al. (2001) in the purified PM28 fraction from leaves. Our Northern- and Western-blot analyses suggest that SoPIP1;1 is expressed at much lower levels than SoPIP1;2 and SoPIP2;1. In addition to the already identified PIP genes, the genome of spinach contains at least one PIP1 and three PIP2 genes (Figure 3).

Sequence–structure–function relationships in PIPs

As revealed by the transmission electron microscopy of solubilized PM28 proteins and by atomic force

Figure 6 | SoPIP1;1 is present in stomatal guard cells

Immunogold labelling was performed on cross-sections of leaves using polyclonal antibodies that recognize SoPIP1;1. **(A)** The epitopes are present in guard cells (gc) and in phloem cells (Ph) as indicated by arrows. Non-specific labelling is seen in xylem vessels (X). Weak non-specific labelling was also detected in palisade mesophyll cells (me). **(B)** Higher magnification of the leaf epidermis and palisade layer of **(A)**. **(C)** A pair of guard cells in a closed stomata is specifically labelled with SoPIP1;1 antibodies (arrow). Weak non-specific labelling can be seen in spongy parenchyma cells (sp). **(D)** Arrows point to the localized epitopes in guard cells in an open stomata. Scale bars, 40 μm .



microscopy of PM28 two-dimensional crystals, SoPIP1;2 and SoPIP2;1 form homotetramers of discrete sizes and shapes (Fotiadis et al., 2001). Thus, the relatively few differences in the primary sequences of the PIP members (Figure 1) have profound effects on their tetrameric structure.

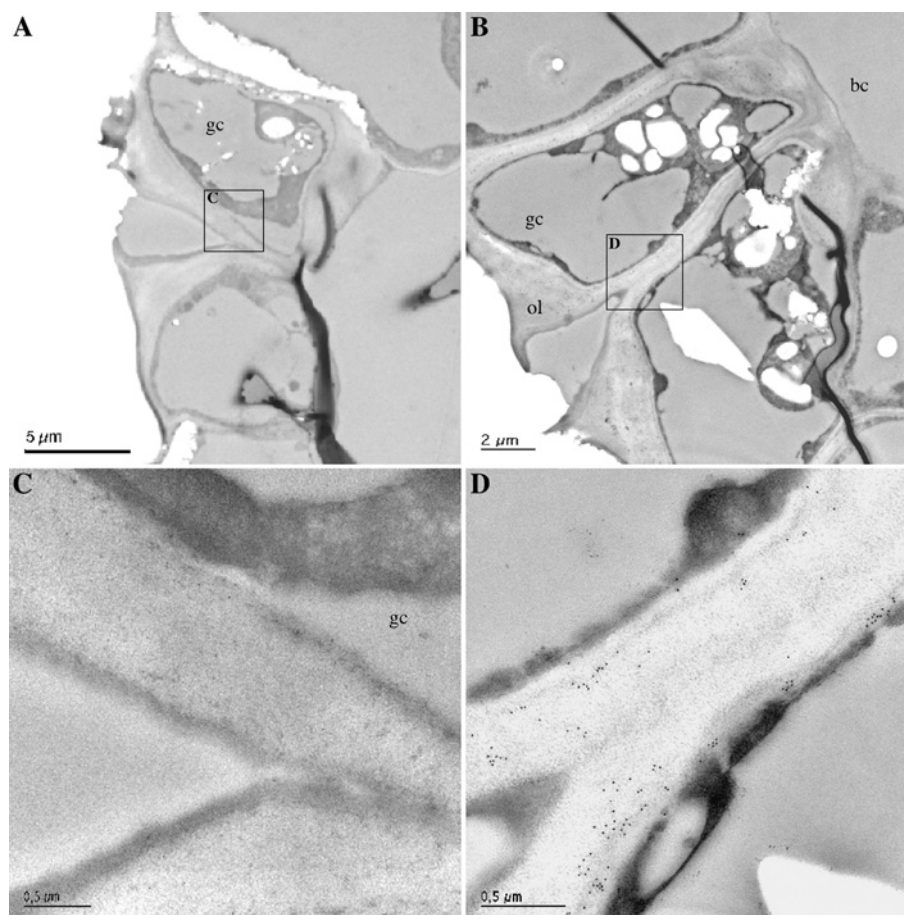
To separate plasma membrane proteins in to their monomeric form, the solubilization was performed in a two-step procedure, first with OTG and then with SDS. Solubilization was executed at room temperature (20°C) for 15 min since aggregation of MIPs frequently occurs at higher temperatures. Despite these precautions, SoPIP1;1 migrated mainly as

dimers, as seen in the leaf plasma membrane fraction [Figure 2B(ii)]; SoPIP2;1 monomeric and dimeric forms were equally abundant, whereas SoPIP1;2 was resolved almost solely as monomers [Figures 2B(iv) and 2B(ii)]. These results suggest structural differences not only between PIP1 and PIP2 members but, more surprisingly, between the more closely related SoPIP1;1 and SoPIP1;2. Only a minor amount of antigens remained in the pellet after OTG solubilization (results not shown).

Consequently, comparative structural studies of PIP1 and PIP2 isoforms should provide insights into the function and regulation of these proteins.

Figure 7 | Immunogold labelling of SoPIP1;1 in stomatal guard cells visualized by electron microscopy

Ultrathin cross-sections of leaves were challenged with SoPIP1;1 antibodies (dilution 1:100, v/v) (**B, D**) or with the corresponding preimmune serum (dilution 1:10, v/v) (**A, C**). Stomatal guard cells are heavily labelled by SoPIP1;1 antibodies on the extracellular side of the plasma membrane. The rectangles in (**A**) and (**B**) indicate regions that are shown at higher magnifications in (**C**) and (**D**). gc, guard cell; ol, outer ledge; bc, back cavity.



Purification of single isoforms from plant plasma membranes has proven to be difficult (Fotiadis et al., 2001). However, SoPIP2;1 has been successfully over-expressed in the yeast *Pichia pastoris* (Karlsson et al., 2003) and crystallized (W. Kukulski, A. Schenk, U. Johanson, T. Braun, D. Fotiadis, P. Kjellbom and A. Engel, unpublished data), offering new perspectives for structural studies of PIP homologues in plants.

Spinach PIP1 homologues are expressed in guard cells and enucleate sieve elements

Having cloned the cDNA encoding SoPIP1;2 (Figure 1), we investigated the organ and cell-type distribution of this protein by immunolocalization using

isoform-specific antibodies. SoPIP1;1 had previously been identified by cDNA cloning (Johansson et al., 1996) but the protein could not be detected by MS (Fotiadis et al., 2001). It was therefore of interest also to investigate the localization of SoPIP1;1.

We used 3'-UTR-specific RNA probes in combination with stringent washes to ensure that the signals observed in Northern blots were specific for *SoPIP1;1* and *SoPIP1;2*. For the generation of SoPIP1;1 and SoPIP1;2 antibodies, peptides in the less conserved loop C were chosen (Figure 3).

Densitometry measurements of bands on Western blots correlated well with the relative levels of transcripts from Northern-blot analysis for SoPIP1;2 (Figures 2C). The SoPIP1;2 epitopes were restricted

Table 1 | Cellular localization of PIP homologues in various plant species

Techniques: GUS, promoter:GUS fusion; IC, immunocytochemistry; IH, *in situ* hybridization; NB, Northern blot; TPI, tissue print immunoblot. Abbreviations: cc, companion cells; se, sieve elements; 3' (or 5'), use of 3' (or 5') UTR-specific probe; cDNA, use of full-length cDNA probe; 3 kb, length of promoter region in GUS.

Species	Name	Protein GenBank® accession no.	At closest homologue	Localization*	Technique
<i>Arabidopsis thaliana</i>	AtPIP1;2	AAC28529		Cotyledons, elongating roots, elongating and differentiating tissues, lateral root buds, guard cells and neighbouring epidermal cells of cotyledons, flower buds, stamens, young siliques (1) Guard cells, parenchyma cells around vascular bundles, proto-xylem and -phloem (1) Leaves: guard cells, mesophyll (weak) (1)	GUS IC (N-terminal) IH (cDNA)
	AtPIP1s			Leaves: mesophyll (plasmalemmasomes), transfer cells surrounding vascular tissue (2)	IC (N-terminal)
	AtPIP2;2	AAD18142		Leaves and roots: vascular tissues (3) Seedlings: root (central part of differentiated tissues) Leaves: vascular tissues and hydathodes Roots: cortex, endodermis, stele (strongest in endodermis and outer cell layers of stele); low in epidermis and apex (4)	GUS (3 kb) GUS (3.7 kb)
	AtPIP2;3 (RD28)	AAD18141		Leaves and roots: vascular tissues (3)	GUS (3 kb)
	<i>Brassica campestris</i>	MOD	AAB61378	AtPIP1;2	Flowers: stigma and distal portion of style, papillar cells, low in anthers, not in pollen grain, sepals. Cotyledons and leaves. Lateral roots: low. (5)
<i>Brassica oleracea</i>	Bo-PIP1b1 and BoPIP1b2	AAG23179 and AAG23180	AtPIP1;2	Flowers: stigma, ovary, petal, sepal, anther (Bo-PIP1b1), pollen (very low). In stigma: cell underlying the papillar cell layer and tissues of style (not in papillae) (6)	NB (3' and cDNA), IH (cDNA), IC (N-terminal)
<i>Mesembryanthemum crystallinum</i>	MIP-A	AAB09747	AtPIP1;4	Roots: endodermis of expanding zones, cells layers surrounding xylem strands of fully expanded zones. Leaves: low in mesophyll (7) Roots and stems: phloem, epidermis of young root, innermost cortex cell layer (8)	NB (cDNA), IH (cDNA) IC (loop C)
	MIP-B	AAA93521	AtPIP1;2	Roots: tip, before differentiation of vascular bundle (7) Young roots: innermost cortex (high), root hairs (low). Older roots: central part of xylem vessels, layer of cell outside phloem ring (8) Root and apical meristems. Xylem parenchyma and cells surrounding vascular tissues. Stems and petioles: subepidermal cells. Trichomes. Immature flowers: sepals, petals, stamen and pistil (9)	NB (cDNA), IH (cDNA) IC (loop C) GUS
	MIP-C	AAB18227	AtPIP2;4	Immature roots: all cells, most strongly in epidermis and endodermis (8)	IC (loop C)

<i>Nicotiana tabacum</i>	NtAQP1	CAA04750	AtPIP1;3	Roots: apex, elongating and differentiating zone (10) Younger stems: developing xylem vessels, internal phloem (cc). Older stems: outer xylem border, internal phloem. Young petioles: in and close to xylem. Older petioles: phloem, xylem and parenchyma. Petioles: longitudinal rows of parenchymatic cells + xylem parenchyma. Leaves: spongy parenchyma (lower) (10)	NB (5'), IH (5') TPI (N-terminal)
<i>Pisum sativum</i>	trg31	CAA38241	AtPIP1;4	Germinating seedlings: high in root tip, above root cap, cotyledons. Root to shoot junction. Hypocotyl to cotyledon junction (high) Roots: vascular region (low), root tip (low), cortex ('patchwork'), secondary phloem, vascular rays, cambium Leaves: marginal meristem (high), trichomes (high), phloem Stems: highest in phloem, least in xylem and epidermis Flowers: calyx (trichomes), anthers, mature pollen, stigma, style. Ovule and pods (11)	GUS (tobacco)
<i>Raphanus sativus</i>	RsPIP1-1/RsPIP1-2/ RsPIP1-3	BAA32777/BAA92258/ BAA92259	AtPIP1;1/AtPIP1;2/ AtPIP1;2	Younger taproots: phloem, cambium, xylem parenchyma, central part, periderm and sub-periderm. Petioles: vascular bundles, periderm, subperiderm Seedling roots and hypocotyls: cambium of central cylinder, endodermis; lower in pericycle and cells around metaxylem Taproots: vascular cambium, xylem. Petioles: vascular bundles (12)	IC (N-terminal) IC (N-terminal) TPI (N-terminal)
	RsPIP2-1	BAA32778	AtPIP2;1	Taproots: vascular cambium, xylem. Petioles: vascular bundles Younger taproots: phloem, cambium, xylem parenchyma, central part. Petioles: vascular bundles. Seedling roots and hypocotyls: cambium of central cylinder, endodermis; lower in pericycle and cells around metaxylem (12)	TPI (N-terminal) IC (N-terminal)
<i>Spinacia oleracea</i>	SoPIP1;1/ SoPM28B	CAB56217	AtPIP1;3	Guard cells (13)	IC (loop C)
	SoPIP1;2/ SoPM28C	AAR23268	AtPIP1;4	Sieve elements (roots, petioles, leaves) (13)	IC (loop C)
<i>Vicia faba</i>	PIP1			Guard cells (14)	IH (on epidermal peels)

*References: (1) Kaldenhoff et al. (1995); (2) Robinson et al. (1996) and Schäffner (1998); (3) S. Törnroth, K. Hedfalk, U. Johanson, M. Karlsson, R. Neutze and P. Kjellbom (unpublished data); (4) Javot et al. (2003); (5) Dixit et al. (2001); (6) Marin-Olivier et al. (2000); (7) Yamada et al. (1995); (8) Kirch et al. (2000); (9) Yamada et al. (1997); (10) Otto and Kaldenhoff (2000); (11) Jones and Mullet (1995); (12) Suga et al. (2003); (13) the present study; and (14) Sun et al. (2001).

to phloem sieve elements in leaves, roots and petioles (Figures 4A–4C and 5). This result was unexpected, considering that SoPIP1;2 is presumably the most abundant PIP homologue in spinach. Cell-type specificity has been documented for other plasma membrane MIPs from different plants and is summarized in Table 1. Several members of the PIP1 subgroup have been shown to be expressed in companion cell–sieve-element complexes in both monocot and dicot species: MIP-A from *M. crystallinum* (Yamada et al., 1995; Kirch et al., 2000), PIP1 homologues in *Arabidopsis* (Schäffner, 1998), the Trg31 gene product in pea (*Pisum sativum*) (Jones and Mullet, 1995), a PIP1 isoform from barley (*Hordeum vulgare*) (Doering-Saad et al., 2002) and tobacco NtAQP1 (Otto and Kaldenhoff, 2000). However, to our knowledge, a restricted localization to sieve elements as observed for SoPIP1;2 has not been reported before.

The SoPIP1;1 antibodies strongly labelled not only guard cells but also sieve elements (Figures 4D–4F and 6). In view of the low intensity of signals in Western blots, cross-reactivity of these antibodies with another PIP isoform, perhaps SoPIP1;2, is suspected. Our results indicate that SoPIP1;1 is localized in guard cells. The presence of PIP1 homologues in guard cells has also been reported in *Arabidopsis* (Kaldenhoff et al., 1995; Table 1) and in *Vicia faba* (Sun et al., 2001; Table 1).

In contrast, PIP2 family members have not been shown to be expressed either in sieve elements or in guard cells.

Functional implications of the cell specificity of PIP1s

The observations above raise the question of the functional role(s) of members of the PIP1 subclass of PIPs in sieve elements and/or companion cells and guard cells. A role for aquaporins as osmotic and turgor sensors has recently been suggested (Hill et al., 2004). This model tries to reconcile the ubiquitous presence and abundance of aquaporins in living organisms, with the apparent superfluous contribution of MIPs to water (and solutes) membrane permeability. Evidence from knockout mutants and characterization of antisense lines favour the hypothesis that PIP1 homologues are water channels. Our immunolocalization results and microarray analyses of MIP expression in leaves of drought-stressed plants (E. Alexandersson and L.C. Fraysse, unpublished data) are consistent

with this function. However, combined functions of MIPs as channels and sensors should not be neglected.

PIP1 isoforms are not (or very little) active as water channels in the *X. laevis* oocyte heterologous system (Chaumont et al., 2000). This observation suggests either that PIP1 homologues transport molecules other than water or that their water channel activity is subject to post-translational regulation. A recent report has proposed carbon dioxide as a substrate for the tobacco PIP1 isoform NtAQP1 (Uehlein et al., 2003). The mRNA levels in leaves, roots and petioles show a similar pattern of expression for NtAQP1 compared with SoPIP1;2 (Figure 2A), with the highest level in roots and the lowest in leaves. Immunolocalization with an antiserum raised against the N-terminal part of NtAQP1 indicates that the protein may be present in vascular tissues (xylem and phloem) in roots, petioles and leaves and in leaf spongy parenchyma cells (Otto and Kaldenhoff, 2000; Table 1). Since the N-terminal region of PIP1 isoforms are highly conserved within species and even between species, it is difficult to immunolocalize specific PIP1 isoforms using an antiserum raised against an N-terminal peptide. A function of PIP1 members as carbon dioxide transporters is difficult to rationalize in phloem sieve elements and companion cells, although it is plausible that changes in phloem fluxes may indirectly affect the rate of production of photosynthetic assimilates in leaves. However, the changed stomatal conductances in NtAQP1 antisense and overexpressing plants (Uehlein et al., 2003) could explain the influence on net photosynthesis. As in other species, such as maize and *Arabidopsis* (Chaumont et al., 2001; Johanson et al., 2001), it is likely that at least four additional highly homologous PIP1 isoforms (with as high as 97% amino acid identity) are expressed in tobacco. Thus, the influence on net photosynthesis seen in the NtAQP1 antisense and ectopically overexpressing lines could be due to an effect on the H₂O transport across the guard cell plasma membrane, resulting in a decreased or increased stomatal conductance respectively.

We favour the idea that the SoPIP1;2 isoform in sieve elements of source (leaves), sink (young leaves and roots) and transport organs (petioles) is needed to ensure water exchange between symplastically isolated phloem conducting cells and the surrounding cells.

Stomatal opening and closure results from turgor changes in guard cells, driven by ion fluxes across the plasma membrane and the tonoplast. Aquaporins in the plasma membrane and the tonoplast are believed to ensure the high membrane water permeability required for these rapid cell volume fluctuations. Based on our observations, we propose a role for the SoPIP1;1 isoform in the high water permeability of the plasma membrane in guard cells.

Guard cells are a favourite model for the study of regulation of molecular processes. Transcriptional control of guard cell-specific *PIP1* genes in response to ABA and blue light has been observed (Kaldenhoff et al., 1993). However, we did not observe any effect of ABA or light on the abundance of the *SoPIP1;1* transcript but rather a high constitutive level of expression in guard cells. Our results also show that SoPIP1;1 epitopes are concentrated in some regions of the guard cell plasma membrane (Figures 6C and 6D). A possible explanation may be that water efflux and influx need to be directed to specific zones in these cells. Vesicle trafficking is thought to play an important role in guard cell function (Blatt, 2002) and could be a possible mechanism of control of the sorting of PIP1 aquaporins to particular regions of the plasma membrane.

Other post-translational regulation mechanisms for plasma membrane aquaporins have been suggested. These include phosphorylation at sites in the C-terminal region and the intracellular loop B (Johansson et al., 1998, 2000), depicted in Figure 1. Members of the PIP1 group have been suggested to be phosphorylated at a site in the N-terminus (Santoni et al., 2003). We propose a conserved threonine residue in the N-terminal region of PIP1s (Figure 1) as a possible phosphorylation site. Cytosolic pH could be another signal for the regulation of PIPs since a conserved histidine residue in loop D (Figure 1) has been identified as playing a role in pH sensing (Tournaire-Roux et al., 2003). Finally, a mechanism of activation of PIP1 aquaporins on interaction with PIP2 paralogues has been suggested (Fetter et al., 2003). Co-localization studies of PIP1 and PIP2 isoforms may give insights into possible interactions between these proteins in plant cells. Also in sieve elements, regulation of water channels may be necessary to adapt to fluctuations in apoplastic water potential due to variations in transpiration (Patrick

et al., 2001) and is an interesting question that remains to be addressed.

Materials and methods

Plant material

S. oleracea Carambole F1 seeds were used. Plants were grown hydroponically in a growth chamber at 20°C with 12 h of light with an intensity of 250 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The nutrient solution contained the macronutrients, 4 mM KNO_3 , 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 and 1.2 mM KCl, and the micronutrients, 180 μM Fe/EDTA, 54 μM H_3BO_3 , 10 μM MnSO_4 , 1 μM ZnSO_4 , 0.35 μM CuSO_4 , 0.3 μM NH_4VO_3 and 0.09 μM MoO_3 .

For salt stress, osmotic stress and ABA treatment, two or three hydroponically grown plants were transferred to a beaker containing fresh nutrient solution to which NaCl, PEG or ABA was added to a final concentration of 250 mM, 10% and 100 μM respectively. Plant tissues were collected after 1–4 h and frozen in liquid N_2 . To generate drought stress, plants were transferred to an empty beaker instead, but harvested in the same way.

cDNA isolation and sequencing

Degenerate primers were designed based on the following internal peptide sequences of the SoPIP1;2 isoform: GFQPGPY-QVGGGGSNYVHHGYTK and QINNWNNDHWIFWVGPF-IGA (Fotiadis et al., 2001). The forward and reverse primers were as follows: 5'-CCGGAATTCTA(C/T)GTNCA(C/T)CA(C/T)-GGNTA(C/T)ACNAA-3' and 5'-CCGCTCGAG(G/A)TG-(G/A)TC(G/A)TTCCA(G/A)TT(G/A)TT(T/G/A)AT(T/C)TG-3'. The EcoRI and XhoI sites used for subsequent cloning are underlined. Using these primers, an approx. 300 bp long fragment was amplified by RT-PCR (reverse transcriptase-PCR) performed on total RNA from spinach leaves. This fragment was cloned into pBluescript II KS+ (Stratagene, La Jolla, CA, U.S.A.) and sequenced. It was subsequently used as a probe to screen a spinach leaf cDNA library constructed in UniZAP XR (Stratagene). The largest isolated cDNA contained approx. 570 bp of the expected 860 bp long coding sequence. To obtain the missing 5'-end of the coding sequence, RACE (rapid amplification of cDNA ends) was performed using the SMART RACE cDNA amplification kit (Clontech BD Biosciences, Palo Alto, CA, U.S.A.). The primer pair used was composed of the universal primer mix provided in the kit and the gene-specific primer 5'-AATCTCAGCACCAAGGCTGATCCC-3'. PCR with a nested primer and sequencing confirmed that the generated, 650 bp long PCR fragment corresponded to the 5'-end of the cDNA coding for SoPIP1;2. This PCR product extended 45 bp upstream of the start codon and contained a 93 bp overlapping region of the partial cDNA clone. An NcoI restriction site present in the overlapping region permitted the subcloning of the 5'-end into the partial cDNA clone, yielding a clone containing the full-length *SoPIP1;2* coding region. To confirm that the RACE product and the partial cDNA clone correspond to the same mRNA, RT-PCR was performed using 5'-UTR- and 3'-UTR-specific primers. Sequencing was performed on an ABI Prism genetic analyser using an AmpliTaq DNA sequencing kit (PerkinElmer, Boston, MA, U.S.A.).

Preparation of epidermal peels

Fully expanded spinach leaves were harvested, midribs were excised and the leaflets were collected in distilled water chilled with ice in a Waring blender. The leaves were homogenized four times for 15 s at maximal speed. The homogenate was filtered through a 200 µm nylon mesh (Spectrum Medical Laboratories, Los Angeles, CA, U.S.A.) and rinsed with cold distilled water. The retentate was homogenized and filtered as before. The final retentate was examined using a light microscope and used for RNA isolation.

RNA blotting and design of probes

Total RNA was isolated from leaves, roots and petioles of 4–5 week-old spinach plants as described by Reymond et al. (2000). RNAs were separated on a 1.5% (w/v) agarose gel containing formaldehyde and blotted on to positively charged nylon membranes (Roche Diagnostics, Rotkreuz, Switzerland). Specific RNA probes were generated and labelled by *in vitro* transcription with T3 RNA polymerase using the DIG Northern Starter Kit (Roche Diagnostics, Rotkreuz, Switzerland). The membranes were hybridized and washed according to the manufacturer's instructions. Stringent washes were performed as follows: 2 × 15 min with 0.1 × SSC (1 × SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.5% (w/v) SDS at 68°C for SoPIP1;1- and SoPIP2;1-specific probes and with an additional washing step with 0.1 × SSC and 1% (w/v) SDS at 72°C for the SoPIP1;2-specific probe.

To limit cross-hybridization, probes corresponding to the 3'-UTR of the mRNAs for SoPIP1;1, SoPIP1;2 and SoPIP2;1 were chosen for these analyses. The regions chosen were amplified by PCR using the primers with the following sequences: SoPIP2;1, 5'-GGCGAATTCCTTCAGAAGCAACCCT-3' (forward) and 5'-GGCCTCGAGCAATAATACACCATTGTC-3' (reverse); SoPIP1;1, 5'-GGCGAATTCCTTTTCATTGGAGCTGCT-3' (forward) and 5'-GGCCTCGAGCACGCCGAAGGTTATG-3' (reverse); and SoPIP1;2, 5'-GGCGAATTCATGGGTGGATGTTGTTGT-3' (forward) and 5'-GGCCTCGAGTACTTAATATACCCGG-3' (reverse). The forward and reverse primers contained EcoRI and XhoI recognition sites respectively for further cloning of the PCR products into pBluescript II KS+. The labelling of the antisense riboprobes was done by the incorporation of digoxigenin-dUTP. The maximal theoretical density of labelling (ratio of number of U to total number of bases) for both *SoPIP1;1* and *SoPIP1;2* probes was 0.24. The probe specificity was assessed by Southern blotting and no cross-hybridization was observed (results not shown).

Cloning of partial open reading frames of different spinach PIP isoforms, containing the sequence encoding loop C

To amplify the regions of the spinach genome corresponding to loop C of different PIP isoforms, we aligned the known spinach *PIP* cDNA sequences with *Arabidopsis PIP* cDNA sequences. Assuming that the positions of introns were conserved between the two species, we designed highly degenerate forward and reverse primers in helices 3 and 4 respectively. To amplify *PIP1* gene fragments, the following primer containing an XhoI restriction site (underlined) was used: 5'-GGCCTCGAGTACATN(A/G)TNATGCA(A/G)TG(C/T)(C/T)TNGG-3'. The corresponding *PIP2* primer was as follows: 5'-GGCCTCGAG-

TACATN(A/G)TNGC(C/T)CA(A/G)TG(C/T)(C/T)TNGG-3'. A common reverse primer containing an EcoRI restriction site (underlined) was used for *PIP1* and *PIP2* gene amplification: 5'-GGCGAATTCAC(A/G)AA(A/G/T)GT(A/G/T)CC(A/G)-A(C/T)(A/G/T)AT(C/T)TC(A/G/T)GC-3'. The fragments amplified by PCR with these primers were subcloned into pBlue-script II KS+ and sequenced.

Antibodies

The peptides (NH₂)-CGPYQVGGGGSNYVHHGYTKGS-(CONH₂) and (NH₂)-CNIYEMQGGGANVVNHGYTRGD(CONH₂) corresponding to loop C of SoPIP1;2 and SoPIP1;1 respectively were conjugated to Keyhole Limpet haemocyanin by their N-terminal cysteine residue. The peptide conjugates were used to produce rabbit antisera. The peptide-specific antibodies were affinity-purified from the sera using a SulfoLink kit (Pierce, Rockford, IL, U.S.A.). The buffer of the eluted fractions was exchanged with PBS, 0.02% NaN₃ using PD-10 gel filtration columns (Amersham Biosciences, Uppsala, Sweden) and concentrated 10-fold in Microcon 10 microconcentrators (Amicon, Beverly, MA, U.S.A.).

SDS/PAGE, immunoblotting and quantification of bands

SDS/PAGE was performed as described by Laemmli (1970), with minor modifications. A 5% (w/v) acrylamide stacking gel and a 12% (w/v) acrylamide resolving gel were used. Plasma membrane samples containing equal amounts of proteins were solubilized for 30 min at room temperature in 10 mM phosphate buffer (pH 7.8), containing 3% OTG. The membranes were pelleted at 4°C by centrifugation at maximal speed in an Eppendorf table centrifuge. SDS sample buffer was added to the supernatant containing the solubilized proteins. The final concentration of dithiothreitol was 200 µM. Samples were incubated for 15 min at room temperature before loading on to the SDS/PAGE gel. After electrophoresis, gels were either stained with Coomassie Brilliant Blue R-250 or transferred on to PVDF membranes for immunodetection.

The Coomassie-stained gels and films obtained by immunodetection were scanned. Band intensities were quantified using ImageQuant software (Molecular Dynamics, Little Chalfont, Bucks., U.K.). To correct for the uneven protein amounts in the different lanes, the intensities on the Coomassie gel were measured for each lane in a rectangle including proteins of molecular mass from 12 to 97 kDa. A correction factor was deduced from the values obtained and applied to correct the intensities of the 29 kDa band in the leaf, petiole and root fractions obtained by immunoblotting with the SoPIP1;2 antiserum.

Tissue preparation and immunogold labelling

Pieces of leaves, roots and petioles were sampled from 5 week-old spinach plants. The tissue preparation was performed as described previously (Bush and McCann, 1999). Briefly, 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.2) was used to fix the plant material. After dehydration in an ethanol series, the tissue pieces were processed for low temperature embedding in L.R. White resin (Wells, 1985; Hills et al., 1987).

For light microscopy, 0.5 µm thick sections were collected on multiwell glass slides. For electron microscopy, ultrathin sections were collected on formvar-carbon-coated 200 mesh gold grids.

The sections were preincubated in 1% (w/v) acetylated Aurion BSA C, 0.1% (v/v) Tween 20 in PBS at room temperature to block non-specific labelling. The antibodies were applied to sections overnight at 4°C. Antibodies were diluted to 1:10, 1:100 or 1:1000 (v/v) in 0.1% (w/v) BSA, 0.01% Tween 20 and PBS. The sections were then washed several times with PBS before incubation for 3 h at room temperature with 10 nm gold conjugates (BioCell, Cardiff, U.K.), diluted 1:30 in the same buffer as for the primary antibodies. The sections were washed in PBS as above and antibody complexes were cross-linked with 1% (v/v) glutaraldehyde in phosphate buffer for 5 min. The sections were washed thoroughly in water before silver enhancement and/or counterstaining. As negative controls, sections were treated using the preimmune sera or omitting the primary antibody.

Microscopy

For light microscopy, silver enhancement was performed with the BioCell silver enhancement kit (BioCell) for 4 min following the manufacturer's instructions. The sections were then counterstained with basic fuchsin. A Leica TCS NT laser scanning confocal microscope was used to examine the sections, with excitation and emission wavelengths of 488 and > 515 nm respectively using an RT 30/70 mirror. Images of autofluorescence and epi-reflectance were recorded separately and processed with Adobe Photoshop 7.0. The sections were counterstained with uranyl acetate and lead citrate and imaged using JEOL-1200 and JEOL-1230 transmission electron microscopes.

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