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Plasma Membrane Intrinsic Proteins from Maize Cluster in Two Sequence Subgroups with Differential Aquaporin Activity[1]

François Chaumont, François Barrieu, Rudolf Jung, and Maarten J. Chrispeels*

Department of Biology, University of California, San Diego, La Jolla, California 92093–0116 (F.C., F.B., M.J.C.); Physiological Biochemistry Unit, Université Catholique de Louvain, B–1348 Louvain-la-Neuve, Belgium (F.C.); and Trait and Technology Development, Pioneer Hi-Bred International Inc., 7300 N.W. 62nd Avenue, Johnston, Iowa 50131–1004 (R.J.)

The transport of water through membranes is regulated in part by aquaporins or water channel proteins. These proteins are members of the larger family of major intrinsic proteins (MIPs). Plant aquaporins are categorized as either tonoplast intrinsic proteins (TIPs) or plasma membrane intrinsic proteins (PIPs). Sequence analysis shows that PIPs form several subclasses. We report on the characterization of three maize (*Zea mays*) PIPs belonging to the PIP1 and PIP2 subfamilies (ZmPIP1a, ZmPIP1b, and ZmPIP2a). The ZmPIP2a clone has normal aquaporin activity in *Xenopus laevis* oocytes. ZmPIP1a and ZmPIP1b have no activity, and a review of the literature shows that most PIP1 proteins identified in other plants have no or very low activity in oocytes. Arabidopsis PIP1 proteins are the only exception. Control experiments show that this lack of activity of maize PIP1 proteins is not caused by their failure to arrive at the plasma membrane of the oocytes. ZmPIP1b also does not appear to facilitate the transport of any of the small solutes tried (glycerol, choline, ethanol, urea, and amino acids). These results are discussed in relationship to the function and regulation of the PIP family of aquaporins.

The plasma membrane regulates the movement of solutes and water and maintains a cellular solute composition very different from that of the external environment. Ions, nutrients, and water are transported through integral membrane proteins (Chrispeels et al., 1999) classified into several families, the members of which are found in all living species. One of these transport families is the major intrinsic protein (MIP) family, named after the first member described, which was found in mammalian lens tissue (Gorin et al., 1984). MIPs have a mass of approximately 30 kD and are characterized by six transmembrane-spanning helices, cytosolic amino and carboxy termini, and the signature sequence SGxHxNPAVxT, which is repeated in the second half of the protein as NPA (Park and Saier, 1996; Maurel, 1997; Agre et al., 1998). Many MIP proteins are aquaporins, so named because they enhance the permeability of the membrane for water, and some MIPs form channels for small neutral solutes, including glycerol and urea (Agre et al., 1998).

Evidence that aquaporins form water channels is mostly derived from experiments with heterologous expression systems: *Xenopus laevis* oocytes, the yeast *Saccharomyces cerevisiae*, the slime mold *Dictyostelium discoideum*, and insect cells. Heterologous cells that express aquaporins swell more rapidly in hypoosmotic medium compared with control cells. Because *X. laevis* oocytes are large cells, the rate of volume increase in these cells can easily be measured and used to calculate the osmotic water membrane permeability (Pf). Expression of aquaporins in this heterologous system increases the Pf value of the plasma membrane considerably (Preston et al., 1992; Maurel et al., 1993), but aquaporins show a range of Pf values, with bovine lens MIP (AQP0) and soybean NOD26 showing a water transport activity at least one order magnitude less than other mammalian and some plant AQPs (Mulders et al., 1995; Dean at al., 1999). While the term "aquaporin" is a functional definition, it appears that some of these channels have multiple specificities. For instance, mammalian AQP3 and AQP7 are permeable to water, glycerol, and urea (Ishibashi et al., 1994, 1997), and have been classified in a subgroup of aquaglyceroporins (Agre et al., 1998). This subgroup also includes the *Escherichia coli* homolog GlpF, which transports glycerol specifically by a pore-like mechanism (Maurel et al., 1994). Interestingly, substitutions of two highly conserved residues in aquaporin by amino acids located at the same positions in the glycerol channel leads to a switch in the selectivity from water to glycerol (Lagrée et al., 1999).

A great number of MIP homologs have been identified in plant species (Weig et al., 1997; Tyerman et al., 1999; for review, see Schäffner, 1998). They are classified in two main groups according to their sequence identity with MIPs localized in the plasma membrane (plasma membrane intrinsic proteins or PIPs) or in the vacuolar membrane (tonoplast intrinsic proteins or TIPs). In addition, a MIP from soybean, NOD26, was found in the symbiosome membrane surrounding nitrogen-fixing bacteria (Rivers et al., 1997). In Arabidopsis, 23 MIP cDNAs have been iden-

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* Corresponding author; e-mail mchrispeels@ucsd.edu; fax 858–534–4052.

tified, and sequence comparison enabled us to assign a putative subcellular membrane location (11 TIPs, 11 PIPs, 1 NOD26-like protein) (Weig et al., 1997). Ten of these have so far been identified as active aquaporins after expression in *X. laevis* oocytes (Kammerloher et al., 1994; Weig et al., 1997). Tobacco NtTIPa and NtAQP1 and soybean NOD26 transport glycerol in addition to water (Rivers et al., 1997; Biela et al., 1999; Dean et al., 1999; Gerbeau et al., 1999). However, because the primary sequences of MIPs do not allow us to deduce which solute is being transported, functional testing is always necessary. In addition to MIP selectivity, expression in *X. laevis* oocytes permits investigation of MIP regulation by phosphorylation. The water channel activity of bean tonoplast α-TIP and the plasma membrane aquaporin PM28A from spinach is regulated by phosphorylation (Maurel et al., 1995; Johansson et al., 1998). For instance, mutation of a conserved cAMP-dependent phosphorylation site located at position 99 in α-TIP and 115 in PM28A decreased the water transport activity, indicating its importance in protein regulation.

Maize (*Zea mays*) is an important crop that has been extensively used to study plant-water relations. Recently, we reported the characteristics of the maize tonoplast aquaporin ZmTIP1 (Barrieu et al., 1998; Chaumont et al., 1998). Here we show the properties of three PIPs, ZmPIP1a, ZmPIP1b and ZmPIP2a, that belong to two subgroups of PIP proteins according to their amino acid sequences and functional assays. Oocytes injected with *ZmPIP2a* cRNAs exhibited a high Pf value, whereas those injected with *ZmPIP1a* and *ZmPIP1b* cRNAs exhibited low Pf values. The subcellular localization of ZmPIP1a, ZmPIP1b, and ZmPIP2a in oocytes and plant cells is documented. Our results indicate that maize cells contain several MIPs in their plasma membrane that have different functions or are differentially regulated.

MATERIALS AND METHODS

RNA Extraction and Gel-Blot Analysis

For maize (*Zea mays*) *ZmPIP1b* cDNA cloning and northern-blot analysis, total RNA was obtained from seeds, embryos, and endosperm at 19 d post-pollination, from shoots and roots of germinating seedlings, from leaves from 1- to 2-week-old plants, from developing ears, and from tassels about 2 cm in size, as described previously (Chaumont et al., 1998). Poly(A^+) RNA was isolated from total RNA using the poly(A^+) Tract Kit (Promega, Madison, WI) following the manufacturer's instructions. RNA blots and hybridizations were as described previously (Chaumont et al., 1998).

cDNA Libraries and Expressed Sequence Tag (EST) Databases

RNA was isolated from various maize tissues using TriZol Reagent (Gibco-BRL, Cleveland), and cDNA synthesis was performed using SuperScript Choice System (Gibco-BRL) and cloned into *Not*I/*Sal*I sites of pSORT1 vector (Gibco-BRL). Clones were picked randomly and inserts were sequenced by Human Genome Systems (Rockville, MD) from the 5′ end to obtain ESTs.

ZMPIP1b cDNA Cloning

cDNA was synthesized from 0.5 μg of seed mRNA using oligo$(dT)_{12-18}$ as a primer and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). Partial *ZMPIP1b* cDNA was amplified using TIP4 (Weig et al., 1997) and ZMTIP2 (5′-GG[GC] CC[GC] ACCCAG[TA] AGATCCA-3′) primers. The reaction products were separated and cloned as described previously (Weig et al., 1997). Full-length cDNAs of *ZMPIP1b* were obtained using the 5′/3′ RACE kit (Boehringer Mannheim, Basel) following the instructions of the manufacturer. Three antisense and one sense primer (MRACE 21: 5′-GCAAGGATAGGAACAT-GGGAG-3′; MRACE22: 5′-TGGCATTCCTCTTGGCATC-3′; MRACE23: 5′- TAAAGTCCCTGCTGGAAGCC-3′; MRACE20: 5′-ATGTTGTCGCACCTGGCTAC-3′) were used. The PCR products were cloned into pCRII (TA cloning kit, Invitrogen, Carlsbad, CA) and sequenced. The full-length *ZMPIP1 b* cDNA was amplified using Pfu polymerase (Stratagene, La Jolla, CA) with proofreading activity and specific primers to the 5′ and 3′ noncoding regions (ZMPIP1b-1: 5′-CGGAATTCGACACACGCCGCTGCTCC-3′; ZMPIP1b-2: 5′-CGGAATTCTTTCTTGCCAACATCCC-3′), incorporating *Eco*RI sites at both ends, and subcloned into *Eco*RI site of Bluescript II SK+ (Stratagene).

ZmPIP2a cDNA Identification

ZmPIP2a EST (CRTBB63R) was identified by its annotation (Arabidopsis pip 2a homolog; accession no. X75883) using the IRIS database interface software (Human Genome Systems). Both strands of the entire insert of *ZmPIP2a* were sequenced by primer walking. The sequencing data were edited and assembled using Sequencher software (Genes Codes Corp., Ann Arbor, MI).

Plasmid Constructions and in Vitro RNA Synthesis

cDNA encoding ZMPIP1a, ZMPIP1b, and ZMPIP2a were amplified by PCR with specific primers (ZMPIP1a-1: 5′-GG-TAACAGATCTGGCATGGAGGGG-3′;ZMPIP1a-2:5′-CCG-GCAAGATCTCGAAGCAGCAGC-3′; ZMPIP1b-3: 5′-CGG-GGATCCAAACAATGGAGG-3′; ZMPIP1b-4: 5′-GGCAGA-TCTCGGCAVGGCCAC-3′; ZMPIP2a-1: GGCTAGATCT-AGAATGGCCAAG; ZMPIP2a-2: CTTCTCGAGCCTCTG-GTATATC), incorporating *Bam*HI and/or *Bgl*II sites on both ends, and subcloned into the *Bgl*II site of a pSP64T-derived Bluescript vector carrying 5′ and 3′ untranslated sequences of a β-globin gene from *Xenopus laevis* (Preston et al., 1992). Clones with an insert in the correct orientation were determined by restriction mapping and sequencing. Capped complementary RNA encoding ZMPIP1a, ZMPIP1b, ZMPIP2a, and AtRD28 (Daniels et al., 1994) were synthesized in vitro by using T3 RNA polymerase and purified as described by Preston et al. (1992).

The 3′-untranslated regions of *ZMPIP1a*, *ZMPIP1b*, and *ZMPIP2a* were amplified by PCR with specific primers

(ZMPIP1a-1: 5′-TAAAGGATCCGATGCTGCTG-3′; ZMPIP 1a-2: 5′-GGATGAATTCTTAAAGCTTG-3′; ZMPIP1b-5: 5′-CAGGTCTAGAGCTGCCGTGG-3′; ZMPIP1b-2; ZMPIP2a-4: 5′-TGGGATCCCGCCGGACAAGGAC-3′; ZMPIP2a-5: 5′-CGTGGATCCGTCGTCACGGAT-3′), incorporating *Bam*HI and *Eco*RI sites (*3′ZMPIP1a*), *Xba*I and *Eco*RI sites (*3′ZMPIP1b*) or *Bam*HI sites (*3′ZMPIP2a*) at the ends, and subcloned into the corresponding sites of pBluescript II SK+ (Stratagene).

ZmPIP1a and ZmPIP2a cDNA coding regions were PCR-amplified with specific primers (ZmPIP1b-6: 5′-GGCAGC-CATGGACCTGCTCTTGAA-3′; ZmPIP1b-7: 5′-GGAGCAA-AACCATGGAGGGG-3′; ZmPIP2a-6: 5′-CGCGACCAT-GGCCAAGGACATC-3′; ZmPIP2a-8: 5′-GCCATCCATG-GAGCGGCTGAAGGA-3′), incorporating *Nco*I sites at both ends, and subcloned into the corresponding site of the *35SC4PPDK-sGFP*(*S65T*) plasmid (Chiu et al., 1996).

35SC4PPDK-sGFP(S65T) and *35SC4PPDK-ZmPIP1b-sGFP*(*S65T*) were cloned with *Hin*dIII/*Eco*RI into the *Agrobacterium tumefaciens* transformation vector pDE1001 (Plant Genetic System, Gent, Belgium).

Osmotic Water Permeability Assay

X. laevis oocytes were prepared and injected as previously described (Daniels et al., 1996). The osmotic water permeability assay was conducted as previously described (Weig et al., 1997).

Labeling of Oocyte Proteins, Subcellular Fractionation, and Protein Analysis

Oocytes were transferred 4 to 6 h after cRNA injection into Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.4) supplemented with 6.0 MBq/mL [^{35}S]Met (555 MBq/mL; 37 TBq/mmol) or [^{35}S] protein labeling mix containing mostly [^{35}S] Met (293 MBq/mL; 4305 TBq/mmol; New England Nuclear, Boston). After 16 h, the oocytes were washed with Barth's solution and lysed in a phosphate buffer (10 mM KH_2PO_4, 5 mM EDTA, 5 mM EGTA, pH 7.5) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 5 μM pepsatin, 2 μg/mL leupeptin, and 1 μg/mL aprotinin. The lysate was centrifuged for 5 min at 150*g* to remove yolk proteins and subsequently for 30 min at 14,000*g*. The microsomal pellet was washed with phosphate buffer and resuspended in denaturation buffer (1% [w/v] SDS, 20 mM Tris [pH 8.6], 8% [w/v] glycerol, 0.3% [v/v[β-mercaptoethanol, and 0.001% [w/v] bromphenol blue). The samples were counted using a scintillation counter, and proteins corresponding to 100,000 cpm were electrophoresed through a 15% SDS-polyacrylamide gel. For fluorography, gels were equilibrated with dimethylsulfoxide, impregnated by immersion in 20% 2,5-diphenyloxazole in dimethylsulfoxide, soaked in water, dried and exposed to Kodak X-Omat film at −70°C. Plasma membrane complexes were prepared as described previously (Wall and Patel, 1989).

Tobacco plants were homogenized as described previously (Chaumont et al., 1994). Plasma membranes were purified from the microsomal fraction by partitioning at 4°C in an aqueous polymer two-phase system as described previously (Larsson et al., 1994).

Radiolabeled Solute Uptake Assay

Two to 3 d after water or mRNA injection, groups of five oocytes were incubated at 21°C in 100 μL of Barth's solution containing 0.1 to 0.5 MBq/mL of the radiolabeled solutes ([^{14}C]glycerol, 0.288 GMBq/mmol; [^{14}C]choline, 2.0 GBq/mmol; [^{14}C]ethanol, 0.144 GBq/mmol; [^{14}C]urea, 2.0 GBq/mmol or ^{14}C labeled amino acid mixture without Met, 1.85 MBq/mL). Non-radioactive solutes were added to give a 1 mM final concentration. After 0, 15, and 30 min, the oocytes were washed rapidly three times in ice-cold Barth's solution, and individual oocytes were dissolved in 5% (w/v) SDS for scintillation counting. Results are shown as the percentage uptake of water-injected oocytes.

Plant Transformation and Transient Gene Expression

Tobacco (*Nicotiana tabacum* cv Xanthii) was transformed using *A. tumefaciens*-mediated transformation as described previously (Rogers et al., 1986) and selected on 100 mg/mL kanamycin. Highly fluorescent plants were selected by epifluorescence microscopy.

Fluorescent Microscopy

Leaf and root samples were sliced with razor blades and mounted between slides and coverslip in Murashige and Skoog medium. Samples were examined using a confocal laser-scanning microscope (model MRC-1024, Bio-Rad Laboratories, Hercules, CA). Focal planes were scanned with the 488 nm argon laser using a 550-nm barrier filter and a ×40, 4.4 numerical aperture oil immersion objective.

RESULTS

Isolation of ZmPIP1b and ZmPIP2a cDNA

The use of degenerate oligonucleotide primers designed from conserved regions of plant aquaporins (HI/VNPAVT and WI/VY/FWVGP) enabled us to clone several cDNAs from different species by reverse transcriptase-PCR (Weig et al., 1997; Chaumont et al., 1998). Using this strategy with cDNAs prepared from maize seeds 19 d after pollination, we obtained a PCR-amplified fragment (0.44 kb) containing a sequence homologous to plant PIP aquaporins. The corresponding full-length cDNA was recovered by 5′/3′ RACE with RNA from maize seeds and named *ZmPIP1b* (accession no. AF131201). The *ZmPIP1b* cDNA consists of 1,280 bp upstream from the poly(A^+) tail, which includes a 60-bp leader sequence, followed by 870 bp of open reading frame encoding 289 amino acids, and, finally, a 350-bp 3′ noncoding region. The *ZmPIP1b* cDNA encodes a protein that has a high sequence identity (95.8%) with the maize transmembrane protein encoded by pZSS4 (accession no.

X82633; Chevalier et al., 1995). Because of the high percentage identity between these clones, we decided to characterize them in parallel, and pZSS4 is hereafter referred to as ZmPIP1a.

The *ZmPIP2a* EST was identified in maize EST databases using the IRIS database interface software (Human Genomic Systems) by its annotation "Arabidopsis pip2a homolog (accession no. X75883)". The entire insert of *ZmPIP2a* was sequenced by primer walking (accession no. AF130975). The *ZmPIP2a* cDNA consists of 1217 bp upstream from the poly(A^+) tail, which includes an 84-bp leader sequence, followed by a 858-bp open reading frame encoding 285 amino acids, and finally, a 275-bp 3′ noncoding region.

ZmPIP1a, ZmPIP1b, and ZmPIP2a have a calculated M_r of 30,677, 30,795, and 29,835, respectively, and contain the MIP family signature sequence SGxHxNPAVT, which is repeated in the second half of the protein as NPA (Fig. 1). A comparison of the amino acid sequences with plant MIPs revealed clearly that the three proteins are PIPs (data not shown). They differ from TIPs in having a longer amino-terminal extension and several amino acid residues throughout the sequence and around the conserved NPA motifs (for review, see Schäffner, 1998). If the amino acid sequence alignment of ZmPIP1a, ZmPIP1b, and ZmPIP2a is restricted to other known plant PIPs, two main subgroups are observed on a dendogram (Fig. 2). They correspond to the PIP1 and PIP2 subfamilies, previously introduced to categorize the first plant plasma membrane-located members discovered in Arabidopsis (Kammerloher et al., 1994). The PIP1 subfamily proteins include ZmPIP1a and ZmPIP1b and are characterized by a longer amino-terminal extension and a shorter carboxy-terminal end compared with the PIP2 subfamily proteins, including ZmPIP2a (Fig. 1). The latter is also characterized by an additional stretch of 8 amino acids predicted to be located in the first extracytosolic loop. It was also interesting that in both subfamilies, ZmPIPs had the highest sequence identity with monocot PIPs from rice and barley and clustered together on the dendogram (Fig. 2).

Functional Studies of ZmPIP1a, ZmPIP1b, and ZmPIP2a in *X. laevis* Oocytes

The biological activity of aquaporins is usually determined with *X. laevis* oocyte-swelling assays (Maurel et al., 1993). MIP cRNA is injected into oocytes and after a 3-d incubation, the oocytes are moved to a hypotonic medium. If water channel proteins are present in the plasma membrane, the oocytes swell and burst much faster than the control oocytes. In these conditions, oocytes injected with the plasma membrane AtRD28 aquaporin (Daniels et al., 1994) or *ZmPIP2a* cRNAs rapidly increased in volume, indicating the presence of a facilitated water transport pathway (Fig. 3). The Pf of the oocyte membrane increased 8-fold over the control. Mercurial compounds are characteristic inhibitors of many water channel proteins. Water transport trough ZmPIP2a is inhibited 50% by 1 mM mercuric chloride, and this inhibition is reversed by the reducing agent β-mercaptoethanol (Fig. 3). Surprisingly, oocytes injected with *ZmPIP1a* and *ZmPIP1b* cRNAs swelled very slowly and had a Pf similar to the water-injected oocyte (Fig. 3A).

The lack of a swelling response by *ZmPIP1a* and *ZmPIP1b* cRNA-injected oocytes could reflect the failure to express the corresponding protein or disturbed trafficking to the plasma membrane. To investigate the expression and presence of ZmPIP1a and ZmPIP1b in the plasma membrane, proteins of oocytes injected with water or cRNAs were labeled with [^{35}S]amino acids. Total membrane proteins and plasma membrane fractions were then extracted, separated by SDS-PAGE, and visualized by fluorography. The fluorogram shown in Figure 4 reveals that the labeling pattern of the oocytes injected with *AtRD28*, *ZmPIP1a*, and *ZmPIP1b* cRNAs differed from the profile of water-injected oocytes by the presence of additional polypeptides of 28 to

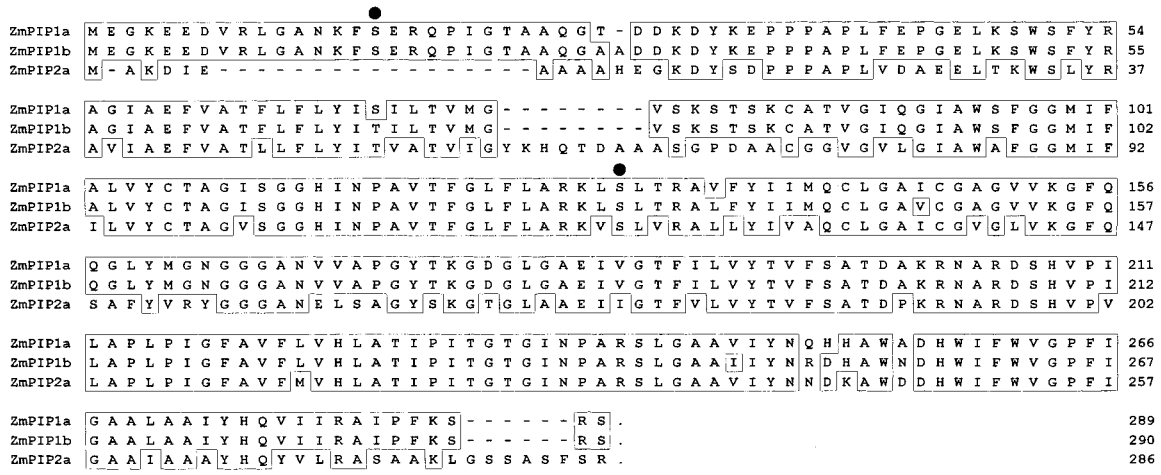

Figure 1. ZmPIP1a (accession no. X82633), ZmPIP1b, and ZmPIP2a sequence comparison. Amino acid sequences were compared with the MEGALIGN program (DNASTAR, Madison, WI). Identical amino acid residues to at least two sequences are in a box. Numbering refers to the respective amino acid sequence. Black dots indicate the putative phosphorylation sites.

29 kD (Fig. 4, lanes 1, 2, 4, and 6). As expected, the plasma membrane fraction was characterized by a protein profile different from the total membrane fraction, and the fluorogram shows that AtRD28, ZmPIP1a, and ZmPIP1b were clearly present in the plasma membrane. The corresponding bands were not enriched in the plasma membrane fraction (Fig. 4, lanes 3, 5, and 7) compared with total membrane preparations (lanes 2, 4, and 6), suggesting that these proteins are also associated with other membranes. This conclusion was confirmed by analyzing the intracellular localization of ZmPIP1b fused to a green fluorescence protein (GFP) in oocytes: the images showed fluorescence at the plasma membrane and in the cytoplasm (data not shown). These results indicate that *X. laevis* oocytes were capable of synthesizing and targeting AtRD28, ZmPIP1a, and ZmPIP1b proteins into the plasma membrane.

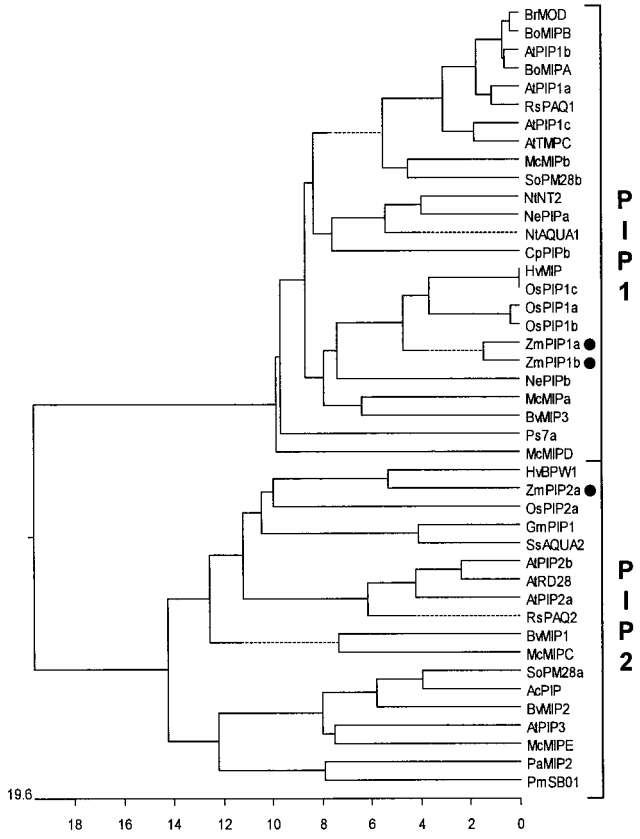

Figure 2. Dendogram of the comparison between 43 plant PIPs, including ZmPIP1a, ZmPIP1b, and ZmPIP2. Amino acid sequences were compared using the MEGALIGN program (DNASTAR). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. A dotted line indicates a negative branch. The black dots indicate the maize ZmPIPs. Accession numbers are (in parentheses): BrMOD (X95640), BoMIPB (AF004293), AtPIP1b (X68293), BoMIPA (X95639), AtPIP1a (X75881), RsPAQ1 (AB012044), AtPIP1c (X75882), AtTMPC (D26609), McMIPb (L36097), SoPM28b (I. Johansson and P. Kjellbom, personal communication), NtNT2 (U62280), NePIPa (AB002149), NtAQUA1 (AJ001416), CpPIPb (AJ001293), HvMIP (S41194), OsPIP1c (AF022737), OsPIP1b (AB009665), ZmPIP1a (X82633), ZmPIP1b(AF131201), NePIPb (AB002147), McMIPa (L36095), BvMIP3 (U60149), Ps7a (X54357), McMIPD (U26537), HvBPW1 (AB009307), ZmPIP2a (AF130975), OsPIP 2a (AF062393), GmPIP1 (U27347), SsAQUA2 (AF067185), AtPIP2b (X75884), AtRD28 (D13254), AtPIP2a (X75883), RsPAQ2 (AB012045), BvMIP1 (U60147), McMIPC (U73466), SoPM28a (L77969), AcPIP (U18403), BvMIP2 (U60148), AtPIP3 (U78297), McMIPE (U73467), PaMIP2 (Z93764), and PmSB01 (AF051202).

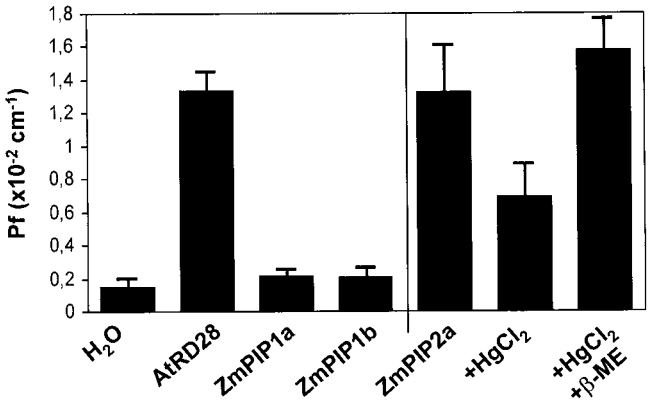

Figure 3. Pf values of individual oocytes injected with water (H_2O) or cRNA encoding AtRD28, ZmPIP1a, ZmPIP1b, or ZmPIP2a, derived from volume change measurements. When indicated, the assay was performed in the presence of 1 mM $HgCl_2$ with a 10-min preincubation ($HgCl_2$) or mercaptoethanol after mercuric chloride treatment ($HgCl_2$ + β-ME: 10 min preincubation with 1 mM $HgCl_2$ followed by a 15-min preincubation and the assay, both in presence of 5 mM mercaptoethanol). Data are expressed as the means ± SE of data from five to 12 cells.

A number of studies have indicatedthat the activity of aquaporins may be regulated by phosphorylation. For instance, α-TIP, a seed-specific vacuolar protein from bean, is phosphorylated in vivo and in vitro and the phosphorylation regulates its water channel activity in *X. laevis* oocytes (Maurel et al., 1995). In the same way, plasma membrane PM28A aquaporin from spinach was demonstrated to be regulated by phosphorylation (Johansson et al., 1996, 1998). A detailed study of ZmPIP1b primary sequence revealed 2 potential phosphorylation sites on the cytoplasmic side: S16, protein kinase C site and S131, protein kinase A site (dots in Fig. 1). The latter is located at a position comparable to bean α-TIP (Ser-99) and spinach PM28A (Ser-115) that have been demonstrated to regulate water transport in oocytes. We investigated the water channel activity of ZmPIP1b in the presence of (1) cAMP agonists (8-bromoadenosine 3′, 5′-cyclic monophosphate, forksolin and 3-isobutyl-1-methylxanthine) to increase the cytosolic cAMP concentration and activate cellular PKA and (2) the protein phosphatase inhibitor okadeic acid. None of these treatments affected the Pf of ZmPIP1b- expressing oocytes (Pf = 0.18–0.20 $\times$ 10^{-2} cm/s) (data not shown).

Although many MIP proteins are aquaporins, some of them can transport small neutral solutes, exclusively or in addition to water. We performed transport essays in ZmPIP1b-expressing oocytes with a series of labeled solutes. The oocytes showed no enhanced uptake of [^{14}C]glycerol, [^{14}C]choline, [^{14}C]ethanol, [^{14}C]amino acids and [^{14}C]urea compared with control water-injected oocytes (<1.2-fold increase) (data not shown).

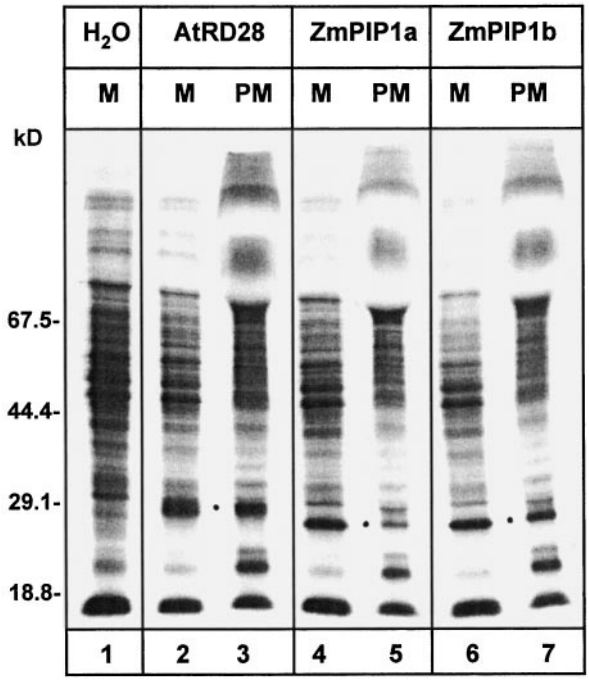

Figure 4. Plasma membrane localization of AtRD28, ZmPIP1a, and ZmPIP1b in cRNA-injected oocytes. In vivo-labeled proteins contained in total membrane fraction (M) and plasma membrane fraction (PM) of water or cRNA-injected oocytes were prepared as described in "Materials and Methods." Dots indicate the polypeptides resulting from cRNA injection. The positions of the molecular mass standards are indicated on the left.

Altogether, these data indicate that when ZmPIP1a and ZmPIP1b are expressed in *X. laevis* oocytes, they arrive at the plasma membrane but do not function as water channels on their own, suggesting that either they are transporters for solutes not yet identified, or they need to be regulated by components not present in *X. laevis* oocytes.

Expression of *ZmPIP1a, 1b*, and *2a* in Different Tissues during Development

To analyze the expression pattern of *ZmPIP1a, 1b,* and *2a*, DNA fragments from the 3′-untranslated region of *ZmPIP1a, 1b,* and *2a* cDNAs were used as probes. The specificity of the probes was tested by Southern hybridization. For this experiment, only restriction enzymes that do not cut the 3′-untranslated sequence (*Eco*RI, *Hin*dIII, and *Xba*I) were used to digest genomic DNA samples. Hybridization at high-stringency conditions (0.1× SSC, 0.1% [w/v] SDS, and 60°C) revealed only one band for each of the restriction digests (data not shown). This result suggests that the probes are likely to be specific for each gene.

The expression patterns of *ZmPIP1a, 1b,* and *2a* were characterized by gel-blot analysis of total RNA from different maize tissues. Transcripts with a size of 1.20 kb were observed with the three specific probes but the expression profiles were distinct (Fig. 5). *ZmPIP1a* was highly expressed in developing shoots and roots and to a lesser extent in tassels. *ZmPIP1a* transcripts were also detected in the embryo and the leaves. *ZmPIP1b* was mainly expressed in the developing tassels and had a basal expression in all the tissues studied. Finally, *ZmPIP2a* transcripts were only present in roots. We previously documented a possible role for the tonoplast aquaporin ZmTIP1 in tissue expansion (Chaumont et al., 1998). For instance, the *ZmTIP1* transcript level was higher in the youngest expanding leaf of a plantlet (corresponding to leaf no. 3 in Fig. 5) in comparison with that fully expanded one (leaf no. 1 in Fig. 5). *ZmPIP1a* and *ZmPIP1b* transcript abundance was low and did not vary in leaves at different developmental stages (Fig. 5, lanes 6–8) suggesting that the corresponding ZmPIP1a and ZmPIP1b proteins are not required for leaf expansion.

Subcellular Localization of ZmPIPs

Amino acid sequence comparisons enable us to identify ZmPIP1a, 1b and 2a as putative plasma membrane proteins. This analysis relies entirely on previously located plasma membrane PIPs and needs to be demonstrated for ZmPIPs. We fused *ZmPIP1b* cDNA to the 5′ end of the plant-adapted *sGFP(S65T)* gene in *35SC4PPDK-sGFP(S65T)* plasmid (Chiu et al., 1996) and introduced these constructs into tobacco cells, using a binary vector and *A. tumefaciens* (see "Materials and Methods"). sGFP(S65T) alone was used as a control. Transgenic tobacco plants, exhibiting good fluorescent signals in the leaves, were selected for protein localization by confocal microscopy.

The location of ZmPIP1b::sGFP(S65T) and sGFP(S65T) was investigated in root tip sections. Cells from the apical meristem and the elongation zone are characterized by numerous small vacuoles fusing together and a large area of cytosol. This allowed us to distinguish cytosolic from plasma membrane fluorescence, which is not possible in fully expanded cells containing a thin ring of cytoplasm located directly underneath the plasma membrane. In the small dividing cells expressing *ZmPIP1b::sGFP*(*S65T*), strong green fluorescence signals were observed around the cell and, to a lesser extent, around the nucleus and between both structures (Fig. 6A). In the cell elongation

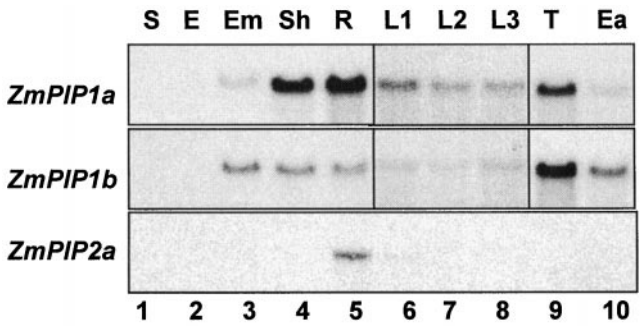

Figure 5. Gel-blot analysis of ZmPIP1a, ZmPIP1b, and ZmPIP2a mRNA in different vegetative and reproductive organs. Total RNA (20 μg) was extracted from seeds (S), endosperm (E), embryos (Em), shoots (Sh), roots (R), 10-d-old maize plantlet leaves (L1, L2, and L3), developing tassels (T), and ears (Ea) and separated by gel electrophoresis. After transfer, the blots were hybridized with the indicated probes.

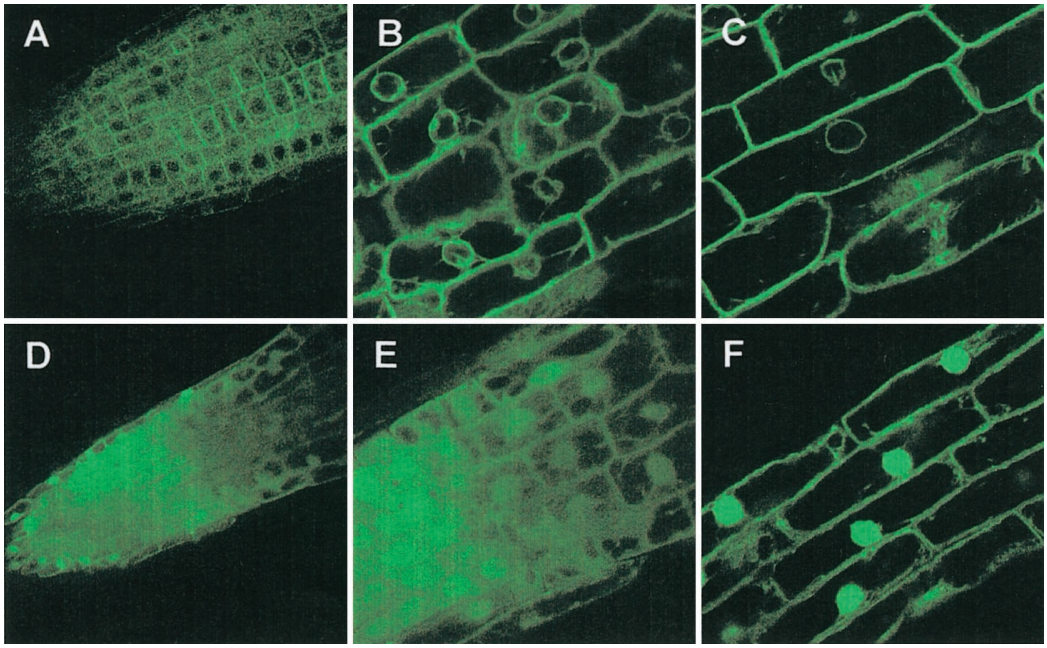

Figure 6. Localization of ZmPIP1b::sGFP(S65T) and sGFP(S65T) in root tip cells. Confocal microscopic images of transgenic tobacco root tips expressing ZmPIP1b::sGFP(S65T) (A–C) and sGFP(S65T) (D–F). A and D, Root tips; B and E, zone of cell elongation; C and F, elongated cells.

zone, strong green fluorescence was located in the plasma membrane surrounding the cell (Fig. 6, B and C). The perinuclear region and transcellular strands extending from the nucleus to the plasma membrane were also labeled and might correspond to the ZmPIP1b::sGFP(S65T) protein routing through the secretory pathway.

In control root tips expressing the soluble sGFP(S65T), the dividing cells appeared uniformly labeled (Fig. 6D). In the elongation zone, the fluorescence signal was observed in the nucleoplasm and the cytoplasm surrounding the fusing vacuoles of sGFP(S65T) cells (Fig. 6E). Finally, more distal from the root tip, green fluorescence was visible in the nucleoplasm, the peripheral cytoplasm, and cytoplasmic strands in the cells (Fig. 6F).

The localization of ZmPIP1b::sGFP(S65T) and GFP was also investigated by immunodetection of subcellular fractions from leaf extracts using a GFP antiserum. Plasma membranes were isolated from microsomal fractions by partitioning in an aqueous polymer two-phase system (Larsson et al., 1994), and their purification was tested with a plasma membrane H^+-ATPase antiserum (Morsomme et al., 1996). Figure 7 shows that the control sGFP(S65T) was present in the supernatant, indicating that the fluorescence observed in epidermal cells was indeed due to the peripheral cytoplasm located directly underneath the plasma membrane (Fig. 6, A and B). Monomeric and dimeric forms of ZmPIP1b::sGFP(S65T) were enriched in the plasma membrane fraction (Fig. 7, lane 8). The observation that ZmPIP1b::sGFP(S65T) appears more enriched in the plasma membrane fraction than the H^+-ATPase (Fig. 7, lanes 7 and 8) indicates that the fusion protein might be

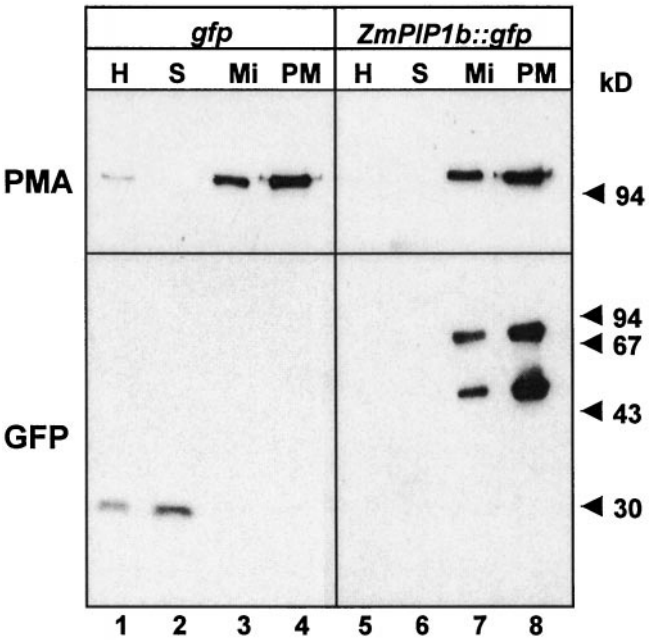

Figure 7. Immunodetection of ZmPIP1b::sGFP(S65T) and sGFP (S65T) in transgenic tobacco plants. Subcellular fractions of transgenic plants expressing ZmPIP1b::sGFP(S65T) and sGFP(S65T) were obtained as described in "Materials and Methods," fractionated by SDS-PAGE, transferred to nitrocellulose, and immunostained using *N. plumbaginifolia* H^+-ATPase (PMA) or GFP antisera. H, Homogenate; S, cytosolic supernatant; Mi, microsomal fraction; PM, plasma membrane-enriched fraction. The positions of the molecular mass standards are indicated on the right.

associated with contaminating endomembranes present in the fraction and confirms the fluorescence distribution detected by microscopy.

DISCUSSION

Intrinsic and peripheral plasma membrane proteins play important roles in regulating numerous cellular activities, and members of the MIP family are abundantly present in the plasma membrane (Johansson et al., 1996). Although abundant, the function of these proteins is not yet clearly understood. The presence of highly conserved motifs in plant MIPs permitted us to identify and clone by reverse transcriptase-PCR and RACE a plasma membrane PIP cDNA from maize, *ZmPIP1b*, which has a high sequence identity with *ZmPIP1a* (*pZSS4*), a previously identified MIP (Chevalier et al., 1995). In addition, computational sequence comparison of maize EST clones allowed us to identify a third maize PIP clone, *ZmPIP2a*. The plant PIP family is clearly divided into two subfamilies named PIP1 and PIP2 by Kammerloher et al. (1994) to classify Arabidopsis plasma membrane-located MIPs. On a dendogram, the ZmPIP1a and ZmPIP1b amino acid sequences cluster with the Arabidopsis PIP1 subfamily members, whereas the ZmPIP2a sequence is closely related to the PIP2 subfamily (Fig. 2). Proteins from both subfamilies are divergent according to the length of their amino and carboxy termini and several single conservative and non-conservative amino acid residue substitutions (Fig. 1). Do these two PIP subfamilies have other distinguishing characteristics related to their function?

ZmPIP1a, ZmPIP1b, and ZmPIP2 Have Widely Divergent Aquaporin Activities

MIP function has mostly been tested by transient expression in *X. laevis* oocytes and subsequent analysis of plasma membrane permeability to water or other metabolites. Many of the plant MIPs for which results have been reported so far increase the Pf of the oocytes and are therefore considered to be aquaporins (for reviews, see Maurel, 1997; Schäffner, 1998). Nearly all of these proteins belong to the plasma membrane PIP2 subfamily (Arabidopsis PIP2a, PIP2b, PIP2c, RD28, and PIP3; and spinach PM28A), and they increased the Pf of the oocyte membrane 5- to 20-fold over the control value (Daniels et al., 1994; Kammerloher et al., 1994; Weig et al., 1997; Johansson et al., 1998). In the same way, maize ZmPIP2a clustering with the PIP2 subfamily on a dendogram increased the Pf of *X. laevis* oocyte membranes 8-fold; $HgCl_2$, a well-known aquaporin blocker, reversibly inhibited its water channel activity (Fig. 3).

When tested in oocytes, members of the PIP1 family have given quite different results. *Mesembryanthemum crystallinum* MipA and MipB proteins and *N. tabacum* NtAQP1 induced only a 2-fold increase in Pf (Yamada et al., 1995; Biela et al., 1999). We observed no change in the Pf of the oocytes that express ZmPIP1a and 1b (Fig. 3). Interestingly, other proteins belonging to the plant PIP1 subfamily also did not show any water channel activity in *X. laevis* oocytes. Spinach PM28B, identified in leaf plasma membranes (Johansson et al., 1996), and the *Brassica campestris* MOD protein, postulated to be involved in the self-incompatibility response (Ikeda et al., 1997), did not significantly modify the Pf of injected oocyte (I. Johansson, P. Kjellbom, and M.J. Chrispeels, unpublished data; R. Dixit, M.E. Nasrallah, and M.J. Chrispeels, unpublished data). Because these are negative data, we have not reported them previously. Nevertheless, the first PIP1 members to be identified (Arabidopsis PIP1a, PIP1b, and PIP1c) increased the water membrane permeability of *X. laevis* oocytes 5- to 8-fold (Kammerloher et al., 1994). These Pf values are somewhat smaller than the Pf induced by Arabidopsis PIP2 proteins (11- to 20- fold Pf increase over the control) (Kammerloher et al., 1994). Thus, most but not all members of the PIP1 family are inactive in *X. laevis* oocytes. Could we be using the wrong heterologous system?

In addition to *X. laevis* oocytes, other heterologous systems have been used to demonstrate aquaporin activity of PIPs. We previously showed that expression of Arabidopsis plasma membrane AtRD28 protein, which belongs to the PIP2 family, in *Dictyostelium discoideum* cells resulted in sensitivity of the cells to hypoosmotic shock, causing the cells to burst (Chaumont et al., 1997). However, *D. discoideum* cells expressing ZmPIP1a were insensitive to low-osmotic-strength buffer, confirming the functional data obtained with *X. laevis* oocytes (F. Chaumont and M.J. Chrispeels, unpublished data).

Possible Causes of the Inactivity of PIP1 Proteins

The lack of swelling response was not due to any failure to express both proteins or to target them properly to the plasma membrane, as was previously reported with several animal aquaporins (Mulders et al., 1997; Bonhivers et al., 1998). ZmPIP1a and ZmPIP1b proteins were indeed synthesized in *X. laevis* oocytes and targeted to the plasma membrane, as indicated by subcellular fractionation experiments (Fig. 4). In addition, the fluorescence distribution in oocytes expressing ZmPIP1b-GFP confirmed the presence of the chimeric protein in the plasma membrane (data not shown).

The failure to detect any water channel activity associated with ZmPIP1a and ZmPIP1b could indicate either that both proteins are transporters specific for other substrates or that they need to be positively regulated. Bean α-TIP and spinach PM28A aquaporins were shown to be regulated by phosphorylation (Maurel et al., 1995; Johansson et al., 1998). Treatment of ZmPIP1b-expressing oocytes with cAMP agonists or phosphatase inhibitors did not alter the swelling responses. Functional testing defines three groups of MIP proteins. Some of them transport only water, others transport small solutes and water, and still others transport only small neutral solutes. In plants, three MIP proteins have recently been identified as aquaglyceroporins: tobacco NtTIPa and NtAQP1 and soybean NOD26 transport glycerol and water (Rivers et al., 1997; Biela et al., 1999; Dean et al., 1999; Gerbeau et al., 1999). However, like bovine lens AQP0, NOD26 expressed in oocytes or functionally reconstituted in liposomes showed a water transport activity 10- to 50- fold lower than mammalian AQP1–5

(Chandy et al., 1997; Rivers et al., 1997; Dean et al., 1999) and resembling the water channel activities of some PIP1 proteins (see above). No plant MIP protein transporting exclusively small neutral solutes has been identified so far. ZmPIP1a and ZmPIP1b might be members of this group, but assays with different labeled solutes to determine the channel specificity were unsuccessful. Finally, voltage clamp experiments with ZmPIP1a and ZmPIP1b cRNA-injected oocytes indicate that neither protein transports ions (S. Thomine and F. Chaumont, unpublished data).

Amino acid sequence comparisons of plant PIPs belonging to PIP1 and PIP2 subfamilies allowed us to identify some amino acid residues specific for each subfamily. Directed mutagenesis could perhaps be used to switch channel selectivity. It was recently demonstrated that two amino acid substitutions in an aquaporin led to a switch in the selectivity of the channel from water to glycerol, and this process may be related to the ability of the subunits to form oligomers (Lagrée et al., 1999). Interestingly, red beet plasma membranes contain two prominent MIP proteins that belong to the PIP1 and PIP2 subfamilies (BvMIP2 and BvMIP3, Fig. 2) and exhibit distinct biochemical and topological properties (Barone et al., 1998). The possibility that these differences are correlated with different channel specificities has yet to be determined.

Subcellular Localization

Amino acid sequence comparison of ZmPIPs with MIP homologs of known localization (such as Arabidopsis RD28; Daniels et al., 1994) allowed us to assign these maize PIPs to the plasma membrane. Our assignment was partially confirmed by the fluorescence distribution of ZmPIP1b::sGFP(S65T) in tobacco plants. In addition to the plasma membrane surrounding the cell, green fluorescence was detected in the perinuclear region and filaments extending from the nucleus to the plasma membrane. We suggest that ZmPIP1b::sGFP(S65T) is associated with organelles of the secretory pathway. We selected transgenic tobacco plants with a high fluorescence signal for confocal microscopy observations; the fusion protein might be overproduced and therefore saturate the secretory pathway, leading to a non-specific green fluorescence labeling. However, recent subcellular fractionation analysis of *M. crystallinum* PIPs suggests a complex location and a possible association with different endomembranes (Barkla et al., 1999). In the same way, Arabidopsis PIP1 subfamily proteins were demonstrated to be present in plasma membrane invaginations called plasmalemmasomes (Robinson et al., 1996). These data suggest a possible mechanism for trafficking regulation through intracellular vesicles, as observed for mammalian AQP2 aquaporin (for review, see Agre et al., 1998). The use of specific antibodies against ZmPIP1b and ZmPIP2a will help to prove this hypothesis.

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