FRET imaging in living maize cells reveals that plasma membrane aquaporins interact to regulate their subcellular localization

Enric Zelazny*, Jan Willem Borst†, Mélanie Muylaert*, Henri Batoko*, Marcus A. Hemminga‡, and François Chaumont*§

*Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 5-15, B-1348 Louvain-la-Neuve, Belgium; and †Laboratory of Biochemistry and ‡Laboratory of Biophysics, MicroSpectroscopy Centre, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Edited by Maarten J. Chrispeels, University of California at San Diego, La Jolla, CA, and approved June 13, 2007 (received for review February 8, 2007)

***Zea mays* plasma membrane intrinsic proteins (ZmPIPs) fall into two groups, ZmPIP1s and ZmPIP2s, that exhibit different water channel activities when expressed in *Xenopus* oocytes. ZmPIP1s are inactive, whereas ZmPIP2s induce a marked increase in the membrane osmotic water permeability coefficient, P_f. We previously showed that, in *Xenopus* oocytes, ZmPIP1;2 and ZmPIP2;1 interact to increase the cell P_f. Here, we report the localization and interaction of ZmPIP1s and ZmPIP2s in living maize cells. ZmPIPs were fused to monomeric yellow fluorescent protein and/or monomeric cyan fluorescent protein and expressed transiently in maize mesophyll protoplasts. When expressed alone, ZmPIP1 fusion proteins were retained in the endoplasmic reticulum, whereas ZmPIP2s were found in the plasma membrane. Interestingly, when coexpressed with ZmPIP2s, ZmPIP1s were relocalized to the plasma membrane. Using FRET/fluorescence lifetime imaging microscopy, we demonstrated that this relocalization results from interaction between ZmPIP1s and ZmPIP2s. Immunoprecipitation experiments provided additional evidence for the association of ZmPIP1;2 and ZmPIP2;1 in maize roots and suspension cells. These data suggest that PIP1–PIP2 interaction is required for *in planta* PIP1 trafficking to the plasma membrane to modulate plasma membrane permeability.**

protein interaction | trafficking | *Zea mays* plasma membrane intrinsic protein

Movement of water across cellular membranes is facilitated by the presence of water channels named aquaporins (AQPs). These proteins are composed of six transmembrane domains linked by five loops, the N and C termini being cytosolic. Three-dimensional structures of archaeon AqpM (1), bacterial AqpZ (2, 3), mammalian AQP0, AQP1, AQP2, and AQP4 (4–8), and *Spinacia oleracea* plasma membrane intrinsic protein 2;1 (SoPIP2;1) (9, 10) have been obtained, and all show that AQPs form homotetramers in the membrane, each monomer being an independent water channel. In plants, homotetramerization might not be the only way AQPs assemble, because interaction between different plasma membrane and tonoplast AQPs has been reported (11–13).

In plants, plasma membrane intrinsic proteins (PIPs) are divided into two sequence-related groups, PIP1 and PIP2, the members of which exhibit different water channel activities when expressed in *Xenopus* oocytes (for review, see ref. 14). Of the 13 currently known maize PIPs, six are PIP1s and seven are PIP2s (15). When expressed alone in *Xenopus* oocytes, *Zea mays* PIP1s (ZmPIP1s) are inactive, whereas ZmPIP2s cause a marked increase in the osmotic water permeability coefficient, P_f (16). When coexpressed, ZmPIP1;2 and ZmPIP2;1 or ZmPIP2;5 interact *in vivo*, leading to an increase in P_f compared with oocytes expressing ZmPIP2s alone (12). In addition, coexpression of ZmPIP2s with ZmPIP1;2 fused to green fluorescent protein (GFP) improves the targeting of ZmPIP1;2 to the plasma membrane and/or its stability in the membrane (12). However, ZmPIP1;1, which shares 97% identity with ZmPIP1;2, does not induce any increase in the P_f of the oocyte plasma membrane when coexpressed with ZmPIP2;5 (12), indicating either that the two proteins do not interact or that the resulting interaction does not modify the cell P_f. Interaction between different plasma membrane AQPs is not restricted to maize, because *Mimosa pudica* PIP1;1 (MpPIP1;1) and MpPIP2;1 also interact when coexpressed in COS cells and increase the oocyte P_f (13).

According to these results, it seems that interaction between PIP1s and PIP2s could be a means of regulating their activity, as observed for several other membrane proteins for which multimerization is an important regulation mechanism (17–19). Because this interaction of plant PIP1s and PIP2s was observed in heterologous expression systems (*Xenopus* oocytes or COS cells) with different water requirements and protein regulations, it is essential to determine whether such interactions occur in plant cells.

An elegant and powerful means of demonstrating protein–protein interactions in living cells is based on FRET (20, 21). FRET is a process in which energy from an excited donor molecule is transferred to an acceptor molecule when the donor fluorescence overlaps with the absorption spectrum of the acceptor and when both molecules are in close proximity (between 2 and 10 nm). FRET can be quantified by recording the decrease in the fluorescence lifetime, τ, of the donor by using fluorescence lifetime imaging microscopy (FLIM) (22, 23). A comparison of the τ value for the donor fluorophore alone with the τ value in the presence of the acceptor molecule makes it possible to tell whether the two proteins interact.

Here, various ZmPIP1 and ZmPIP2 isoforms were fused to the monomeric cyan fluorescent protein (mCFP) and monomeric yellow fluorescent protein (mYFP), respectively the donor and acceptor molecules, that have been used successfully for *in planta* FRET studies (24–26). The fusion proteins were transiently expressed in maize mesophyll protoplasts. We report here that, in contrast to ZmPIP2s, ZmPIP1s expressed alone in protoplasts were not targeted to the plasma membrane but were retained intracellularly. However, when ZmPIP1s and ZmPIP2s were coexpressed, FRET/FLIM analysis showed that they interacted, resulting in ZmPIP1 relocalization to the plasma membrane. In addition, evidence for interaction in maize roots and suspension cells was obtained in immunoprecipitation experiments by using isoform-specific antibodies.

Author contributions: E.Z., J.W.B., H.B., M.A.H., and F.C. designed research; E.Z. and M.M. performed research; E.Z. and J.W.B. contributed new reagents/analytic tools; E.Z., J.W.B., M.M., H.B., M.A.H., and F.C. analyzed data; and E.Z., M.A.H., and F.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: AQP, aquaporin; PIP, plasma membrane intrinsic protein; FLIM, fluorescence lifetime imaging microscopy; mCFP, monomeric cyan fluorescent protein; mYFP, monomeric yellow fluorescent protein; ER, endoplasmic reticulum; BMS, Black Mexican Sweet.

§To whom correspondence should be addressed. E-mail: chaumont@fysa.ucl.ac.be.

This article contains supporting information online at www.pnas.org/cgi/content/full/0701180104/DC1.

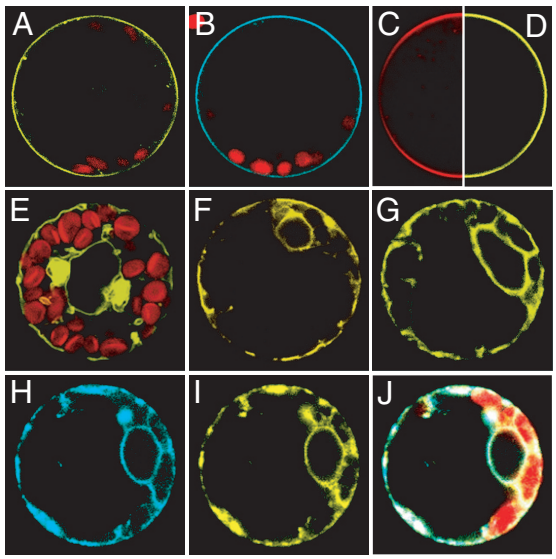

Fig. 1. Subcellular localization of ZmPIPs fused to mYFP or mCFP in maize cells. (*A–G*) Maize mesophyll protoplasts transiently expressing mYFP::ZmPIP2;1 (*A*), mCFP::ZmPIP2;5 (*B*), ROP6::YFP (*D*), mYFP::ZmPIP1;2 (*E*), mYFP::ZmPIP1;1 (*F*), or mYFP::ZmPIP1;6 (*G*). In *C*, the protoplast plasma membrane was labeled with the FM4-64 steryl dye. (*H–J*) Coexpression of mCFP::ZmPIP1;2 with the ER marker, YFP::HDEL. (*H*) mCFP::ZmPIP1;2 fluorescence. (*I*) YFP::HDEL fluorescence. (*J*) Merged images. Chlorophyll autofluorescence (red) in chloroplasts is seen in *A*, *B*, *E*, and *J*. The diameters of the maize mesophyll protoplasts ranged from 25 to 35 μm.

Results

In Contrast to ZmPIP2s, ZmPIP1s, When Expressed Alone, Are Not Targeted to the Plasma Membrane of Maize Mesophyll Protoplasts. ZmPIP1;1, ZmPIP1;2, ZmPIP1;6, ZmPIP2;1, and ZmPIP2;5 were fused to the C terminus of mCFP and/or mYFP. mCFP and mYFP have an A206K mutation that abolishes the tendency of these fluorophores to dimerize when present at high concentration and especially when confined in two dimensions close to a membrane (27). This feature is crucial in preventing fluorophore aggregation-dependent interactions during FRET/FLIM experiments. The fusion of ZmPIPs with GFP does not affect the activity of the ZmPIPs or their ability to interact (12).

The subcellular localization of each ZmPIP fusion protein was determined in transfected maize protoplasts by using confocal microscopy (Fig. 1). Cells transiently expressing mYFP::ZmPIP2;1 or mCFP::ZmPIP2;5 showed sharp fluorescent signals at the plasma membrane (Fig. 1 *A* and *B*), as confirmed by colocalization with the membrane marker, FM4-64 (Fig. 1*C*, red fluorescence). mYFP::ZmPIP2;1-derived fluorescence was occasionally detected in punctate structures in the vicinity of the plasma membrane (Fig. 1*A*) and, more commonly, around the nucleus, probably corresponding to proteins transiting the endoplasmic reticulum (ER) en route to the plasma membrane (data not shown). In contrast, mYFP::ZmPIP1;1, mYFP::ZmPIP1;2, and mYFP::ZmPIP1;6 (the most divergent of the ZmPIP1 isoforms) were not found in the plasma membrane, but in internal structures around the nucleus, within the cytosol, and near the plasma membrane (Fig. 1 *E–G*). Similar results were obtained for ZmPIP1;3 (data not shown). The lack of plasma membrane targeting of PIP1 fusion proteins was not a consequence of overexpression, because the patterns described above were seen even when the overall protoplast fluorescence signal was quite weak (data not shown). To identify the internal structures containing ZmPIP1 fusion proteins, mCFP::ZmPIP1;2 was coexpressed in maize protoplasts with the YFP::HDEL protein, an ER marker (28, 29). As shown in Fig. 1 *H–J*, mCFP::ZmPIP1;2 and YFP::HDEL colocalized, demonstrating that mCFP::ZmPIP1;2 was retained in the ER of maize protoplasts.

Coexpression of ZmPIP2s and ZmPIP1s Results in Relocalization of ZmPIP1s to the Plasma Membrane. We previously showed that, in *Xenopus* oocytes, ZmPIP1;2 and ZmPIP2s interact to control the amount and/or stability of ZmPIP1;2 in the plasma membrane (12). We therefore investigated the localization of ZmPIP1 fusion proteins in maize protoplasts when coexpressed with ZmPIP2s. Interestingly, when mCFP::ZmPIP2;1 was present, mYFP::ZmPIP1;1, mYFP::ZmPIP1;2, or mYFP::ZmPIP1;6 was found in the cell plasma membrane, like mCFP::ZmPIP2;1 (Fig. 2 *A–I*). As shown in the merged images (Fig. 2 *Bottom*), the mYFP::ZmPIP1s were perfectly colocalized with mCFP::ZmPIP2;1. Similar results were obtained with ZmPIP1;3 (data not shown), indicating that PIP1 relocalization to the plasma membrane in the presence of ZmPIP2;1 is an isoform-independent mechanism. Similarly, coexpression of other ZmPIP2s with any of the ZmPIP1s tested resulted in plasma membrane localization of the latter. For instance, ZmPIP2;5, which shares 82% identity with ZmPIP2;1, induced the relocalization of mYFP::ZmPIP1;1 to the plasma membrane (Fig. 2 *J–L*). Several unidentified structures in the vicinity of the plasma membrane containing only one type of fluorophore were also visible (Fig. 2*C Inset*).

Finally, we investigated the localization of mCFP::ZmPIP1;2 and mYFP::ZmPIP1;1 when coexpressed in mesophyll protoplasts. Both proteins were found in intracellular organelles, most probably the ER, as shown above for mCFP::ZmPIP1;2 alone, and not in the plasma membrane (Fig. 2 *M–O*). This result emphasizes the fact that ZmPIP2s are required for ZmPIP1 routing to the plasma membrane and that coexpression of two different ZmPIP1s does not trigger this process.

Relocalization of ZmPIP1s Is a Consequence of Physical Interaction with ZmPIP2s. The subcellular relocalization of ZmPIP1s and their colocalization with ZmPIP2s suggested that these proteins might interact. Evidence for a physical interaction was obtained by using FRET/FLIM analyses of mesophyll protoplasts expressing the fusion proteins described above. In these experiments, the fluorescence lifetime τ of the donor mCFP-labeled ZmPIP was measured in the absence or presence of the acceptor molecule, and a molecular interaction between the two was detected as a decrease in the τ of the donor molecule, which is depicted as a false color code image (Fig. 3).

We first calculated τ for mCFP-labeled ZmPIP2;1 in the absence of the acceptor molecule, mYFP. In the FRET/FLIM analyses presented in Fig. 3, τ is visualized as a color code from dark blue (τ of $\approx$2.4 ns) to yellow (τ of $\approx$1.5 ns). mCFP::ZmPIP2;1 alone was found in the plasma membrane, with a τ of 2.36 ns (Fig. 3*A* and Table 1). As a control for the interaction between ZmPIPs, we coexpressed ZmPIP2;1 fused separately to mCFP and to mYFP, which should interact within a homotetramer, as reported for spinach SoPIP2;1, which shares 73% identity with ZmPIP2;1 (9, 10). In the presence of mYFP::ZmPIP2;1, τ for mCFP::ZmPIP2;1 decreased markedly to 1.77 ns (Table 1), as shown by a change in plasma membrane color from dark blue to green (Fig. 3 *A* and *B*), demonstrating that ZmPIP2;1 subunits interact in living maize cells probably within homotetramers (see *Discussion*). As a negative control, when mCFP::ZmPIP2;1 was coexpressed with YFP fused to ROP6, a GTPase protein localized in the plasma membrane (Fig. 1*D*) (30), no significant decrease in τ was seen (2.27 ns) (Fig. 3*C* and Table 1), showing that the measured interaction is dependent on the presence of a cognate protein and not on the anchoring of the acceptor fluorophore to the plasma membrane.

The interactions between mCFP::ZmPIP2;1 and mYFP::ZmPIP1;1, mYFP::ZmPIP1;2, or mYFP::ZmPIP1;6 were then analyzed. When mCFP::ZmPIP2;1 was coexpressed

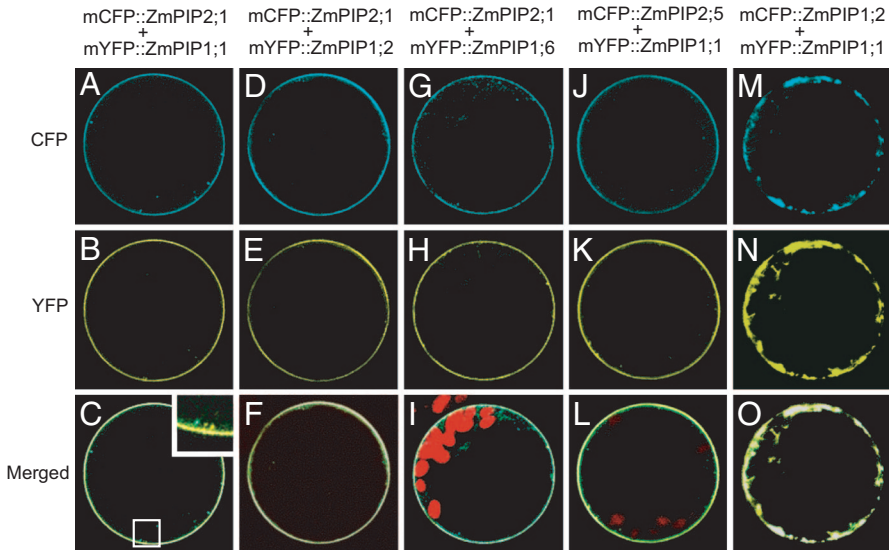

Fig. 2. The subcellular localization of mYFP::ZmPIP1s is modified by coexpression of mCFP::ZmPIP2s. Protoplasts coexpressing mCFP::ZmPIP2;1 and mYFP::ZmPIP1;1 (*A–C*); mCFP::ZmPIP2;1 and mYFP::ZmPIP1;2 (*D–F*); mCFP::ZmPIP2;1 and YFPm::ZmPIP1;6 (*G–I*); mCFP::ZmPIP2;5 and mYFP::ZmPIP1;1 (*J–L*); or mCFP::ZmPIP1;2 and mYFP::ZmPIP1;1 (*M–O*). The CFP channel (*Top*), the YFP channel (*Middle*), and the superimposed image for the two channels (*Bottom*) are shown. Chlorophyll autofluorescence (red) from chloroplasts can be seen in *I* and *L*. (*C Inset*) Magnified image showing unidentified structures in the vicinity of the plasma membrane containing only one type of fluorophore.

with mYFP-labeled ZmPIP1;1 or ZmPIP1;2, both proteins were found in the plasma membrane, and a strong decrease in τ from 2.36 to 1.58 ns was seen (Fig. 3 *D* and *E* and Table 1), demonstrating physical interaction between the isoforms. As shown in Fig. 3*F* and Table 1, mYFP::ZmPIP1;6 also interacted with mCFP::ZmPIP2;1 in the plasma membrane, with a reduction in τ from 2.36 to 1.70 ns. These results suggest that the relocalization of ZmPIP1s to the plasma membrane results from interaction with ZmPIP2;1.

To investigate whether ZmPIP1s interact with ZmPIP2s other than ZmPIP2;1, FRET/FLIM analysis was performed on protoplasts coexpressing mCFP::ZmPIP2;5 and mYFP::ZmPIP1;1 (Fig. 3*H*). As compared with mCFP::ZmPIP2;5 expressed alone (Fig. 3*G*), a strong reduction in τ from 2.39 to 1.51 ns was observed (Table 1) in the plasma membrane, resulting in the highest FRET efficiency (37%) recorded with any of the PIP combinations. A similar interaction was detected between mCFP::ZmPIP2;5 and mYFP::ZmPIP1;2, again allowing the latter to be targeted to the plasma membrane (data not shown).

ZmPIP1;2 Assembles as Homomers in the ER and Interacts with ZmPIP1;1 When Coexpressed. The data above suggest that, in maize mesophyll cells, mYFP::ZmPIP1;2 is present in the ER when expressed alone and in the plasma membrane after its interaction with ZmPIP2s. We wondered whether the failure of ZmPIP1;2 to be transported to the plasma membrane could be due to its failure to associate as homotetramers, as reported previously for silent voltage-gated K^+ channels in mammals (17). However, FRET/FLIM studies on maize protoplasts showed that coexpression of mCFP::ZmPIP1;2 and mYFP::ZmPIP1;2 induced a reduction in τ from 2.38 to 1.94 ns compared with mCFP::ZmPIP1;2 alone (Fig. 3*J* and Table 1), showing that ZmPIP1;2 existed as homomers within the maize cell ER.

FRET/FLIM was then performed on protoplasts coexpressing mCFP::ZmPIP1;2 and mYFP::ZmPIP1;1 to test whether ZmPIP1;2 and ZmPIP1;1, which were retained in the ER (Fig. 2 *M–O*), interacted. A significant decrease in τ for mCFP::ZmPIP1;2 from 2.38 to 1.90 ns was seen in the presence of mYFP::ZmPIP1;1 (Fig. 3*K* and Table 1), indicating that an interaction between ZmPIP1;1 and ZmPIP1;2 occurred in the ER but did not result in the transport of the complex to the plasma membrane.

ZmPIP1;2 and ZmPIP2;1 Are Coimmunopurified from Root and Suspension Cells. The physical interaction between ZmPIP1;2 and ZmPIP2;1 in maize cells was also demonstrated by coimmunopurification experiments using octyl-β-D-thioglucopyranoside-solubilized plasma membrane proteins from maize roots and Black Mexican Sweet (BMS) suspension cultured cells. As shown in Fig. 4, when ZmPIP2;1 was precipitated by using anti-ZmPIP2;1 antibodies (31) and Protein A Sepharose beads, ZmPIP1;2 was coprecipitated, showing that these isoforms interact in maize plasma membranes under physiological conditions. The negative control, H^+ ATPase, was not found in the precipitated sample (Fig. 4 *A* and *C*). In a reverse experiment, ZmPIP2;1 was coimmunopurified by using ZmPIP1;2 antibodies, confirming the interaction between the proteins [see supporting information (SI) Fig. 5].

Discussion

Plant PIP1s and PIP2s differ not only in the length of their N and C termini but also in terms of their water channel activity when expressed in *Xenopus* oocytes (12, 16). Whereas PIP1s are generally silent in this heterologous host, PIP2s show high water channel activity. Recent data obtained in this heterologous expression system indicate that ZmPIP1;2 interacts with ZmPIP2s, resulting in an increase in the amount of ZmPIP1;2 in the plasma membrane and also in the membrane osmotic water permeability (12). Here, we report the localization and interaction of PIP1s and PIP2s in maize cells.

When transiently expressed as translational mCFP or mYFP fusions in maize mesophyll protoplasts, all of the tested ZmPIP1 isoforms were retained in the ER (Fig. 1), indicating a conserved mechanism for retention. This localization could be due to ER retention signals, a lack of export signals, and/or a requirement for prior assembly. The addition of the fluorophore to the N terminus of the PIP1s might have interfered with trafficking. This is unlikely because (*i*) similar ER retention was seen when GFP was fused to the C terminus of ZmPIP1;2 and expressed in maize mesophyll cells (data not shown) and (*ii*) ZmPIP2;5 and ZmPIP2;1 fused, respectively, to mCFP and mYFP were localized to the plasma membrane

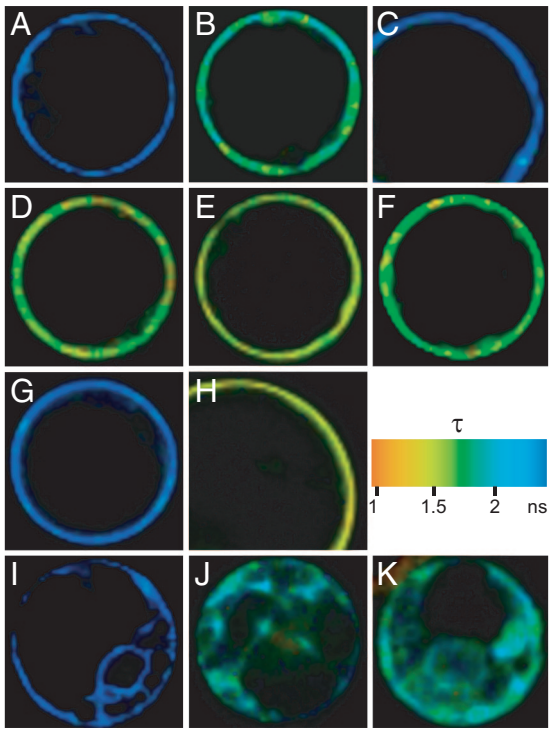

Fig. 3. FRET/FLIM analyses of maize protoplasts transiently expressing different combinations of tagged ZmPIP1s and ZmPIP2s. (*A–F*) mCFP::ZmPIP2;1 alone (*A*) or coexpressed with mYFP::ZmPIP2;1 (*B*), ROP6::YFP (negative control) (*C*), mYFP::ZmPIP1;1 (*D*), mYFP::ZmPIP1;2 (*E*), or mYFP::ZmPIP1;6 (*F*). (*G* and *H*) mCFP::ZmPIP2;5 alone (*G*) or coexpressed with mYFP::ZmPIP1;1 (*H*). (*I–K*) mCFP::ZmPIP1;2 alone (*I*) or coexpressed with mYFP::ZmPIP1;2 (*J*) or mYFP::ZmPIP1;1 (*K*). The donor fluorescence lifetime τ was calculated as described in *Methods* and is indicated by a color code from red for τ values of $\approx$1 ns to blue for τ values of $>$2 ns.

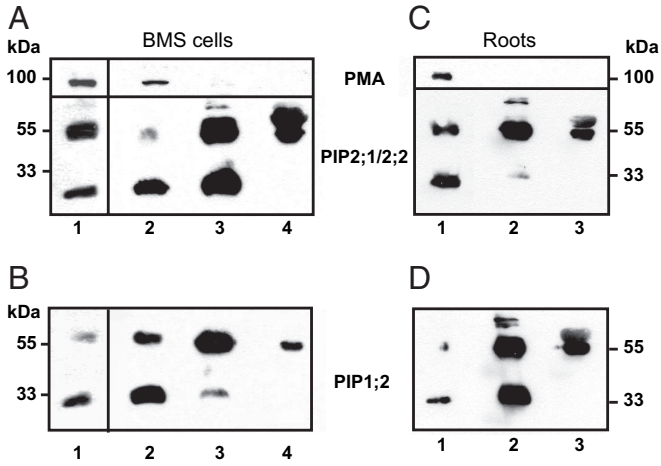

Fig. 4. Coimmunopurification of ZmPIP1;2 and ZmPIP2;1 from maize BMS cells and roots. Proteins from BMS suspension cell plasma membranes (*A* and *B*) or maize root plasma membranes (*C* and *D*) were solubilized and subjected to immunoprecipitation with anti-ZmPIP2;1 antiserum and Protein A Sepharose as described in *Methods*, and different fractions were analyzed by using anti-ZmPIP2;1/2;2 (*A* and *C*) or anti-ZmPIP1;2 (*B* and *D*) antibodies. Monomer and dimer forms of each protein are shown (at $\approx$29 kDa and 55 kDa, respectively). (*A* and *B*) Lanes: 1, purified plasma membranes; 2, solubilized proteins; 3, immunoprecipitated proteins; and 4, anti-ZmPIP2;1 antibodies (negative control). (*C* and *D*) Lanes: 1, solubilized proteins; 2, immunoprecipitated proteins; and 3, anti-ZmPIP2;1 antibodies. (*A* and *C Upper*) H^+ ATPase (PMA) was used as a negative control for immunoprecipitation and interaction. The positions of the molecular mass markers are indicated on the *y* axis.

(Fig. 1), suggesting that the presence of the fluorophore does not affect PIP trafficking. When transiently coexpressed with ZmPIP2s in maize cells, ZmPIP1s were routed to the plasma membrane and perfectly colocalized with the PIP2s (Fig. 2). Similar protein relocalization from the ER or Golgi apparatus to the plasma membrane as a consequence of a heteromerization process has been reported in mammalian and plant cells for different channels or receptors (17, 32, 33), but not for AQPs.

The plasma membrane relocalization of ZmPIP1s is a result of their interaction with ZmPIP2s, as demonstrated by using FRET/FLIM, which makes it possible to study interactions with a high spatial and temporal resolution (Fig. 3) (23). Using this method, we showed that ZmPIP1;1, ZmPIP1;2, or ZmPIP1;6 physically interacted with ZmPIP2;1 and/or ZmPIP2;5 in the plasma membrane. Because AQPs assemble in tetramers in biological membranes (1–6, 9, 10), the most obvious way to interpret the FRET data is to assume that ZmPIP1s and ZmPIP2s assemble as heterotetramers. However, we cannot exclude the possibility that ZmPIP1 and ZmPIP2 homotetramers interact to form larger packed complexes, leading to the targeting of ZmPIP1 homotetramers to the plasma membrane. Interaction between AQP0 tetramers has been reported in lens cells, but, in this case, it consists in interaction between AQP0 molecules from adjoining membranes, forming thin junctions between fiber cells (34, 35). Hereafter, the term "heteromer" will be used to define a complex formed by interactions

Table 1. Fluorescence lifetime τ and FRET efficiency for the ZmPIP interaction studies

Protein	$\tau \pm$ SD, ns	FRET efficiency, %	No. of protoplasts analyzed
mCFP::ZmPIP2;1	2.36 ± 0.02	—	47
mCFP::ZmPIP2;5	2.39 ± 0.02	—	15
mCFP::ZmPIP1;2	2.38 ± 0.04	—	23
mCFP::ZmPIP2;1 + ROP6::YFP	2.27 ± 0.07	—	30
mCFP::ZmPIP2;1 + mYFP::ZmPIP2;1	1.77 ± 0.06	25	25
mCFP::ZmPIP2;1 + mYFP::ZmPIP1;1	1.58 ± 0.05	33	33
mCFP::ZmPIP2;1 + mYFP::ZmPIP1;2	1.58 ± 0.08	33	29
mCFP::ZmPIP2;1 + mYFP::ZmPIP1;6	1.70 ± 0.07	28	28
mCFP::ZmPIP2;5 + mYFP::ZmPIP1;1	1.51 ± 0.08	37	29
mCFP::ZmPIP1;2 + mYFP::ZmPIP1;1	1.90 ± 0.09	20	22
mCFP::ZmPIP1;2 + mYFP::ZmPIP1;2	1.94 ± 0.04	18	22

For each analysis, an average value of τ and the FRET efficiency was determined as described in *Methods*. — indicates no FRET.

between different ZmPIPs either within a heterotetramer or between homotetramers.

Among the four PIP1–PIP2 combinations tested by FRET/FLIM analysis, the FRET efficiency, *E*, which decreases with increased distance between the donor and acceptor fluorophores, was lowest for mCFP::ZmPIP2;1/mYFP::ZmPIP1;6 (Table 1). This may be due to the longer N terminus (six extra amino acids) in ZmPIP1;6 compared with those in ZmPIP1;1 and ZmPIP1;2, which could increase the distance between the two fluorophores. Alternatively, ZmPIP2s may interact preferentially with ZmPIP1;1 and ZmPIP1;2. Even lower FRET efficiencies were obtained with the combinations of mCFP::ZmPIP2;1/mYFP::ZmPIP2;1, mCFP::ZmPIP1;2/mYFP::ZmPIP1;2, and mCFP::ZmPIP1;2/mYFP::ZmPIP1;1, suggesting either that the distance between the fluorophores may be affected by PIP conformation, association, and packing or that there is a higher donor homomer to heteromer ratio. Although the reduction in τ in the plasma membrane was fairly homogeneous, some patches with different τ values were observed. This phenomenon could also be due to the presence in the measured area of some donor homomers that did not lead to any FRET.

Because ZmPIP1;2 subunits interact in the ER to probably form homotetramers (Fig. 3), the lack of targeting to the plasma membrane is not due to a lack of oligomerization of this protein but to the presence of potential exposed retention signals and/or the lack of export signals. Interaction between ZmPIP1;2 and ZmPIP1;1 was also observed in the ER, without either isoform being relocalized (Fig. 3*K*), suggesting that interaction with ZmPIP2s is required for ZmPIP1s to be targeted to the plasma membrane. Association with ZmPIP2s might mask the retention signals or provide an export signal, allowing the channels to leave the ER and reach the plasma membrane. Construction of chimeric proteins between ZmPIP1s and ZmPIP2s and analysis of their subcellular localization will allow the domain(s) containing trafficking information to be identified.

In maize protoplasts, endogenous AQPs could interact with transiently expressed ZmPIPs, creating a competitive effect that could potentially modify the FRET measurements. However, when expressed alone, ZmPIP1 fusion proteins were not targeted to the plasma membrane, suggesting that endogenous ZmPIP2s were very lowly expressed. To confirm this hypothesis, we quantified the mRNA of the 13 endogenous *ZmPIP*s in mesophyll protoplasts by quantitative RT-PCR and observed a very low expression level of all of the genes tested. Comparatively, values obtained, for instance, in the mature zones of maize leaves or roots were substantially higher (C. Hachez, R. Heinen, E.Z., and F.C., unpublished data and ref. 31). Therefore, the low expression level of endogenous ZmPIPs would have marginal interference in the interaction study in the presence of overexpressed mCFP- or mYFP-labeled ZmPIPs.

Because interaction between ZmPIP1;2 and ZmPIP2;1 in *Xenopus* oocytes results in an increase in P_f (12), we investigated whether this also occurred in protoplasts transiently coexpressing both proteins using the protoplast swelling assay described previously (36, 37). Although the expression of mCFP::ZmPIP2;1 in protoplasts induced an increase in P_f compared with that in untransformed cells or cells expressing mYFP::ZmPIP1;2, we obtained very variable data for protoplasts coexpressing both fusion proteins (data not shown). This observation could be due to (*i*) the protoplast transient expression approach in which, in contrast to the oocyte system, the level of coexpressed proteins varies from cell to cell; (*ii*) the counterselection of protoplasts with a high PIP activity in the plasma membrane; or (*iii*) potential multiple posttranslational mechanisms regulating AQP activity in plant cells (reviewed in refs. 14 and 38).

Experiments in *Xenopus* oocytes showed that coexpression of ZmPIP2;5 and ZmPIP1;1 does not lead to an increase in membrane P_f, whereas replacing the extracellular loop E of ZmPIP1;1 with that of ZmPIP1;2 resulted in a functional interaction with ZmPIP2;5 as detected by a significant P_f increase (12). It was concluded that ZmPIP1;1 and ZmPIP2;5 do not interact functionally in *Xenopus* oocytes and that loop E plays an important role in ZmPIP1–ZmPIP2 interactions (12). However, FRET/FLIM analysis in living maize cells demonstrated that ZmPIP2;5 interacts with ZmPIP1;1, allowing the routing of the latter to the plasma membrane. A possible explanation for this apparent discrepancy between *Xenopus* oocytes and plant cells could be that ZmPIP1;1 and ZmPIP2;5 expressed in oocytes do physically interact, but this heteromerization does not lead to a P_f increase because of the fact that ZmPIP1;1 is not functional (unless the ZmPIP1;1 loop E is swapped with that of ZmPIP1;2). Molecular dynamics simulation of the structure of ZmPIP1;1 containing loop E of ZmPIP1;2 indicated that the position of the loop is changed, affecting the structure of the pore and possibly the channel specificity (14). Another difference between the results in oocytes and maize cells is seen on coexpression of ZmPIP1;1 and ZmPIP1;2, which resulted in an increase in membrane P_f in oocytes (12), whereas, in maize protoplasts, such a synergetic effect is not expected, as neither ZmPIP1;1 nor ZmPIP1;2 was detected in the plasma membrane after coexpression.

Importantly, in addition to the PIP interactions observed in maize protoplasts coexpressing the tagged isoforms, immunoprecipitation experiments using root and cultured cell-derived plasma membranes showed that ZmPIP1s and ZmPIP2s interacted *in vivo* in their native environment in the absence of any overexpression. This observation is in accordance with expression data showing that different ZmPIP1 and ZmPIP2 mRNAs and proteins are expressed in the same cell type in normal roots (12, 31) and nontransfected BMS cells (M. Moshelion, C. Hachez, and F.C., unpublished data). The physiological implication of these interactions in plant cells remains to be demonstrated, but this process could possibly increase the plasma membrane P_f, as seen for ZmPIP1;2/ZmPIP2;1 or ZmPIP1;2/ZmPIP2;5 in *Xenopus* oocytes (12). In addition, we cannot exclude the possibility that heteromerization increases the plasma membrane permeability to other solutes, depending on the channel specificity of the PIP1 isoforms. For instance, *Nicotiana tabacum* AQP1 (NtAQP1), a PIP1, facilitates the transport of glycerol and CO_2 (39, 40).

Our data showed that ZmPIP1s need to interact with ZmPIP2s to be targeted to the plasma membrane, providing the cells with an additional mechanism for regulating membrane permeability. The different combinations of PIP1–PIP2 heteromers in specific cell types or in response to different environmental cues would greatly increase the diversity of channel activity in the membrane. This mechanism may be interconnected with other posttranslational modifications of plant AQPs, including phosphorylation, protonation, glycosylation, and methylation, which might control the gating and trafficking of the channels (14, 38, 41, 42). This interconnection is suggested both by the modeling of SoPIP2;1 gating, which is dependent on protonation and phosphorylation status (10), and by the activity of MpPIP1–MpPIP2 heteromers in oocytes, which might depend on PIP1 phosphorylation (13). Further experimental work is needed to reveal the exact mechanism of heteromer formation and regulation and its physiological importance in plant cells.

Methods

Isolation and Transfection of Maize Protoplasts. Maize B73 seedlings were grown on an 8-h dark/16-h light regime at 22°C for 7 days and then were transferred to the dark for 7 days. To obtain mesophyll protoplasts, the middle parts of etiolated leaves were abraded with glass paper and digested in D medium (0.645 M sorbitol/10 mM KCl/8 mM Mes/1 mM $CaCl_2$, pH 5.5) containing 0.6% cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan), 0.1% pectolyase (Sigma, St. Louis, MO), 0.1% BSA, and 0.1% polyvinylpyrrolidone K30 for 3 h at 24°C on a rotating shaker (90 rpm). The leaf pieces were then transferred to fresh D medium, and protoplasts were

released by vigorous manual shaking. After filtration through a 65-μm nylon mesh, the protoplasts were collected by centrifugation at 90 $\times$ *g* for 3 min and washed in D medium.

The transfection protocol was adapted from that developed for *Arabidopsis thaliana* protoplasts (43). Protoplasts (≈50,000) were resuspended in 100 μl of transfection medium (0.6 M mannitol/15 mM $CaCl_2$/4 mM Mes, pH 5.7), and 5–10 μg of plasmid was added. The suspension was then mixed with 110 μl of PEG medium (40% PEG 4000/0.1 M $Ca(NO_3)_2$/0.3 M mannitol) and incubated for 2 min at room temperature (RT). After the addition of 440 μl of W5 medium (154 mM NaCl/125 mM $CaCl_2$/5 mM KCl/4 mM Mes, pH 5.7), the protoplasts were collected by centrifugation at 110 $\times$ *g* for 1 min, resuspended in 1.1 ml of incubation buffer (0.6 M mannitol/4 mM KCl/4 mM Mes, pH 5.7), and kept in the dark at 20°C for 16–24 h in the presence of 90 μg/ml cefotaxime. RNA extraction and quantitative RT-PCR were performed as described previously (31).

Plasmid Constructions. All constructs used for protoplast transfection were prepared in pCAMBIA-35S-EYFP-C1 containing the 35S promoter, enhanced *YFP-C1* (*EYFP-C1*) cDNA, and the nopaline synthase transcription terminator (44). The constructs are described in detail in *SI Methods*.

Confocal Microscopy. CFP and YFP fusion proteins were detected by using a Zeiss (Jena, Germany) confocal laser scanning microscope 510 as described previously (45). FM4-64 dye (Molecular Probes, Carlsbad, CA) was excited at 543 nm, and its fluorescence was recorded by using a 650-nm long pass filter. A $\times$ 40 oil immersion objective (numerical aperture of 1.3) was used for scanning. The pinhole was set at 1 Airy unit. Images were analyzed by using Zeiss LSM software, version 3.2.0.70.

FLIM. FLIM analyses were performed by using a Bio-Rad (Hercules, CA) Radiance 2100 MP system in combination with a Nikon (Tokyo, Japan) TE300 inverted microscope. After two-photon excitation, FLIM measurements were made as described previously (25). For each protoplast, the fluorescence lifetime was calculated as the average of 20 τ values randomly measured in the cell. The values obtained for *n* protoplasts (see Table 1) were used to determine the average value of τ for each pair of proteins analyzed. FRET efficiency E was calculated by using the formula $E = 1 - (\tau_{DA}/\tau_D)$, where τ_D corresponds to the fluorescence lifetime of the donor alone and τ_{DA} to that in the presence of the acceptor.

Immunoprecipitation. BMS suspension cells were cultivated as described previously (37). Roots were collected from seedlings grown aeroponically as described previously (31). Plasma membrane fractions were prepared as described previously (16).

Plasma membrane proteins (150 μg) were solubilized in 250 μl of TBS [0.02 M Tris and 0.136 M NaCl (pH 7.6) containing 3.5% *n*-octyl-β-D-thioglucopyranoside] (solubilization buffer) for 4 h at RT on a rotating wheel (20 rpm). The sample was then centrifuged at 169,000 $\times$ *g* for 40 min at 4°C, and the supernatant was incubated overnight at 4°C with 1 μl of anti-ZmPIP2;1/2;2 or anti-ZmPIP1;2 antiserum (31) on a rotating wheel (20 rpm). Then 100 μl of Protein A Sepharose CL-4B resin (GE Healthcare Life Sciences, Piscataway, NJ) was added, and incubation continued for 2 h at RT in a Micro Bio-Spin Chromatography column (Bio-Rad). After four washes with 400 μl of solubilization buffer and eight washes with 400 μl of TBS, the resin was recovered and incubated in 60 μl of SDS/PAGE sample buffer (containing 15 mM DTT) for 1 h at RT. Proteins (25 μl) were separated by SDS/PAGE and transferred to PVDF membranes as described previously (31). Western blot analysis was performed by using antisera raised against the N-terminal peptides of ZmPIP1;2 and ZmPIP2;1/2;2 previously described (31) and against H^+ ATPases (46).

This work was supported by grants from the Belgian National Fund for Scientific Research (Fonds National de la Recherche Scientifique), the Interuniversity Attraction Poles Program–Belgian Science Policy, and the Communauté Française de Belgique–Actions de Recherches Concertées. E.Z. and M.M. were research fellows at the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture. H.B. is a research associate of the Fonds National de la Recherche Scientifique.

1. Lee JK, Kozono D, Remis J, Kitagawa Y, Agre P, Stroud RM (2005) *Proc Natl Acad Sci USA* 102:18932–18937.
2. Ringler P, Borgnia MJ, Stahlberg H, Maloney PC, Agre P, Engel A (1999) *J Mol Biol* 291:1181–1190.
3. Savage DF, Egea PF, Robles-Colmenares Y, O'Connell JD, III, Stroud RM (2003) *PLoS Biol* 1:E72.
4. Murata K, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y (2000) *Nature* 407:599–605.
5. Sui H, Han BG, Lee JK, Walian P, Jap BK (2001) *Nature* 414:872–878.
6. Schenk AD, Werten PJ, Scheuring S, de Groot BL, Muller SA, Stahlberg H, Philippsen A, Engel A (2005) *J Mol Biol* 350:278–89.
7. Gonen T, Sliz P, Kistler J, Cheng Y, Walz T (2004) *Nature* 429:193–197.
8. Hiroaki Y, Tani K, Kamegawa A, Gyobu N, Nishikawa K, Suzuki H, Walz T, Sasaki S, Mitsuoka K, Kimura K, *et al.* (2006) *J Mol Biol* 355:628–639.
9. Kukulski W, Schenk AD, Johanson U, Braun T, de Groot BL, Fotiadis D, Kjellbom P, Engel A (2005) *J Mol Biol* 350:611–616.
10. Tornroth-Horsefield S, Wang Y, Hedfalk K, Johanson U, Karlsson M, Tajkhorshid E, Neutze R, Kjellbom P (2006) *Nature* 439:688–694.
11. Harvengt P, Vlerick A, Fuks B, Wattiez R, Ruysschaert JM, Homble F (2000) *Biochem J* 352:183–190.
12. Fetter K, Van Wilder V, Moshelion M, Chaumont F (2004) *Plant Cell* 16:215–228.
13. Temmei Y, Uchida S, Hoshino D, Kanzawa N, Kuwahara M, Sasaki S, Tsuchiya T (2005) *FEBS Lett* 579:4417–4422.
14. Chaumont F, Moshelion M, Daniels MJ (2005) *Biol Cell* 97:749–764.
15. Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R (2001) *Plant Physiol* 125:1206–1215.
16. Chaumont F, Barrieu F, Jung R, Chrispeels MJ (2000) *Plant Physiol* 122:1025–1034.
17. Ottschytsch N, Raes A, Van Hoorick D, Snyders DJ (2002) *Proc Natl Acad Sci USA* 99:7986–7991.
18. Etxeberria A, Santana-Castro I, Regalado MP, Aivar P, Villarroel A (2004) *J Neurosci* 24:9146–9152.
19. Torvinen M, Marcellino D, Canals M, Agnati LF, Lluis C, Franco R, Fuxe K (2005) *Mol Pharmacol* 67:400–407.
20. Hink MA, Bisselin T, Visser AJ (2002) *Plant Mol Biol* 50:871–883.
21. Wouters FS, Verveer PJ, Bastiaens PI (2001) *Trends Cell Biol* 11:203–211.
22. Borst JW, Hink M, Van Hoek A, Visser AJWG (2003) in *Multiphoton Microscopy in the Biomedical Sciences III*, eds Periasamy A, So PT (SPIE, Bellingham, WA) Vol 4963, pp 231–238.
23. Chen Y, Mills JD, Periasamy A (2003) *Differentiation* (*Berlin*) 71:528–541.
24. Immink RG, Gadella TW, Jr, Ferrario S, Busscher M, Angenent GC (2002) *Proc Natl Acad Sci USA* 99:2416–2421.
25. Russinova E, Borst JW, Kwaaitaal M, Cano-Delgado A, Yin Y, Chory J, de Vries SC (2004) *Plant Cell* 16:3216–3229.
26. Tonaco IA, Borst JW, de Vries SC, Angenent GC, Immink RG (2006) *J Exp Bot* 57:33–42.
27. Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) *Science* 296:913–916.
28. Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) *Proc Natl Acad Sci USA* 94:2122–2127.
29. Batoko H, Zheng HQ, Hawes C, Moore I (2000) *Plant Cell* 12:2201–2217.
30. Bischoff F, Vahlkamp L, Molendijk A, Palme K (2000) *Plant Mol Biol* 42:515–530.
31. Hachez C, Moshelion M, Zelazny E, Cavez D, Chaumont F (2006) *Plant Mol Biol* 62:305–323.
32. Xicluna J, Lacombe B, Dreyer I, Alcon C, Jeanguenin L, Sentenac H, Thibaud JB, Cherel I (2007) *J Biol Chem* 282:486–494.
33. Uberti MA, Hague C, Oller H, Minneman KP, Hall RA (2005) *J Pharmacol Exp Ther* 313:16–23.
34. Gonen T, Cheng Y, Sliz P, Hiroaki Y, Fujiyoshi Y, Harrison SC, Walz T (2005) *Nature* 438:633–638.
35. Gonen T, Cheng Y, Kistler J, Walz T (2004) *J Mol Biol* 342:1337–1345.
36. Volkov V, Hachez C, Moshelion M, Draye X, Chaumont F, Fricke W (2007) *J Exp Bot* 58:377–390.
37. Moshelion M, Moran N, Chaumont F (2004) *Plant Physiol* 135:2301–2317.
38. Luu DT, Maurel C (2005) *Plant Cell Environ* 28:85–96.
39. Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R (1999) *Plant J* 18:565–570.
40. Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R (2003) *Nature* 425:734–737.
41. Santoni V, Verdoucq L, Sommerer N, Vinh J, Pflieger D, Maurel C (2006) *Biochem J* 400:189–197.
42. Hachez C, Zelazny E, Chaumont F (2006) *Biochim Biophys Acta* 1758:1142–1156.
43. Sheen J (2002) *Science* 296:1650–1652.
44. Preuss ML, Serna J, Falbel TG, Bednarek SY, Nielsen E (2004) *Plant Cell* 16:1589–1603.
45. Shah K, Russinova E, Gadella TW, Jr, Willemse J, De Vries SC (2002) *Genes Dev* 16:1707–1720.
46. Morsomme P, de Kerchove d'Exaerde A, De Meester S, Thines D, Goffeau A, Boutry M (1996) *EMBO J* 15:5513–5526.