Running Head: A transmembrane motif to sort aquaporins

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A new LxxxA motif in the transmembrane helix 3 of maize PIP2 aquaporins is required for their trafficking to the plasma membrane

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Summary: Two amino acids located in a transmembrane helix regulate the trafficking of endoplasmic reticulum- and plasma membrane-localized aquaporins along the secretory pathway.
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Abstract

Aquaporins play important roles in maintaining plant water status under challenging environments. The regulation of aquaporin density in cell membranes is essential to control transcellular water flows. This work focuses on the maize (Zea mays) plasma membrane intrinsic (PIP) aquaporin subfamily, which is divided into two sequence-related groups, ZmPIP1s and ZmPIP2s. When expressed alone in mesophyll protoplasts, ZmPIP2s are efficiently targeted to the plasma membrane, whereas ZmPIP1s are retained in the endoplasmic reticulum (ER). A protein domain-swapping approach was utilized to demonstrate that the transmembrane domain 3 (TM3), together with the previously identified N-terminal ER export diacidic motif, account for the differential localization of these proteins. In addition to protoplasts, leaf epidermal cells transiently transformed by biolistic particle delivery were used to confirm and refine these results. By generating artificial proteins consisting of a single transmembrane domain, we demonstrated that the TM3 of ZmPIP1;2 or ZmPIP2;5 discriminates between ER and plasma membrane localization, respectively. More specifically, a new LxxxA motif in the TM3 of ZmPIP2;5, which is highly conserved in plant PIP2s, was shown to regulate its anterograde routing along the secretory pathway, and more particularly its export from the ER.
Introduction

Aquaporins are of major importance to plant physiology, being essential for the regulation of transcellular water movement during growth and development (Maurel et al., 2008; Gomes et al., 2009; Heinen et al., 2009; Prado and Maurel, 2013; Chaumont and Tyerman, 2014). Aquaporins are small membrane proteins consisting of six transmembrane domains (TMs) connected by five loops (A to E), and N- and C-termini facing the cytosol (Figure 1A). They assemble as homo- and/or heterotetramers in the membrane, with each monomer acting as an independent water channel (Murata et al., 2000; Fetter et al., 2004; Gomes et al., 2009). Aquaporins form a highly divergent protein family in plants (Chaumont et al., 2001; Johanson et al., 2001), and this work focuses on the maize (Zea mays, Zm) plasma membrane intrinsic protein (PIP) family (Chaumont et al., 2001). The regulation of the subcellular localization of these proteins is a key process controlling their density in the plasma membrane and, hence, their physiological roles (Hachez et al., 2013).

PIP aquaporins cluster in two groups, PIP1s and PIP2s, which are highly conserved across species (Kammerloher et al., 1994; Chaumont et al., 2000; Chaumont et al., 2001; Johanson et al., 2001; Anderberg et al., 2012). We previously showed that the maize PIP1 and PIP2 isoforms exhibit different water channel activities when expressed in Xenopus laevis oocytes, with only PIP2s increasing the membrane water permeability coefficient (Pf) (Chaumont et al., 2000). However, when ZmPIP1 and ZmPIP2 are co-expressed, the isoforms physically interact to modify their stability and trafficking to the oocyte membrane, and synergistically increase the oocyte Pf (Fetter et al., 2004). Similar synergistic interactions between PIP1s and PIP2s have been reported in numerous plant species (Temmei et al., 2005; Mahdieh et al., 2008; Vandeleur et al., 2009; Bellati et al., 2010; Ayadi et al., 2011; Horie et al., 2011; Yaneff et al., 2014).

PIPs were originally thought to be exclusively localized in the plasma membrane and were named accordingly (Kammerloher et al., 1994). However,
recent experiments have shown that not all PIPs are located to the plasma membrane under all conditions, and that regulation of PIP subcellular localization is a highly dynamic process involving protein interactions (Boursiac et al., 2005; Zelazny et al., 2007; Boursiac et al., 2008; Uehlein et al., 2008; Zelazny et al., 2009; Besserer et al., 2012; Luu et al., 2012). When expressed singly in maize leaf mesophyll protoplasts, fluorescently-tagged ZmPIP1s and ZmPIP2s differ in their subcellular localization. ZmPIP1s are retained in the endoplasmic reticulum (ER), whereas ZmPIP2s are targeted to the plasma membrane (Zelazny et al., 2007). However, upon co-expression, ZmPIP1s are re-localized from the ER to the plasma membrane where they perfectly co-localize with ZmPIP2s. This re-localization results from their physical interaction as demonstrated by Förster Resonance Energy Transfer/Fluorescence Lifetime Imaging Microscopy and immunoprecipitation experiments (Zelazny et al., 2007). These results indicate that ZmPIP2s, but not ZmPIP1s, possess signals which allow them to be delivered to the plasma membrane, and that hetero-oligomerization is required for ZmPIP1 trafficking to the plasma membrane. Interestingly, an N-terminal diacidic motif (DxE, aspartate - any amino acid - glutamate) located in the N-terminus of ZmPIP2;4, ZmPIP2;5, and Arabidopsis thaliana AtPIP2;1 has been shown to be required to exit the ER (Zelazny et al., 2009; Sorieul et al., 2011). Diacidic motifs interact with Sec24, which is thought to be the main cargo-selection protein of the COPII coat protein complex which mediates vesicle formation at ER export sites (Miller et al., 2003). However, not all plasma membrane-localized PIP2s contain a diacidic ER export signal (Zelazny et al., 2009). In addition, swapping the N-terminal region of ER-retained ZmPIP1;2 with that of plasma membrane-localized ZmPIP2;5, which contains the functional di-acidic motif, is not sufficient to trigger ER export of the protein (Zelazny et al., 2009). This result suggests that (i) other export signals might be present in PIP2s and/or (ii) ER retention signals might be present in PIP1s elsewhere than in the N-terminus.

To identify new signals regulating ZmPIP1 and ZmPIP2 protein trafficking
along the secretory pathway, we used a protein domain swapping-based approach and identified the TM3 as an important region which discriminates between ER-retained ZmPIP1;2 and plasma membrane-localized ZmPIP2;5. Specific mutations in the TM3 region of ZmPIP2;5 allowed the identification of a new PIP2-conserved LxxxA motif, which regulates its export from the ER.

Results

TM3 plays a role in the delivery of ZmPIP2s to the plasma membrane

To identify new trafficking signals, the loops and termini of plasma membrane-localized ZmPIP2;1 were first replaced, individually or in combinations, by the corresponding portions of ER-retained ZmPIP1;2 (Figure S1A). The loop and TM regions were defined on the basis of a multiple alignment of all of the ZmPIPs sequences, and on the topological model of ZmPIP1;2 (Chaumont et al., 2001). Maize mesophyll protoplasts were transiently transformed with genetic constructs encoding the chimeric ZmPIPs fused downstream of the monomeric yellow fluorescent protein (mYFP) (see Experimental procedures). Previous studies demonstrated that tagging ZmPIPs with GFP variants at their N-terminus does not affect their activity (Fetter et al., 2004; Besserer et al., 2012; Bienert et al., 2012), nor does it prevent N-terminal trafficking motifs to be functional (Zelazny et al., 2009). In plant cells, PM, ER and cytosol are sometimes difficult to distinguish because of the presence of the central vacuole that occupies most of the cell volume and pushes all other cell compartments against the PM. For this reason, mCFP:HDEL and FM4-64 have been used throughout this study as widely accepted markers of the ER and the PM, respectively. The use of those markers confirmed previous results obtained in protoplasts demonstrating that ZmPIP1s are retained in the ER, while ZmPIP2s reach the PM (Figure S1B panels 1-8; Figure
Even though some chimeric proteins strongly labeled various intracellular structures, the plasma membrane remained clearly stained by all of them (Figure S1B). This led us to conclude that the N- and C-termini and the intra- and extracellular loops of ZmPIP2;1 and ZmPIP1;2 did not contain dominant trafficking signals. However, one chimeric protein, mYFP:ZmPIP2;1-PIP1;2Mix, which contains the TM3 and TM4 of ZmPIP1;2 (Figure S1A), was completely retained in intracellular structures (Figure S1B, panels 33-36), co-localizing with the ER marker mCFP:HDEL. This observation points to a possible role for the TM domains in the routing of ZmPIPs to the plasma membrane.

A similar domain swapping approach was used to assess the possible involvement of TMs in the regulation of ZmPIP trafficking to the plasma membrane. In this case, ZmPIP2;5 was used instead of ZmPIP2;1, as the former exhibited a more defined plasma membrane localization when compared to ZmPIP2;1. Indeed, when expressed in protoplasts, mYFP:ZmPIP2;5 strongly labeled the plasma membrane, and the signal was weaker in the intracellular structures than that of mYFP:ZmPIP2;1 (compare Figure 1B, panels 1-4, to Figure S1B, panels 1-4; (Zelazny et al., 2007).

The six TMs of ZmPIP2;5 were individually replaced by those of ZmPIP1;2 (Figure 1A). Because the length of the TM is known to influence the localization of type I membrane proteins along the secretory pathway (Brandizzi et al., 2002), the swapped regions were slightly longer than the predicted TMs. Figure 1B shows the subcellular localization of the mYFP-tagged chimeric proteins transiently expressed in maize mesophyll protoplasts. Strikingly, ZmPIP2;5, whose TM3 was replaced by that of ZmPIP1;2 (mYFP:ZmPIP2;5-TM3<sub>P</sub>IP1;2; Figure 1A) was unable to reach the plasma membrane (Figure 1B, panels 13-16), which is in contrast to the wild-type mYFP:ZmPIP2;5 (Figure 1B, panels 1-4). All of the other chimerae (Figure 1B, panels 5-12 and 17-28) sharply labeled the cell periphery and co-localized with the steryl dye FM4-64, showing that they were still, at least to some extent, able to
reach the plasma membrane. The control experiment showed that the position of the fluorescent tag did not modify the localization of ZmPIP2;5, ZmPIP1;2, and ZmPIP2;5-TM3PIP1;2 (Figure S2).

As shown by the partial co-localization with the ER marker mCFP:HDEL, mYFP:ZmPIP2;5-TM3PIP1;2 was retained both in the ER (Figure 1B, panels 13-16) and in bright punctate intracellular structures (Figure 1B, arrows in panel 13). To investigate the nature of these structures, mYFP:ZmPIP2;5-TM3PIP1;2 was transiently co-expressed with the Golgi marker ST:mCFP (Boevink et al., 1998; Batoko et al., 2000). Figure 1C shows the co-localization of mYFP:ZmPIP2;5-TM3PIP1;2 and ST:mCFP in Golgi stacks (arrowheads), demonstrating that mYFP:ZmPIP2;5-TM3PIP1;2 is retained in the Golgi apparatus in addition to the ER.

The double mutation L127F/A131M is sufficient to retain mYFP:ZmPIP2;5 in intracellular structures

To identify the amino acid residues responsible for the retention of mYFP:ZmPIP2;5-TM3PIP1;2 in intracellular structures, each TM3 residue differing between ZmPIP2;5 and ZmPIP1;2 was individually mutated. For each position, the residue in ZmPIP2;5 was mutated into its ZmPIP1;2 counterpart (Figure 2A). The final three amino acid residues (S158, A159, and F160) of the exchanged region were mutated together (ZmPIP2;5SAF/QGL). These mutated proteins were tagged with mYFP and expressed in protoplasts (Figures 2B and S3). For most of the mutants, the fluorescent signal in the intracellular structures was stronger than for the wild-type protein. However, all showed a sharp signal at the periphery of the cell, which co-localized with FM4-64, and were therefore able to reach the plasma membrane, at least partially. These results indicated that two or more residues were involved in the full intracellular retention observed for mYFP:ZmPIP2;5-TM3PIP1;2 (Figure 1).
To analyze more precisely which amino acid residues of the TM3 could together form a trafficking motif, a previously published homology model of the ZmPIP2;5 homotetramer was utilized (Figure 2C; Bienert et al., 2012). This model showed that the TM3 was located on the outer surface of the ZmPIP2;5 tetramer. Furthermore, among the 10 amino acids of ZmPIP2;5 which differed from ZmPIP1;2, V123, L127, V130, and A131 might possibly be involved in protein-protein or protein-lipid interactions in the membrane, since they were facing the lipid bilayer and were in close proximity to each other. Based on these observations, a mYFP:ZmPIP2;5 variant combining the four point mutations was generated (mYFP:ZmPIP2;5V123T/L127F/V130I/A131M). As hypothesized, this protein was totally absent from the plasma membrane (Figure 2D, panels 1-3). Interestingly, reintroducing either L127 (mYFP:ZmPIP2;5V123T/V130I/A131M) or A131 (mYFP:ZmPIP2;5V123T/L127F/V130I) was sufficient to restore a sharp signal in the plasma membrane (Figure 2D, panels 4-9), indicating that these amino acid residue exchanges were responsible for the full intracellular retention of mYFP:ZmPIP2;5-TM3P1;2. A construct combining both the L127F and A131M mutations was then generated (mYFP:ZmPIP2;5L127F/A131M). As shown in Figure 2D (panels 10-12), this double mutation was sufficient to prevent the protein from reaching the plasma membrane. Quantification of the relative fluorescent signal in the plasma membrane confirmed these results (Figures 2E and S4).

The steady state subcellular localization of ZmPIPs in protoplasts is reached after 16 h

Some of the proteins of interest were localized in intermediate compartments of the secretory pathway (ER and/or Golgi) 16 h after protoplast transformation. To determine whether this localization was their steady state destination or whether it represented intermediate trafficking steps, a three-day time course experiment was performed (Figure S5). Protoplasts were transfected with genetic constructs encoding mYFP:ZmPIP2;5, mYFP:ZmPIP1;2 or mYFP:ZmPIP2;5L127F/A131M, and the subcellular localization of the proteins was observed 16, 40 and 64 h after
transfection. Cycloheximide (CHX) was added after the first observation in order to follow the evolution of the pool of proteins observed after 16h. FM4-64 was added to the protoplast suspension just before observation. Pearson correlation coefficients between the YFP and FM4-64 signals were calculated to measure to which extent the proteins reached the PM. The localization of all three proteins of interest appeared not to change over time (Figure S5). Even 60 h after transfection, mYFP:ZmPIP1;2 and mYFP:ZmPIP2;5L127F/A131M were unable to reach the PM, while ZmPIP2;5 stayed in the PM at all time points.

Transient transformation of maize leaf epidermal cells by biolistic particle delivery

The above-described experiments were repeated using biolistic transient transformation of maize leaf epidermal cells (Figure 3). The major advantage of using this technique was to express the chimeric proteins in intact living cells of known origin, still surrounded by their wall and neighboring tissue. As expected, when expressed alone, mYFP:ZmPIP2;5 labeled the cell periphery (Figure 3A), whereas mYFP:ZmPIP1;2, showed a network-like ER localization (Figure 3B). However, upon co-expression, both proteins co-localized in the plasma membrane (Figure 3D), confirming previous observations performed in maize mesophyll protoplasts that demonstrated a re-localization of ZmPIP1;2 from the ER to the plasma membrane by its physical interaction with ZmPIP2;5 (Zelazny et al., 2007). mYFP:PIP2;1-PIP1;2Mix (Figure 3C), mYFP:ZmPIP2;5-TM3PIP1;2 (Figure 3E), and mYFP:ZmPIP2;5L127F/A131M (Figure 3F) were not able to reach the cell plasma membrane, as observed in protoplasts. mYFP:ZmPIP2;5-TM3PIP1;2 and mYFP:ZmPIP2;5L127F/A131M only partially co-localized with mCFP:HDEL and also accumulated in intense punctate structures closely associated with the ER (Figure 3E and F).

The intracellular retention of PIP2-TM3PIP1 chimerae is conserved for different
isoforms

To investigate whether the essential role of TM3 in regulating PIP plasma membrane localization was conserved among different ZmPIP isoforms, the localization of two additional TM3 chimeric proteins was analyzed using ZmPIP1;6 and ZmPIP2;1 as alternative isoforms to ZmPIP1;2 and ZmPIP2;5, respectively (mYFP:ZmPIP2;5-TM3PIP1;6 and mYFP:ZmPIP2;1-TM3PIP1;2). As expected, the wild-type proteins ZmPIP1;6 and ZmPIP2;1 were targeted to the ER and the plasma membrane, respectively (Figure 4B and D). Similarly to mYFP:ZmPIP2;5-TM3PIP1;2, mYFP:ZmPIP2;5-TM3PIP1;6 and mYFP:ZmPIP2;1-TM3PIP1;2 were fully retained in intracellular structures (Figure 4E and F), demonstrating that the intracellular retention of a ZmPIP2 caused by the replacement of its TM3 by the corresponding region from an ER-retained ZmPIP1 was conserved.

Interestingly, while mYFP:ZmPIP2;5-TM3PIP1;2 was located in punctate structures in addition to the ER (Figure 1B, 1C and 3E), mYFP:ZmPIP2;1-TM3PIP1;2 seemed to label less punctate structures (Figure 4F). To investigate this question further, we quantified these labeled structures when the fusion proteins were expressed in protoplasts (Figure S6). On average three times less fluorescent punctate structures were detected in protoplasts expressing mYFP:ZmPIP2;1-TM3PIP1;2 than in protoplasts expressing mYFP:ZmPIP2;5-TM3PIP1;2 (Figure S6), indicating that mYFP:ZmPIP2;1-TM3PIP1;2 is mostly present in the ER, contrary to mYFP:ZmPIP2;5-TM3PIP1;2.

**TM3 alone is sufficient to discriminate between ER and plasma membrane localization**

To confirm that the TM3 is involved in the regulation of the trafficking of ZmPIPs, artificial single-TM reporter proteins were generated by fusing the TM3 of ZmPIP2;5 or ZmPIP1;2 to the C-terminal end of the mYFP (mYFP:TM3ZmPIP2;5 and mYFP:TM3ZmPIP1;2, respectively). Interestingly, these proteins displayed distinct subcellular localization patterns when expressed in maize leaf epidermal cells.
(Figure 5). While mYFP:TM3ZmPIP2;5 mostly labeled the plasma membrane (Figure 5C), mYFP:TM3ZmPIP1;2 remained in an ER-like structure (Figure 5D). These patterns fully copied the localization of the full length wild-type ZmPIP2;5 and ZmPIP1;2, respectively (Figure 5A and B), confirming that the TM3 of ZmPIP2;5 and ZmPIP1;2 have distinct trafficking abilities.

**Mutated ZmPIP1;2 remains in the ER**

Previous experiments showed that mYFP:ZmPIP1;2 was retained in the ER, even when fused to the N-terminus of ZmPIP2;5 (mYFP:ZmPIP1;2-NPIP2;5), which contains the diacidic ER export motif (Zelazny et al., 2009). Based on the data presented above, the TM3 of ZmPIP1;2 was replaced in mYFP:ZmPIP1;2 (mYFP:ZmPIP1;2-TM3PIP2;5; Figure 6A) or mYFP:ZmPIP1;2-NPIP2;5 (mYFP:ZmPIP1;2-N+TM3PIP2;5; Figure 6A) with that of ZmPIP2;5 in order to test whether the ZmPIP2;5 TM3 was sufficient to bring these proteins to the plasma membrane. When expressed in leaf protoplasts, neither mYFP:ZmPIP1;2-TM3PIP2;5 nor mYFP:ZmPIP1;2-N+TM3PIP2;5 were able to reach the plasma membrane (Figure 6B). Co-localization with mCFP:HDEL showed that both chimeric proteins were retained in the ER. In addition to the ER, mYFP:ZmPIP1;2-N+TM3PIP2;5, but not mYFP:ZmPIP1;2-TM3PIP2;5, was also present in punctate structures closely associated with the ER (Figure 6B, panel 10, arrowheads). However, the labeling of these structures was likely due to the N-terminus of ZmPIP2;5, as the fusion of the N-terminus of ZmPIP2;5 to ZmPIP1;2 (mYFP:ZmPIP1;2-NPIP2;5, Figure 6A) also causes the protein to be partially located in these punctate structures (Figure 6B, panel 4, arrowheads). Identical results were obtained when the double mutant ZmPIP1;2F137L/M141A, with or without the N-terminus of ZmPIP2;5 (ZmPIP1;2F137L/M141A or PIP1;2F137L/M141A-NPIP2;5, respectively) was used (Figure S7).
ZmPIP2;5-TM3PIP1;2 exhibits PIP1-like water channel activity

When expressed in Xenopus oocytes, ZmPIP2s significantly increase the cell Pi, demonstrating their efficient water channel activity (Chaumont et al., 2000; Fetter et al., 2004; Moshelion et al., 2009), whereas ZmPIP1s are inactive, except if they are co-expressed with PIP2s (Fetter et al., 2004; Bienert et al., 2012). Similar behaviors have been described for co-expressed PIP1 and PIP2 proteins from different plant species (Temmei et al., 2005; Mahdieh et al., 2008; Vandeleur et al., 2009; Bellati et al., 2010; Ayadi et al., 2011; Horie et al., 2011; Yaneff et al., 2014). In contrast to oocytes expressing ZmPIP2;5, the Pi of cells expressing ZmPIP2;5-TM3PIP1;2 did not increase compared to control cells (Figure 7). However, when ZmPIP2;5-TM3PIP1;2 and ZmPIP2;5 were co-expressed, the Pi significantly increased above the level of ZmPIP2;5-expressing oocytes.

Discussion

This work aimed at identifying new protein motifs regulating the subcellular localization of ZmPIPs. By analyzing the localization of fluorescently tagged ZmPIP1-ZmPIP2 chimeric or mutated proteins in two different homologous systems, we showed that the TM3 plays an important role in regulating ZmPIP delivery to the plasma membrane. Furthermore, we identified two residues within TM3 which are critical for the routing of ZmPIPs between the ER and the plasma membrane.

TM3 is involved in ZmPIP trafficking

The replacement of the third TM of ZmPIP2;5 with the corresponding region of ZmPIP1;2 strikingly prevented plasma membrane delivery of the chimeric protein (Figure 1). Point-mutation analysis of the diverging residues in the TM3 of
ZmPIP2;5 and ZmPIP1;2 showed that the combination of only two single point mutations (mYFP:ZmPIP2;5L127F/A131M) induced the complete intracellular retention phenotype observed for ZmPIP2;5-TM3_PIP1;2 (Figure 2). Trafficking of those mutants to the PM is fully blocked and not simply delayed, as mYFP:ZmPIP2;5L127F/A131M is still exclusively found in intracellular structures three days after protoplast transfection (Figure S5).

Trafficking signals are usually located in the N- or C-terminal soluble regions of membrane proteins (Kappeler et al., 1997; Nishimura and Balch, 1997; Sevier et al., 2000; Ma et al., 2001; Otte and Barlowe, 2002) or in cytosolic loops (Takano et al., 2010). The accessibility of the sorting motifs from the cytoplasm was previously believed to be crucial for the interaction of membrane cargo proteins with trafficking regulators (Barlowe, 2003; Miller et al., 2003; Mossessova et al., 2003; Contreras et al., 2004; Robinson et al., 2007; Sato and Nakano, 2007; Bassham et al., 2008; Sieben et al., 2008). However, more and more examples of the regulation of membrane protein sorting by alternative mechanisms (Springer et al., 2014), and specifically by TMs (Cosson et al. 2013), have been reported. For instance, the TM6 of the tonoplast aquaporin a-TIP from bean contains sufficient information for its transport to the vacuolar membrane (Hofte and Chrispeels, 1992). The rice secretory carrier membrane protein 1 (SCAMP1) relies on its TM2 and TM3 for Golgi export, and on its TM1 for trans-Golgi network to plasma membrane trafficking (Cai et al., 2011). Together with our data, these results provide evidence for the involvement of TMs in intracellular sorting processes. The exact mechanisms underlying TM-based sorting are not yet fully understood, but likely involve recognition by sorting receptors and targeting to specific membrane subdomains (Cosson et al., 2013).

The length of the TM has been shown to regulate the progression of single TM integral membrane proteins through the secretory pathway in plants, vertebrates, and fungi (Brandizzi et al., 2002; Ronchi et al., 2008; Sharpe et al., 2010). Whether such a TM length-dependent sorting process might, to some
extent, explain the results obtained here with single TM reporters (Figure 5) was investigated. A possible hypothesis could be that the TM3 of ZmPIP1;2 is shorter than that of ZmPIP2;5, resulting in a different progression through the secretory pathway. Interestingly, TM3 (and TM4) of ZmPIP1s are generally predicted to be shorter than their ZmPIP2 counterparts by the algorithm TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/; Table 1). This first prediction seemed to confirm our hypothesis. However, the calculated length of the TM3 of both ZmPIP1;2 and ZmPIP2;5 in the context of the single TM YFP reporters was strictly identical (Table 1). Therefore, the different destination of our single TM proteins is not likely to be the consequence of a different TM length. It has to be noted that the output of the TMHMM 2.0 algorithm differs according to the sequence context of the TM helix. Whether the length of TM regions actually changes depending on their sequence context or whether this is a calculation artifact is not known. The question is even more complicated regarding full-length proteins. TM segments in aquaporins are slightly tilted in the membrane (Murata et al., 2000; Thornroth-Horsefield et al., 2006). In a membrane of a given thickness, aquaporin TM helices are therefore likely to be longer than those of single pass membrane proteins. Even though examples of TM length-based sorting have been reported for single TM proteins, whether and how such a mechanism could occur in multi TM proteins has, to our knowledge, not been addressed. Because TM length can regulate partitioning of single TM proteins in different lipid subdomains of the ER and, therefore, their export to the Golgi (Ronchi et al., 2008), whether similar mechanisms occur in multispansing membrane proteins should be studied.

While at first sight, the shorter predicted TM3 (18 aa) in ZmPIP1s could be responsible for their retention in the ER, the TM3 of ER-retained ZmPIP1;6 (Figure 4) had a predicted length similar to PIP2 TM3 (Table 1). A length effect alone could therefore not by itself explain the intracellular retention of mYFP:ZmPIP2;5-TM3_PIP1;6. Furthermore, in the context of ZmPIP2;5-TM3_PIP1;2, the length of the TM3 was predicted to be 23 amino acid residues (Table 1), however the protein was not
able to reach the plasma membrane (Figures 1 and 3). Together, these predictions indicate that regulation of the subcellular localization of ZmPIPs by the length of the TM3 is unlikely but, as such predictions have to be interpreted with care, a length effect of the TM3 cannot be fully excluded.

**ER retention specifically occurs for mYFP:ZmPIP2;5-TM3\textsubscript{PIP1;2}**

The mutated ZmPIP2;5 proteins might be retained in intracellular structures as a consequence of an improper folding. Water transport activity assays were performed to rule out this possibility (Figure 7). When expressed alone, ZmPIP2;5-TM3\textsubscript{PIP1;2} was unable to induce an increase in oocyte $P_f$. However, when ZmPIP2;5-TM3\textsubscript{PIP1;2} was co-expressed with the wild-type ZmPIP2;5, the $P_f$ was significantly higher than the $P_f$ of ZmPIP2;5 expressing cells, similarly to what was previously reported with ZmPIP1;2 (Fetter et al., 2004). The mutated protein was therefore still able to interact with wild-type ZmPIP2;5 proteins to induce this synergistic $P_f$ increase. A minor conformational change, turning ZmPIP2;5 into a ZmPIP1-like isoform might happen as a consequence of TM3 exchange, but the overall structure and topology must still be consistent with those of a ZmPIP to allow water to be transported. These results, in addition to the fact that the trafficking defects were specifically observed for TM3 and not for other TMs of ZmPIP2;5, suggest that the altered localization of mYFP:ZmPIP2;5-TM3\textsubscript{PIP1;2} is due to specific trafficking properties of the TM3, rather than to a non-specific response retaining the mutated protein inside the cell for degradation. The fact that mYFP:ZmPIP2;5L127F/A131M was still detected in protoplasts three days after transformation and in the presence of CHX (Figure S5) further supports this conclusion.

**The TM3 of ZmPIPs regulates ER export**
The chimeric proteins that were unable to reach the plasma membrane as a consequence of TM3 exchange were located both in the ER and in punctate structures, some of them being Golgi bodies (Figures 1 and 4), raising the question of whether the TM3 regulates ER or Golgi export of ZmPIPs. If PIP2-TM3PIP1 chimeras are impaired in their ER export function, Golgi particles might be partially stained as a consequence of ER leakage, due to the presence of the ER export diacidic motif in the chimeric proteins. In support of this hypothesis, fewer punctate structures were detected for mYFP:ZmPIP2;1-TM3PIP1;2 than for mYFP:ZmPIP2;5-TM3PIP1;2 (compare Figures 3E, F, and 4F), in accordance with the occurrence of a functional ER export motif in the N-terminus of ZmPIP2;5 but not ZmPIP2;1. The localization of mYFP:TM3ZmPIP1;2 in the ER, and not in punctate structures (Figure 5), also sustains this hypothesis. In a similar manner, mutation of the diacidic ER export motif of the Golgi protein GONST1 caused the retention of the protein in the ER, but the protein was still partially able to escape to the Golgi and post-Golgi punctate structures (Hanton et al., 2005). These results suggest that the TM3 regulates ER export rather than Golgi export of ZmPIPs. However, since the intense punctate structures were stained by mYFP:ZmPIP2;5-TM3PIP1;2 and mYFP:ZmPIP2;5L127F/A131M (Figures 1, 2, and 3), it is possible that TM3 might, to some extent, take part in Golgi export regulation.

The observation that some of the punctate structures labeled by mYFP:ZmPIP2;5-TM3PIP1;2 and mYFP:ZmPIP2;5L127F/A131M did not colocalize with the Golgi marker ST:mCFP suggests that those proteins were located in other subcellular compartments, in addition to the ER and Golgi apparatus. This hypothesis is further supported by the difference in the size of the punctate structures observed (Figure 1B, panel 13). As the proteins were retained in the early steps of the secretory pathway, it is particularly tempting to speculate that the proteins accumulate at ERES (endoplasmic reticulum export sites), leading to an intense labeling of these structures, even if we cannot exclude a trans-Golgi network (TGN) location. In that case, the protein would be distributed almost all
along the secretory pathway (ER, Golgi and TGN).

**Export signal of ZmPIP2;5 or retention signal of ZmPIP1;2?**

Our domain-exchange experiments allowed us to identify the critical role of the TM3 in the trafficking of ZmPIPs, but whether this domain contains an anterograde signal of ZmPIP2;5 or a retrograde signal of ZmPIP1;2 remains an open question. The replacement of the TM3 of ZmPIP1;2 with the corresponding region of ZmPIP2;5 or the double mutation F137L/M141A did not cause the export of the protein out of the ER (Figures 6 and S6), as it would be expected if an ER-retention motif was mutated, suggesting that the new trafficking motif is an LxxxA anterograde signal of ZmPIP2;5 rather than an FxxxM ER retention signal for ZmPIP1;2. In support of this hypothesis, mYFP:ZmPIP2;5-TM3<sub>PIP1;6</sub> was absent from the plasma membrane even though ZmPIP1;6 does not contain the ZmPIP1;2 FxxxM motif (Figure 4), demonstrating that the ER retention was due to the absence of the LxxxA motif of ZmPIP2;5. In addition, ZmPIP2;1, which contains the TM3 LxxxA motif but does not contain the N-terminal diacidic motif, was able to reach the plasma membrane, pointing to a role for the LxxxA motif in ER export. Altogether, these results confirm that the trafficking motif identified in this study is an LxxxA ER export motif located in the TM3 of ZmPIP2;5.

Hanton et al. (2005) suggested that diacidic ER export motifs are dominant over TM-based ER retention signals. According to this theory, one would expect that proteins containing the diacidic motif of ZmPIP2;5 and the TM3 of ZmPIP1;2 (mYFP:ZmPIP2;5-TM3<sub>PIP1;2</sub>; mYFP:ZmPIP1;2-N<sub>PIP2;5</sub>; Figures 6 and S6 and Zelazny et al., 2009) would be able to reach the plasma membrane. However, the results showed that these proteins were retained inside the cell, suggesting that the TM3 of ZmPIP2;5 contains an LxxxA ER export signal which is required, together with the N-terminal signal, to reach the plasma membrane. The reason why ZmPIP1;2 did not reach the plasma membrane even when fused to both ER
export motifs is unknown. Another, yet unidentified motif might be necessary, in addition to the diacidic and LxxxA motifs, for ER exit. Alternatively, the trafficking signals present in the N-terminus and the TM3 of ZmPIP2;5 might not be fully functional when present in ZmPIP1;2 due to a non-optimal structural context.

The critical residues identified in the TM3 of ZmPIP2;5 are highly conserved among plant PIPs. The L127 and A131 residues of ZmPIP2;5 are conserved in all maize PIP2s, and all ZmPIP1s contain phenylalanine and methionine residues at these positions, except for ZmPIP1;6 in which the phenylalanine is replaced by a tyrosine residue. The LxxxA motif is present in the TM3 of 18 out of 23 PIP2 isoforms from rice, maize, and Arabidopsis (Bansal and Sankararamakrishnan, 2007). This motif is present in some TIPs and a few NIPs (Nodulin26-like Intrinsic Proteins), but is replaced by LxxxA in all XIPs (Uncharacterized-X Intrinsic Proteins). On the other hand, an LxxxA sequence is also found in the TM3 of some SIPs, which are reported to be localized in the ER (Ishikawa et al., 2005), suggesting that the functionality of LxxxA sequences in intracellular sorting relies on the molecular context.

**Working model for a TM-based LxxxA sorting signal in ZmPIP2;5**

Figure 8 presents four hypothetical models describing how TM-based trafficking motifs, as identified in this study, could integrate into well-known secretory pathway mechanisms. In the first two scenarios (Figures 8A, B), the TM trafficking motif acts as a protein-protein interaction motif with another membrane protein. In a first case (Figure 8A), this interacting protein acts as an intermediate between the protein of interest and a cargo-selecting protein (e.g. Sec24 in the case of ER export). In the second scenario, the interaction induces a conformational change in the protein of interest, making a classical soluble trafficking motif (e.g. the N-terminal diacidic motif of ZmPIP2;5) accessible to the export machinery (Figure 8B). The third scenario relies on protein-lipid interaction. The presence of the TM trafficking signal
causes the protein of interest to segregate in a specific, export competent, domain of the membrane (e.g. The ERES, endoplasmic reticulum export sites, in the case of ER export; Figure 8C). Finally, the fourth model combines scenarios 2 and 3. The protein reaches a specific membrane sub-domain due to a protein-lipid interaction. Upon interaction with lipids, the protein undergoes a conformational change which releases a classical export motif (Figure 8D).

In the case of the ER export of ZmPIP2;5, the second hypothesis seems particularly attractive. Given previous results in regard to ZmPIP trafficking and interaction (Zelazny et al., 2007; Zelazny et al., 2009), it is tempting to assume that ZmPIP homo- or heterotetramerization could be involved in this process. However, as depicted in Figure 2C, the LxxxA motif of ZmPIP2;5 faces the membrane side of the PIP tetramer and therefore cannot be directly involved in tetramerization events. On the other hand, the fourth hypothesis (Figure 8D) could partially explain our results. The LxxxA motif could direct ZmPIP2;5 to an export-competent domain of the ER membrane, most likely the ERES. There, the N-terminal diacidic motif could be released, recruiting COPII particles for export toward the Golgi (Figure 8D). However, no evidence for a ZmPIP2;5-lipid interaction is available. Finally, none of our four scenarios can explain how the single TM reporter mYFP:TM3ZmPIP2;5 is able to reach the plasma membrane in the absence of a diacidic motif (Figure 5). Our current research aims at discriminating between these hypotheses and refining this working model. ZmPIP ER export regulation might depend on other more complex factors, such as the number of export-competent units among a heterotetramer.

Materials and Methods

Genetic constructs

cDNAs encoding ZmPIP1;2, ZmPIP1;6, ZmPIP2;1, and ZmPIP2;5 were amplified
by PCR and cloned into the uracil-excision vectors pCambia2300 35S N-term mYFP and/or pCambia2300 35S N-term mCFP (Nour-Eldin et al., 2006; Bienert et al., 2011). Genetic constructs encoding the chimerical proteins were created using the USER fusion method (Geu-Flores et al., 2007). The same methods were used to clone the sequences of interest into the pNB1u vector (Nour-Eldin et al., 2006) for water transport assays in *Xenopus laevis* oocytes, and to transfer constructs encoding ST:GFP and GFP:HDEL into the mYFP and mCFP USER vectors (Bienert et al., 2011). Point mutations were created by overlapping PCRs. The primers used are listed in Table S1. All constructs were verified by sequencing. The plasmids were amplified in *Escherichia coli* and purified using the Nucleobond PC 500 kit (Macherey-Nagel; Duren, Germany) following the instructions provided by the manufacturer.

**Plant materials**

Maize (*Zea mays*) B73 seeds were germinated at 28°C for 2 days. The seedlings were transferred to soil and grown under an 8 h dark/16 h light regime at 25°C for 24-48 h, and then transferred to dark conditions for 5-7 days. The median portion of the third leaf was used for protoplast preparation or leaf epidermal cell transformation.

**Transient transfection**

Maize mesophyll protoplasts were prepared and transiently transfected as described previously (Zelazny et al., 2007). For maize leaf epidermal cells transfected by biolistic particle delivery, plasmid DNA (1-2 µg) was precipitated on 480 µg of 0.6 µm diameter gold beads (BIORAD; Hercules, California) and the leaves were bombarded under a 27 mm Hg vacuum (950 kPa), using a 1100 PSI (7600 kPa) rupture disk, at a shooting distance of 3 cm with a Biolistic PDS1000/He device (BIORAD). The leaf pieces were transferred on solid Hoagland medium and incubated for 16-40h at 25°C under dark light conditions.
**Microscopy and image processing**

Confocal laser scanning microscopy experiments were performed using a LSM 710 microscope (ZEISS; Jena, Germany). Zen 2010 (Zeiss) was used for image acquisition. mCFP and mYFP were excited at 445 and 514 nm, and detected from 450 to 510 nm and from 520 to 620 nm, respectively. FM4-64 (LIFE TECHNOLOGIES; Carlsbad, California) was added at a concentration of 16 µM for a duration of 5 min for plasma membrane labeling of protoplasts. FM4-64 was excited at 514 nm and detected from 600 nm to 760 nm. A 63 x (N.A. = 1.4) oil immersion and a 40 x (N.A. = 1.2) water immersion objective were used for protoplast and leaf cell experiments, respectively.

ImageJ (http://rsbweb.nih.gov/ij/) and Fiji (http://fiji.sc) were used for image processing. Maximum displayed values were set to 100-200 to ensure an optimal display both on a computer screen and as a printout. When necessary, the images were smoothened by the application of a median filter. The relative protoplast plasma membrane fluorescence signal was calculated as the ratio of plasma membrane to total fluorescence, as described in (Besserer et al., 2012). Briefly, the outer limit of the fluorescent signal was converted into a first region of interest (ROI1), representing the total fluorescence of the cell. A second region of interest (ROI2), reflecting intracellular fluorescence, was created by narrowing ROI1 0,35 µm in diameter. The fluorescent signal in the PM was calculated as the difference between the overall signal from the whole cell (ROI1), and the signal originating from intracellular structures (ROI2).

To measure the evolution of the localization of the proteins of interest over time, Pearson’s R correlation coefficients (above threshold) between YFP and FM4-64 signals were calculated with the Coloc 2 plugin included in the Fiji package (http://fiji.sc). Non-treated samples were observed 16, 40 and 64 h after protoplast transformation. CHX was added at a concentration of 50 µM (from a 200 mM stock in ethanol; Luu et al., 2012) after the first observation (20 h after transfection) to
block protein synthesis. CHX-treated samples were observed 40 and 64 h after protoplast transfection.

**cRNA synthesis and water transport assays in oocytes**

cRNA synthesis, injection of oocytes, oocyte swelling assays, and calculation of the membrane water permeability coefficient ($P_f; \text{m/s}$) were performed as described in (Fetter et al., 2004).

**Statistical analyses**

Relative PM fluorescence, Pearson’s coefficient and oocyte Pf data were submitted to an ANOVA 1 analysis, followed by a Bonferroni post test comparing all datasets, using PRISM 3.0 (http://www.graphpad.com/scientific-software/prism/). Confidence intervals with $\alpha = 0.05$ were calculated and displayed on bar charts using Microsoft Excel. Three independent experiments were performed.

**Supplemental Material**

**Figure S1** TMs are involved in the trafficking of ZmPIPs to the PM.

**Figure S2** Localization of ZmPIP2;5-TM3PIP1;2:mYFP.

**Figure S3** Localization of point-mutated ZmPIP2;5.

**Figure S4** Quantification of the signal in the plasma membrane of protoplasts expressing mutated mYFP:ZmPIP2;5 proteins.
Figure S5 The localization of mYFP:ZmPIP2;5L127F/A131M does not change over time

Figure S6 Punctate structures labelled by mYFP:ZmPIP2;1-TM3PIP1;2.

Figure S7 Point mutations of ZmPIP1;2.

Table S1 PCR primers used to create the genetic constructs encoding chimerical and mutated proteins.

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Figure Legends

Figure 1 Swapping TM3 of ZmPIP2;5 with that of ZmPIP1;2 retains the protein in intracellular structures.

A Cartoons representing the chimeric proteins composed of ZmPIP2;5, where each TM has been replaced by the corresponding TM from ZmPIP1;2. All proteins are drawn with the cytosolic domains facing down. ZmPIP2;5 and ZmPIP1;2 portions are shown in black and white, respectively. All chimeras were fused to the C-terminus of mYFP, which is not displayed for clarity purposes.

B Confocal microscopy images of maize mesophyll protoplasts transiently co-expressing mYFP-tagged ZmPIP2;5-PIP1;2 TM chimeric proteins (green) and the ER marker mCFP:HDEL (cyan). FM4-64 was added as a plasma membrane marker (red). Arrows in panel 13 indicate accumulation of the protein in punctate structures which are not labelled by mCFP:HDEL. The localization patterns of the proteins of interest are representative of a total of at least 22 cells coming from three independent experiments. Scale bars = 5 µm.

C Confocal microscopy images of a maize mesophyll protoplast transiently expressing mYFP:ZmPIP2;5-TM3PIP1;2 (green) and ST:mCFP (magenta). Arrows indicate co-localization in Golgi stacks. The images are representative of a total of 17 cells coming from two independent experiments. Scale bars = 5 µm.

Figure 2 The double mutant mYFP:ZmPIP2;5L127F/A131M is retained in intracellular structures.

A Alignment of the region exchanged between ZmPIP2;5 and ZmPIP1;2. The residues that differ between both proteins are highlighted in grey. Mutations introduced in ZmPIP2;5 are indicated below the alignment. The dashed-line box
highlights the predicted TM3. The alignment was generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

**B** Confocal microscopy images of maize mesophyll protoplasts transiently expressing wild-type or mutated mYFP:ZmPIP2;5 proteins (green). FM4-64 was added as a plasma membrane marker (red). Scale bars = 5 µm.

**C** Homology model of the ZmPIP2;5 homotetramer. One subunit is highlighted in green, with its TM3 shown in blue. The residues of ZmPIP2;5 that differ from ZmPIP1;2 in this region are represented in yellow and labelled.

**D** Confocal microscopy images of maize mesophyll protoplasts transiently expressing YFP-tagged mutated ZmPIP2;5 proteins (green) chosen on the basis of the model **C**. FM4-64 was added as a plasma membrane marker (red). Scale bars = 5 µm.

**E** Quantification of the relative mYFP fluorescence intensity in the plasma membrane of protoplasts expressing wild-type or mutated mYFP:ZmPIP2;5 proteins as shown in **D**. The Y-axis shows the fluorescence ratio between the plasma membrane and the whole cell. A minimum of 29 cells has been analyzed for each construct. Error bars are confidence intervals (a = 0.05). The letter above each bar represents statistical classes determined by a Bonferroni test (p < 0.001).

The localization patterns of the proteins of interest are representative of a total of, at least, 29 cells coming from a minimum of two independent experiments (**B, D**). Fluorescence calculations (**E**) were performed using the same dataset.

**Figure 3** Transient expression in intact leaf epidermal cells confirms localization data obtained in protoplasts.

Maize leaf epidermal cells transiently expressing **A** mYFP:ZmPIP2;5; **B** mYFP:ZmPIP1;2; **C** mYFP:ZmPIP2;1-PIP1;2Mix; **D** mYFP:ZmPIP1;2 (green) and
mCFP:ZmPIP2;5 (magenta); E mYFP:ZmPIP2;5-TM3<sub>PIP1;2</sub> (green) and mCFP:HDEL (magenta); F mYFP:ZmPIP2;5L127F/A131M (green) and mCFP:HDEL (magenta). When necessary, half of the cell is shown as the maximum projection of a Z-stack to better visualise intracellular structures (B, C, E, F). The localization patterns of the proteins of interest are representative of a total of at least 14 cells coming from a minimum of two independent experiments, except for mYFP:ZmPIP2;1-PIP1;2Mix and mYFP:ZmPIP2;5L127F/A131M for which only 4 and 6 cells were observed, respectively. Scale bars = 20 µm.

**Figure 4** Intracellular retention of ZmPIP2s due to the insertion of a PIP1 TM3 is isoform-independent.

Maize leaf epidermal cells transiently expressing A mYFP:ZmPIP2;5; B mYFP:ZmPIP2;1; C mYFP:ZmPIP1;2; D mYFP:ZmPIP1;6; E mYFP:ZmPIP2;5-TM3<sub>PIP1;6</sub> (green) and mCFP:HDEL (magenta); F mYFP:ZmPIP2;1-TM3<sub>PIP1;2</sub> (green) and mCFP:HDEL (magenta). The upper half of each cell is shown as a maximum projection of a Z-stack to visualise intracellular structures. The localization patterns of the proteins of interest are representative of a total of at least 10 cells coming from a minimum of two independent experiments, except for mYFP:ZmPIP2;5-TM3<sub>PIP1;6</sub> and mYFP:ZmPIP2;1-TM3<sub>PIP1;2</sub> for which 7 and 8 cells were observed, respectively. Scale bars = 20 µm.

**Figure 5** The TM3 of ZmPIPs is sufficient to discriminate between ER and plasma membrane localization.

Maize leaf epidermal cells expressing A mYFP:ZmPIP2;5; B mYFP:ZmPIP1;2; C the TM3 of ZmPIP2;5 fused to the mYFP; D the TM3 of ZmPIP1;2 fused to the mYFP. Cartoons in C and D represent the single TM reporter proteins. The upper half of each cell is shown as a maximum projection of a Z-stack to visualise
intracellular structures. The localization patterns of the proteins of interest are representative of a total of at least 15 cells coming from three independent experiments. Scale bars = 20 µm.

**Figure 6** Swapping the TM3 of ZmPIP1;2 with that of ZmPIP2;5 does not allow the protein to reach the plasma membrane.

**A** Cartoons representing ZmPIP1;2-N\textsubscript{PIP2;5}, ZmPIP1;2-TM3\textsubscript{PIP2;5}, and ZmPIP1;2-N+TM3\textsubscript{PIP2;5}. ZmPIP1;2 and ZmPIP2;5 are shown in white and black, respectively. The proteins are drawn with the cytosolic domains facing down. They were fused to the C-terminal end of the mYFP, which is not displayed for clarity purposes.

**B** Confocal microscopy images of maize mesophyll protoplasts transiently co-expressing mYFP:ZmPIP1;2, mYFP:ZmPIP1;2-N\textsubscript{PIP2;5}, mYFP:ZmPIP1;2-TM3\textsubscript{PIP2;5} or mYFP:ZmPIP1;2-N+TM3\textsubscript{PIP2;5} (green) and the ER marker mCFP:HDEL (magenta). Arrows on panels 4 and 7 show punctate structures that are seen only in the YFP channel. The localization patterns of the proteins of interest are representative of a total of at least 27 cells coming from three independent experiments. Scale bars = 5 µm.

**Figure 7** Water transport activity of the ZmPIP2;5-TM3\textsubscript{PIP1;2} chimera.

Water permeability coefficients of Xenopus oocytes injected with water (negative control), or cRNA encoding ZmPIP2;5 (positive control), ZmPIP2;5 and ZmPIP1;2 (positive control for the synergistic effect), ZmPIP2;5-TM3\textsubscript{PIP1;2}, or ZmPIP2;5-TM3\textsubscript{PIP1;2} and ZmPIP2;5. Error bars are confidence intervals (α = 0.05). The letter above each bar represents statistical classes determined by a Bonferroni test (p < 0.001). At least 12 oocytes injected with each cRNA were assayed, except for the
negative control (water), for which the swelling of 8 cells was recorded. Three independent experiments were performed.

**Figure 8** Working model for TM-based trafficking signals.

A Protein-protein interaction: intermediate protein. The protein of interest (green cylinder) interacts with another membrane protein (blue cylinder) via the TM-based motif (red circle), which in turn interacts with a transport protein (orange circle) that exports the complex from its compartment. The membrane of the compartment is shown in light grey.

B Protein-protein interaction: conformational change. The protein of interest interacts with another membrane protein via the TM-based motif. This interaction induces a conformational change, which exposes a classical, cytosol-exposed, export motif (yellow circle). A transport protein is then recruited by classical mechanisms.

C Protein-lipid interaction: membrane domain bulk-flow. The TM-based motif segregates the protein of interest in a specific, export-competent, domain of the membrane (dark grey). All proteins present in this domain are exported thanks to the interaction of a transport protein with classical export signals present on some of the proteins present in this membrane domain.

D Protein-lipid interaction: membrane domain segregation and conformational change. The TM-based motif segregates the protein of interest in a specific, export-competent, domain of the membrane. This induces a conformational change, releasing a classical, cytosol-exposed, sorting signal. The transport machinery is then recruited according to well-known mechanisms.
Table 1 Predicted TM length of ZmPIPs and chimeric proteins by the TMHMM 2.0 algorithm

| Isoform                  | Predicted TM length (in amino acid residues) |
|--------------------------|----------------------------------------------|
|                          | TM1   | TM2   | TM3   | TM4   | TM5   | TM6   |
| ZmPIP1;1                 | 23    | 23    | 18    | 18    | 23    | 23    |
| ZmPIP1;2                 | 23    | 23    | 18    | 18    | 23    | 23    |
| ZmPIP1;3                 | 23    | 23    | 18    | 18    | 23    | 23    |
| ZmPIP1;4                 | 23    | 23    | 18    | 18    | 23    | 23    |
| ZmPIP1;5                 | 23    | 23    | 18    | 18    | 23    | 23    |
| ZmPIP1;6                 | 23    | 23    | 23    | 18    | 23    | 23    |
| ZmPIP2;1                 | 23    | 23    | 23    | 23    | 23    | 23    |
| ZmPIP2;2                 | 23    | 23    | 23    | 23    | 23    | 23    |
| ZmPIP2;3                 | 23    | 23    | 23    | 23    | 20    | 23    |
| ZmPIP2;4                 | 23    | 23    | 23    | 23    | 20    | 23    |
| ZmPIP2;5                 | 23    | 23    | 23    | 23    | 20    | 23    |
| ZmPIP2;6                 | 23    | 23    | 23    | 23    | 20    | 23    |
| ZmPIP2;7                 | 23    | 23    | 23    | 23    | 20    | 23    |
| ZmPIP2;5-TM3ZmPIP1;2     | 23    | 23    | 23    | 20    | 23    | 23    |
| ZmPIP2;5L127F/A131M      | 23    | 23    | 23    | 20    | 23    | 23    |
| mYFP:TM3ZmPIP1;2        | 23    |      |      |      |      |      |
| mYFP:TM3ZmPIP2;5        | 23    |      |      |      |      |      |
A

B

C

```
mYFP: ZmPIP2;5 + FM4-64 + mCFP:HDEL
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```
mYFP: ZmPIP2;5-TM1PIP1;2 + FM4-64 + mCFP:HDEL
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```
mYFP: ZmPIP2;5-TM2PIP1;2 + FM4-64 + mCFP:HDEL
```

```
mYFP: ZmPIP2;5-TM3PIP1;2 + FM4-64 + mCFP:HDEL
```

```
mYFP: ZmPIP2;5-TM4PIP1;2 + FM4-64 + mCFP:HDEL
```

```
mYFP: ZmPIP2;5-TM5PIP1;2 + FM4-64 + mCFP:HDEL
```

```
mYFP: ZmPIP2;5-TM6PIP1;2 + FM4-64 + mCFP:HDEL
```

```
mYFP:ZmPIP2;5-TM3PIP1;2 + ST:mCFP
```
A mYFP: ZmPIP2;5

B mYFP: ZmPIP1;2

C mYFP: TM3\textsubscript{ZmPIP2;5}

D mYFP: TM3\textsubscript{ZmPIP1;2}
