Plant Aquaporin Trafficking

Junpei Takano, Akira Yoshinari, and Doan-Trung Luu

Abstract Aquaporins transport water and small neutral molecules across different membranes in plant cells and thus play important roles in cellular and whole plant physiology. The high diversity of intracellular localization of aquaporin isoforms is dependent on specific trafficking machineries. ER-to-Golgi trafficking of the plasma membrane intrinsic protein (PIP) isoforms has been shown to be dependent on DxE motifs in N-terminal cytosolic region, LxxxA motif in transmembrane domain 3, phosphorylation in C-terminal cytosolic region, and heteromerization. Stress-induced downregulation of the PIPs in the early secretory pathway was uncovered. Subsets of PIPs and Nodulin 26-like intrinsic proteins (NIPs) showed polar localization in the plasma membrane (PM) in certain cell types for directional transport of water and small neutral molecules such as boric acid and silicic acid. Latest techniques to study the mobility of PIPs revealed immobile nature in the plane of the PM and constitutive cycling between the PM and the endosomes. The roles of clathrin- and microdomain-dependent endocytosis for PIPs were uncovered. When challenged by stress conditions, some PIPs and TIPs showed quick relocalization probably to adjust water status. Vacuolar trafficking of different TIPs was shown to follow multiple routes dependent or independent of Golgi apparatus. These findings greatly advanced our understanding of the trafficking machineries of plant aquaporins, as significant models of plant membrane proteins.

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1 Introduction

Aquaporins are membrane channel proteins permeating water and/or small neutral molecules. The aquaporins share a basic structure comprising six transmembrane domains linked by five loops with cytosolic N- and C-termini and assembled as tetramers in which each monomer functions as a channel (Törnroth-Horsefield et al. 2006). In plants, aquaporins in different membrane compartments play different roles in various processes at cellular and whole plant levels. Therefore, how aquaporins are transported to their destination is a fundamental question.

Plant aquaporins are classified to seven subfamilies dependent on sequence similarities (see chapter "Structural Basis of the Permeation Function of Plant Aquaporins"). As indicated by the names, the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) function in the plasma membrane (PM) and the vacuolar membrane, respectively. Many of them function in the transport of water, and some of them transport small uncharged molecules besides water. The prototype of Nodulin 26-like intrinsic proteins (NIPs) was identified in the peribacteroid membrane of nitrogen-fixing nodules of legume roots (Fortin et al. 1985), while NIPs were found in the PM and the endoplasmic reticulum (ER) in non-legume plants (see chapter "The Nodulin 26 Instrinsic Protein Subfamily"). Some members of NIPs have efficient transport activity of small uncharged solutes such as boric acid and silicic acid (Ma et al. 2006; Takano et al. 2006; see chapter "Plant Aquaporins and Metalloids"). The small intrinsic proteins (SIPs) were localized to the ER and shown to facilitate water transport (Ishikawa et al. 2005), although their physiological functions remain unknown. The X intrinsic proteins (XIPs) were identified in moss *Physcomitrella patens* and some dicots including poplar and Solanaceae plants tomato and tobacco (Danielson and Johanson 2008; Lopez et al. 2012; Bienert et al. 2011). Solanaceae XIPs were localized in the PM and shown to facilitate transport of various small neutral solutes (Bienert et al. 2011). In moss *P. patens*, additional two subfamilies GlpF-like intrinsic proteins (GIPs) and hybrid intrinsic protein (HIP) were found, although their subcellular localization remains unstudied (Danielson and Johanson 2008; Gustavsson et al. 2005).

As listed above, the members of each subfamily generally share similar patterns of subcellular localization. However, complex patterns of localization have been reported for some isoforms. For example, dual localization was observed for a PIP (*Nt*AQP1) in the PM and the chloroplast inner membrane in leaf mesophyll cells (Uehlein et al. 2008), for AtTIP3;1 and 3;2 in the tonoplast and the PM during seed maturation and germination (Gattolin et al. 2011), and for TIPs in the tonoplast and the symbiosome membrane in developing root nodule of *Medicago truncatula* (Gavrin et al. 2014). Interestingly, some PIPs and NIPs show polar localization in the PM, which is considered to be important for directional transport of water or small uncharged molecules in specific cell types. Some PIPs and TIPs also show dynamic changes of localization in response to environmental conditions. The differential localization between isoforms and the changes of localization are apparently important for plants to adapt to the changing environment. Considering the

shared basic structure of aquaporins, the variable localization should be determined by signals embedded in amino acid sequences or specific conformations. The present chapter focuses on the mechanisms and physiological significances of intracellular trafficking of plant aquaporins.

1.1 PM Trafficking of PIPs

1.1.1 ER-to-Golgi Trafficking of PIPs Dependent on Trafficking Signals and Heteromerization

The membrane proteins synthesized by ribosomes are co-translationally inserted into the ER in which they are folded with the help of chaperons. Generally, the membrane protein destined to the PM, tonoplast, or other post-Golgi membrane compartments is transported in the vesicular trafficking network starting from ER to Golgi trafficking. The ER-to-Golgi trafficking is regulated by three complementary mechanisms: retention of immature proteins in the ER, selective packaging of mature proteins into COPII vesicle (ER exit), and retrieval from the Golgi apparatus of immature cargo proteins through COPI vesicles (Geva and Schuldiner 2014). The improperly folded proteins in the ER can be transported into the cytoplasm and degraded by the process known as ER-associated degradation (ERAD; Liu and Li 2014). In the step of ER exit, some cargoes are selectively packaged by direct or indirect binding to the Sec24 subunit of the COPII complex. Therefore, the binding sites of cargo proteins to Sec24 or to cargo receptors, which mediate the interaction to Sec24, function as signals for ER exit. The signals in animal and yeast systems range from diacidic DxE motifs (where x is an undetermined amino acid residue) to conformational epitopes and posttranslational modifications (Venditti et al. 2014; Geva and Schuldiner 2014).

In plant systems, PIP is one of the best-studied cargoes for the ER-to-Golgi trafficking. In ZmPIP2;4 and ZmPIP2;5, a diacidic DIE motif is present at residues 4–6 (Zelazny et al. 2009). In maize mesophyll protoplast, ZmPIP2;4 and PIP2;5 were targeted to the PM when fused to fluorescent proteins, while ZmPIP2;4AIA and ZmPIP2;5AIA were retained in ER (Zelazny et al. 2009). A fluorescence resonance energy transfer (FRET) analysis showed that the ZmPIP2;5AIA mutant still had the ability to form oligomers. These results indicated the importance of the DIE motif not for oligomerization but for ER exit (Fig. 1). Furthermore, in root epidermal cells of *Arabidopsis*, replacement of the DVE motif of AtPIP2;1 to AVE, DVA, AVA, EVE, or DVD resulted in ER retention (Sorieul et al. 2011). This result suggested the requirement of the strict DxE motif rather than just a diacidic motif. The DxE motifs were shown to be a direct binding site to Sec24 in yeast Golgi protein Sys1 (Votsmeier and Gallwitz 2001; Mossessova et al. 2003; Miller et al. 2003). In plant cells, the DxE motifs were found to be important in ER-to-Golgi trafficking of the potassium channel KAT1 (Mikosch et al. 2006) and Golgi-localized proteins GONST1 and CASP (Hanton et al. 2005). Importantly, KAT1 was shown to interact

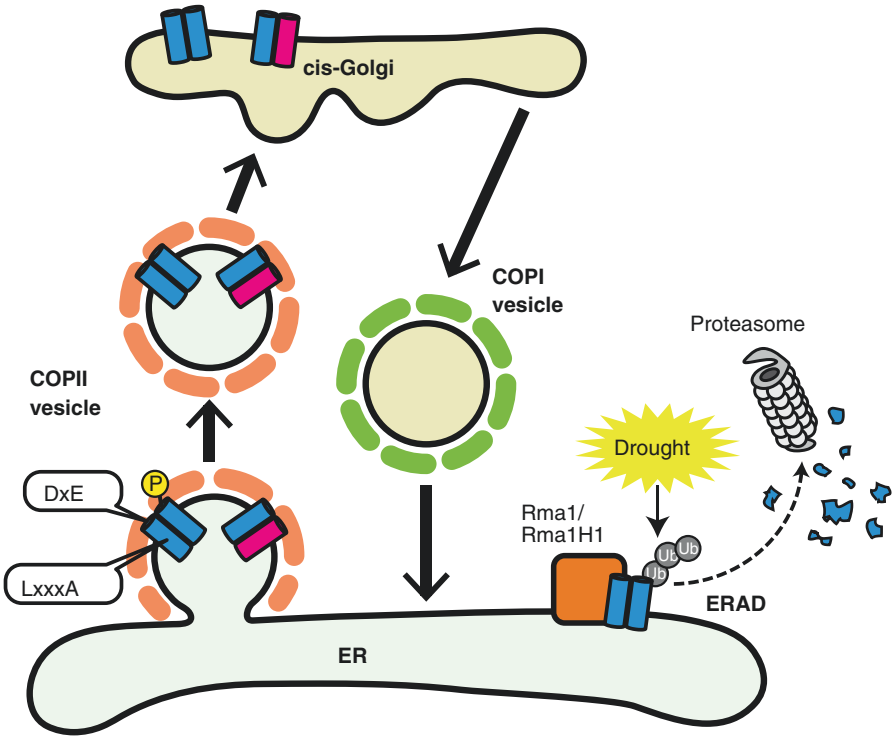


Fig. 1 A model of ER-Golgi trafficking of PIPs. The DxE motif in the N-terminal region, LxxxA motif in the transmembrane domain 3, and phosphorylation of a serine residue in the C-terminal region are implicated for the ER exit of PIP2 isoforms (*magenta cylinders*). PIP1s (*blue cylinders*) alone cannot be targeted to the PM by ER retention/retrieval mechanism, while hetero-oligomers with PIP2 isoforms are targeted to the PM via Golgi apparatus. Under drought stress, E3 ligases Rma1/Rma1H1 in the ER membrane ubiquitinate PIP2s for degradation via the ERAD pathway

with Sec24 by a FRET analysis (Sieben et al. 2008). Therefore, it is likely that some PIP2s are recruited into COPII vesicles via interaction of the DxE motif to Sec24.

The ER-to-Golgi trafficking of PIPs first attracted attention by the finding that the heteromerization of PIP1 and PIP2 isoforms allows ER-to-Golgi trafficking of PIP1s (see chapter "Heteromerization of Plant Aquaporins", Fig. 1). In *Xenopus laevis* oocytes, expression of the maize PIP2s but not PIP1s increased membrane water permeability (P_f) (Chaumont et al. 2000). In this system ZmPIP1;2-GFP was only slightly detected in the PM when expressed alone, while it was significantly increased by co-expression of ZmPIP2;5 (Fetter et al. 2004). ZmPIP1;2 and ZmPIP2;5 were shown to physically interact and synergistically increase the water permeability of the oocytes. The synergistic effect in the oocytes has been reported for PIP1s and PIP2s in various plant species (Chaumont and Tyerman 2014). Subsequently, analysis in maize mesophyll protoplasts showed that ZmPIP1 fusion proteins were retained in the ER when expressed alone, while relocalized to the PM when co-expressed with ZmPIP2s (Zelazny et al. 2007). The physical interactions

of ZmPIP1 and ZmPIP2 were demonstrated by a FRET analysis in the protoplasts and co-immunoprecipitation in maize roots and suspension cells. These results suggested that heteromerization with PIP2s is required for the ER-to-Golgi trafficking of PIP1s. This view was confirmed by experiments in *Arabidopsis* roots using AtPIP2;1-GFP and its E6D (DVD) variant which is retained in ER (Sorieul et al. 2011). Co-expression of AtPIP2;1-GFP with AtPIP1;4-mCherry resulted in colocalization of these fusion proteins in the PM, while AtPIP2;1-GFP DVD variant resulted in ER retention of both fusion proteins. Importantly, *Arabidopsis* plants overexpressing the ER-retained variants of AtPIP2;1-GFP significantly reduced root hydraulic conductivity (Lp_r). The ER-retained AtPIP2;1-GFP probably interacted also with other endogenous PIPs and hampered their trafficking to the PM.

An important question underlying the ER-to-Golgi trafficking of PIP1s is whether PIP1s possess ER retention/retrieval signal or do not possess an ER exit signal. It was tested whether the DxE motif found in PIP2s was sufficient to confer ER-to-Golgi trafficking of ZmPIP1;2. The replacement of N-terminal cytosolic region of ZmPIP1;2 to that of ZmPIP2;5 that contains the DxE motif did not confer PM targeting (Zelazny et al. 2009). This result suggests either DxE alone is not sufficient for ER exit or the existence of an ER retention/retrieval signal in other regions of PIP1s.

To further examine the signal for ER-to-Golgi trafficking of PIPs, a protein domain swapping of ZmPIP1;2 and ZmPIP2;5 was conducted and demonstrated the importance of the transmembrane domain 3 (TM3, Chevalier et al. 2014). Furthermore, a LxxxA motif in the TM3 of ZmPIP2;5 was identified to be required for the PM targeting. In this study, chimeric proteins unable to target to the PM by substitution of TM3 (such as mYFP-ZmPIP2;5-$TM3_{PIP1;2}$) were localized in the ER and in punctate structures. The punctate structures overlapped with a Golgi marker and thus raised a question whether the TM3 is involved in the ER-to-Golgi and/or PM trafficking from the Golgi apparatus. The authors suggested that the partial Golgi localization might be a consequence of ER leakage dependent on the presence of the ER exit DxE motif in the N-terminal region of ZmPIP2;5. Indeed, fewer punctate structures were observed for mYFP-ZmPIP2;1-$TM3_{PIP1;2}$, which has no functional DxE motif. Alternatively, the punctate structures overlapping with a Golgi marker might be actually endoplasmic reticulum export sites (ERESs). The ERES and Golgi are often associated and thus are difficult to be distinguished by fluorescent microscopy (da Silva et al. 2004; Takagi et al. 2013). Taken together, it is most likely that the TM3 is required for ER-to-Golgi but not for later secretory trafficking (Fig. 1). The TM-based signal is possibly recognized by a transmembrane cargo receptor that binds to both cargo and Sec24 for packaging the cargo into the COPII vesicles (Dancourt and Barlowe 2010; Cosson et al. 2013; Chevalier and Chaumont 2014). A recent study identified rice cornichon (OsCNIH1), a homolog of cargo receptors Erv14p in yeast and cornichons in *Drosophila* and mammals, as a possible cargo receptor for the Golgi-localized sodium transporter OsHKT1;3 (Rosas-Santiago et al. 2015). In this study, AtPIP2;1 was used as a negative control for interaction of OsCHIH1 and OsHKT1;3 by bimolecular fluorescence complementation (BiFC) assay in tobacco leaves. There might be a specific cargo receptor recognizing the LxxxA motif in the TM3 of PIP2s.

It should be noted that replacement of both the N-terminal region and TM3 of ZmPIP1;2 to those of ZmPIP2;5 did not confer the PM targeting (Chevalier et al. 2014). One possible explanation is that ZmPIP1;2 possesses an ER retention or Golgi-to-ER retrieval signal in a region other than the N-terminus and TM3. The heteromerization of PIP1s with PIP2s might mask the retention/retrieval signal and promote ER-to-Golgi trafficking. For example, arginine-based retrieval signals were identified in cytosolic regions of ATP-sensitive K^+ channels, GABA receptors, and kainate receptors in mammals (Zerangue et al. 1999; Margeta-Mitrovic et al. 2000; Ren et al. 2003). ATP-sensitive K^+ channels have an octameric stoichiometry consisting of four pore-lining inward rectifier α-subunits and four regulatory β-subunits. The arginine-based retrieval signal of each subunit prevents the PM trafficking of unassembled channels, while the octamerization masks the signal and allows the PM trafficking (Zerangue et al. 1999). The arginine-based signal was shown to be recognized by the COPI vesicle coat at the Golgi apparatus for retrieval to the ER (Michelsen et al. 2007). Similar regulations by heteromerization, and with retrieval signals in transmembrane domain, were reported for the yeast iron transporter Ftr1 and Fet3 and mammalian NMDA receptors (Sato et al. 2004; Horak et al. 2008).

1.1.2 ER-to-Golgi Trafficking of *Arabidopsis* PIP2;1 Dependent on Phosphorylation

Another identified factor for ER-to-Golgi trafficking of PIPs is phosphorylation in the C-terminal region of AtPIP2;1 (Prak et al. 2008). A phosphoproteomic analysis identified two phosphorylation sites (S280 and S283) in the C-terminal region of AtPIP2;1. An S280A mutation did not affect the PM localization of AtPIP2;1; however, S283A mutation caused apparent ER retention. Consistently, a phosphomimic mutation S283D allowed PM localization. These results suggest that the phosphorylation of S283 is involved in ER-to-Golgi transport, although the mechanism is as yet unsolved (Fig. 1). The phosphorylation might increase affinity to a COPII subunit for packaging or mask a signal for ER retention/retrieval.

1.1.3 Downregulation of *Arabidopsis* PIP2s in Early Secretary Pathway

Another posttranslational modification, that of ubiquitination, was shown to control the amount of AtPIP2;1 trafficking to the PM (Lee et al. 2009). Rma1H1 from hot pepper and Rma1 from *Arabidopsis* are homologs of a human RING membrane-anchor 1 E3 ubiquitin ligase. Overexpression in *Arabidopsis* protoplasts of Rma1H1 and Rma1, which are both localized in the ER membrane, provoked retention of AtPIP2;1 in the same compartment and reduced its total amount. This reduction effect was inhibited by MG132, a proteasome inhibitor, and also by RNA interference approach targeting *Rma* homologs. Rma1H1 physically interacted with AtPIP2;1, and overexpression of Rma1H1 in transgenic *Arabidopsis* plants resulted

in decreased total amount and increased ubiquitination of AtPIP2;1. Therefore, it is likely that Rma1H1 and Rma1 ubiquitinate PIPs to induce degradation by the ERAD process (Fig. 1). Various abiotic stresses, including dehydration, increased the mRNA level of *Rma1H1* in hot pepper plants, and its overexpression in *Arabidopsis* plants greatly enhanced tolerance to drought stress (Lee et al. 2009). Therefore, Rma1H1 and Rma1 are important factors controlling the level of PIPs at the ER in response to various stresses.

In addition, abiotic stress-induced multi-stress regulator was shown to reduce the accumulation of AtPIP2;7 in the PM (Hachez et al. 2014b). The regulator tryptophan-rich sensory protein/translocator (TSPO) is localized in the Golgi apparatus and is downregulated through an autophagic pathway (Vanhee et al. 2011). BiFC analysis showed interaction of TSPO with AtPIP2;7 in the ER and the Golgi apparatus. Their co-expression in transgenic plants or induction of expression of endogenous TSPO by ABA treatment decreased the level of AtPIP2;7 (Hachez et al. 2014b). These data suggest that the PM trafficking of AtPIP2;7 is downregulated by TSPO-mediated degradation via the autophagic pathway in response to abiotic stresses. In *Arabidopsis*, PIP transcripts and protein levels were generally downregulated upon drought stress (Alexandersson et al. 2005). The various regulations at ER and/or Golgi levels in addition to the mRNA level can fine-tune the abundance of PIPs in the PM.

1.1.4 PM Trafficking of PIPs Dependent on SNAREs and a Rab GTPase

Recent studies have uncovered the role of molecules involved in the trafficking to the PM of cargoes such as PIPs. For instance, regulation by SNAREs (soluble N-ethylmaleimide-sensitive factor protein attachment protein receptors) of the syntaxin family has been established (Besserer et al. 2012; Hachez et al. 2014a). The PM-localized SNARE isoform SYP121 has been shown to mediate the trafficking of vesicles between intracellular compartments and the cell surface (Geelen et al. 2002; Tyrrell et al. 2007). Interestingly, the overexpression of a dominant-negative cytosolic fragment of SYP121 (SYP121-Sp2) could impair the targeting to the PM of the K^+ channel KAT1, but not of the H^+-ATPase PMA2 (Sutter et al. 2006; Tyrrell et al. 2007). These results show that such a strategy of dominant-negative expression could be informative and indicate that SYP121 syntaxin exhibits a function for targeting to the PM of specific cargoes through vesicle fusion. Indeed, in maize mesophyll protoplasts, co-expression of an mYFP-ZmPIP2;5 construct and the SYP121-Sp2 impaired the targeting to the PM of the aquaporin isoform (Besserer et al. 2012). As a control, the full-length SYP121 did not exhibit any phenotype. Moreover, expression of Sp2 fragment of either the syntaxins in the PM SYP71 and SYP122 or the syntaxin in the prevacuolar compartment SYP21 had no effect on targeting of the ZmPIP2;5 construct, indicating the specific function of SYP121 on PIP targeting. Functional analysis of the role of SYP121 has been performed by using protoplast swelling assays. Here, protoplasts expressing the ZmPIP2;5 construct alone or co-expressed with the full-length SYP121 exhibited P_f values higher

than protoplasts co-expressing ZmPIP2;5 construct with SYP121-Sp2 fragment or expressing only SYP121-Sp2 fragment or mock protoplasts. This series of experiments showed that SYP121 can regulate the function of an aquaporin by controlling the targeting. The authors noticed that although impaired, targeting of the ZmPIP2;5 construct to the PM was not negligible. However, they did not record any increase in P_f consistent with the presence of PIPs in the PM. This observation suggested that SYP121 could have a regulatory function on the intrinsic activity of PIPs. Indeed, the authors used *Xenopus* oocytes swelling assays and observed that when they co-expressed SYP121-Sp2 fragment and ZmPIP2;5 construct, there was reduced P_f values compared to oocytes expressing ZmPIP2;5 construct alone or co-expressed with the full-length SYP121. Importantly, when co-expressed in oocytes with full-length SYP121 or SYP121-Sp2 fragment, the ZmPIP2;5 construct was still localized in the PM, indicating that reduced P_f was related to an inhibition of ZmPIP2;5 intrinsic activity. To go further, affinity chromatography purification, BiFC, and FRET also indicated a direct physical interactions between ZmPIP2;5 and SYP121. This series of experiments shows that SYP121 not only impairs the targeting of PIPs but also physically interacts with them and inhibits their intrinsic activity (Besserer et al. 2012). Such a dual function of SYP121 in trafficking and activity regulation has been also demonstrated on AKT1/KC1 K^+ channel complex (Grefen et al. 2010).

Another SNARE, SYP61, mainly localized in the *trans*-Golgi network/early endosomal compartments (TGN/EE), cycles between this compartment and the PM and has been shown to colocalize with AtPIP2;7 (Hachez et al. 2014a). Physical interactions between SYP61 and AtPIP2;7 have been established. In the *syp61* mutant background, it was observed that a miss targeting of an overexpressed AtPIP2;7 construct to globular or lenticular structures corresponds to ER-derived stacked membrane arrays, suggesting a key function of this SNARE in targeting of PIPs to the PM. Importantly, SYP61 and SYP121 belong to the same complex, and it is believed that they might regulate PIP trafficking (Besserer et al. 2012; Hachez et al. 2014a, b).

As for SNAREs, there is evidence that any molecules with a role in the trafficking between the TGN/EE and the PM might control PIP targeting. For instance, RAS Genes From Rat BrainA1b (RabA1b) is a small GTPase localized in TGN/EE, and the corresponding mutant has been screened for defects in exocytosis (Feraru et al. 2012). Interestingly, the fungal toxin Brefeldin A (BFA) inhibits intracellular trafficking, mainly secretion, and causes accumulation of PM proteins into large aggregates known as BFA compartments or BFA bodies (Geldner et al. 2001). BFA is an inhibitor of a subset of guanine nucleotide exchange factors (GEFs) for the ADP ribosylation factor (ARF) GTPase, which function in recruitment of coat components of vesicles. Washout of BFA allows the gradual disappearance of PM proteins from the BFA bodies and their relocation to the PM. Interestingly, in *bex5*, a mutant of *RabA1b*, washout experiments did not completely suppress the labeling of aggregates by an AtPIP2;1 construct, suggesting a role of this small GTPase in the exocytosis of PIPs (Feraru et al. 2012).

1.2 Dynamic Properties of PIPs

1.2.1 Approaches Developed to Study the Lateral Diffusion of PIPs

Different microscopy approaches such as variable-angle epifluorescence microscopy (VAEM), also named total internal reflection fluorescence microscopy, in combination with fluorescence correlation spectroscopy (FCS) or super-resolution, provided recent quantitative insights into the immobility of AtPIPs in the plane of the PM and their endocytosis ((Hosy et al. 2015; Li et al. 2011), also see (Li et al. 2013) for a review of these techniques in plant cells). However, it has to be mentioned that fluorescence recovery after photobleaching (FRAP) technique was used in pioneer studies to address the mobility of membrane proteins such as AtNIP5;1 and AtPIP2;1 (Takano et al. 2010; Luu et al. 2012; Martinière et al. 2012).

VAEM in combination with single particle tracking (sptVAEM) allowed to track single particles of AtPIP2;1-GFP constructs expressed in epidermal cells of *Arabidopsis* roots at a high spatiotemporal resolution. In a pioneer study using an AtPIP2;1 construct, four types of trajectories and modes of diffusion were examined: Brownian (33.7 ± 3.3 %), directed (27.5 ± 2.4 %), restricted (17.5 ± 2.1 %), or mixed trajectories (21.2 ± 3.1 %) (Li et al. 2011). It was also concluded that the diffusion coefficient of AtPIP2;1-construct particles (2.46×10^{-3} $\mu m^2.s^{-1}$) was ten times lower than that for LTi6a (2.37×10^{-2} $\mu m^2.s^{-1}$), confirming the immobility or the extremely low lateral diffusion for the aquaporins in the PM. FCS, by measuring the intensity of the fluorescence and its variation in a volume, allowed an estimation of the density of the AtPIP2;1-GFP molecules in the PM of 30.3 ± 5.1 μm^{-2} in cells under resting conditions.

VAEM combined with high-density SPT and photoactivated localization microscopy (sptPALM) was used in epidermal cells of *Arabidopsis* roots expressing fusions of mEos2 with PM (AtPIP2;1, LTi6a) or tonoplast (AtTIP1;1) proteins (Hosy et al. 2015). This imaging method provides images with a spatial resolution beyond the diffraction limit, i.e., at the nanometer level, and thus is so-called super-resolution microscopy (Manley et al. 2008). It also provided the diffusion coefficient for each construct: 4.7×10^{-3} μm^2 s^{-1} (AtPIP2;1), 7.7×10^{-2} μm^2 s^{-1} (LTi6a), and 4.8×10^{-1} μm^2 s^{-1} (AtTIP1;1). This analysis revealed that AtPIP2;1 has a mobility seven- to 19-fold lower than that of its mammalian homolog AQP1 (Crane and Verkman 2008) and that LTi6a exhibited a mobility similar to lipids in the PM of plant cells (Dugas et al. 1989). Also, the values obtained for AtPIP2;1 and LTi6a are consistent with previous data by FRAP analysis (Luu et al. 2012) and stressed the immobile nature of the aquaporin. Considering the value of 2.5×10^{-2} μm^2 s^{-1} as a threshold between the immobile and mobile fractions, it was observed that only 22 % of AtPIP2;1 particles were mobile. The authors addressed the molecular mechanisms involved in the high confinement of AtPIP2;1. In an analysis with sptPALM in combination with pharmacological interference, they treated the cells with either oryzalin, latrunculin B, or cytochalasin D, used as an inhibitor of microtubule polymerization, an actin polymerization inhibitor, or an actin depolymerization

inducer, respectively. Whereas treatments with oryzalin did not change the confinement of AtPIP2;1 particles, drugs targeting actin filaments provoked a significant increase in their mobility, suggesting that actin is one of the molecules involved in this confinement (Fig. 2a). Gradual plasmolysis of the cells allowing to progressively separate the PM and the cell wall provoked a gradual increase in the mobility of AtPIP2;1 particles. Since plasmolysis may also disrupt actin filaments, it is suggested that these molecules and the cell wall, by its close association with the PM, can immobilize aquaporin AtPIP2;1.

1.2.2 Approaches Developed to Study the Constitutive Cycling

PIPs as other PM cargoes are subjected to constitutive cycling. As canonical proteins of the PM, they have been used as reference markers in several studies which employed pharmacological interference on the constitutive cycling. For instance, the tyrosine analogue, Tyrphostin A23 (A23), is believed to prevent the interaction between the μ2 subunit of the clathrin-binding adaptor protein AP2 complex and cytosolic motifs of cargo PM proteins (Banbury et al. 2003). Indeed, A23 treatments of *Arabidopsis* root cells prevented the labeling of BFA bodies byAtPIP2;1-GFP construct, suggesting an inhibition of its endocytosis by a clathrin-dependent pathway (Dhonukshe et al. 2007). Consistent with this explanation, A23 treatments also increased the density of AtPIP2;1 constructs in the PM (Li et al. 2011). Next, the synthetic auxin analogue, 1-naphthaleneacetic acid (NAA), has also been shown to inhibit the endocytosis of several PM cargoes including AtPIP2;1 (Dhonukshe et al. 2008; Paciorek et al. 2005; Pan et al. 2009). Wortmannin (Wm), an inhibitor of phosphatidylinositol-3-phosphate and phosphatidylinositol-4-phosphate kinases, induces in *Arabidopsis* root cells clustering, fusion, and swelling of TGN vesicles and late endosomes/multivesicular bodies (LE/MVB) (Jaillais et al. 2006; Niemes et al. 2010; Takáč et al. 2012). The labeling of Wm-induced enlarged endosomes by AtPIP2;1 indicates that this aquaporin traffics between TGN and LE/MVB (Jaillais et al. 2006). The polyene antibiotic filipin that has also sterol fluorescence detection properties has been used to show a role for membrane sterols in the endocytosis of AtPIP2;1 (Kleine-Vehn et al. 2006).

As a complement of the pharmacological interference approach using A23, a dominant-negative mutant strategy was employed to prove the role of clathrin-dependent pathway on AtPIP2;1 endocytosis (Dhonukshe et al. 2007). The clathrin hub corresponding to the C-terminal part of clathrin heavy chain was overexpressed in plant cells, provoking the binding to and titering away the clathrin light chains, thus making them unavailable for clathrin cage formation. AtPIP2;1 was unable to label anymore BFA bodies, indicating a disruption of its endocytosis by the clathrin-dependent pathway.

FRAP and photoactivation of a photoactivatable version of GFP (*pa*GFP) were classical techniques for the analysis of the lateral diffusion of membrane proteins. Their use has been extended to study the cycling of PM aquaporins (Luu et al. 2012). The low lateral mobility of AtPIP2;1 in the plane of the PM compared to a variant retained into the ER membranes has been previously shown (Sorieul et al. 2011). FRAP experiments were performed using *Arabidopsis* root epidermal cells stably

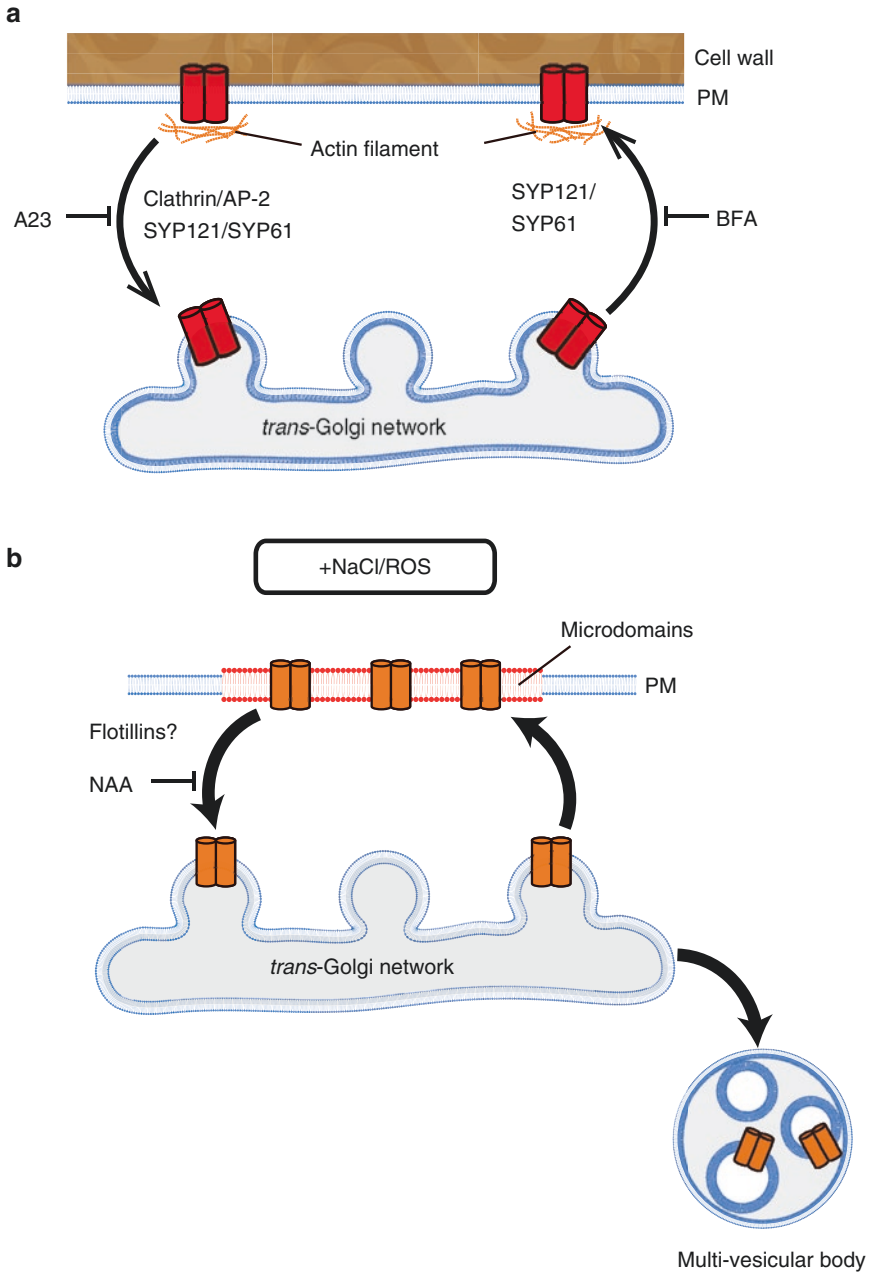


Fig. 2 Dynamic properties of plant PM aquaporins. (**a**) In resting conditions, PIPs undergo constitutive cycling from the TGN to the surface of the cell. SNAREs and clathrin-mediated endocytosis are involved. Their lateral mobility is restricted by a combination of actin filaments and cell wall interactions. (**b**) A marked change in PIP dynamics occurs in stimuli-challenged conditions, where enhanced cycling and higher mobility in the plane of the PM could be observed, concomitant to a trafficking to MVBs. Importantly, a clathrin-independent pathway overcomes the clathrin-mediated pathway, hypothetically involving flotillins. Chemicals used in pharmacological interference approach to block trafficking processes are indicated

expressing fusions of GFP with AtPIP1;2, AtPIP2;1, LTi6a, or AtTIP1;1 proteins. The fluorescence signal of AtPIP constructs had recovered below 60 % even after 30 min, whereas LTi6a construct exhibited a recovery of ~55 % at only 50 sec and almost completely at 7 min after photobleaching. Kymograms, representing as a function of time the recovery of the fluorescence signal along a line which crosses the bleached region, also showed slow dynamics of AtPIP constructs in the plane of the PM compared to LTi6a. Photoactivation of *pa*GFP in LTi6a or AtPIP2;1 constructs confirmed their fast or slow dynamics, respectively. The whole set of data indicates a significant immobility of AtPIP1;2 and AtPIP2;1 constructs in the PM of *Arabidopsis* root epidermal cells. However, a marked FRAP response was observed over the 30 min of records. This cannot be explained only by the lateral diffusion of the AtPIP1;2 and AtPIP2;1 constructs, and other processes must be invoked. Interestingly, the fluorescence recovery curves of AtPIP1;2 and AtPIP2;1 constructs were biphasic, with a fast process up to 60 s and a slower process that developed for up to 30 min and beyond. Thus, the authors suggested that the first process could account for a fast cytoplasmic streaming which drags intracellular compartments containing AtPIP1;2 and AtPIP2;1 constructs into the initially bleached region. Because of the immobility of the AtPIP1;2 and AtPIP2;1 constructs in the PM, it was suggested that constitutive cycling could account for the slower recovery observed during the next 30 min. They came to the conclusion that the early recovery phase could account for an estimation of the intracellular labeling of the constructs, and the constitutive cycling rate could be accounted by the slow recovery phase including sequential steps in endocytosis, sorting in the endosomes, and exocytosis. Pharmacological interference validated this tentative model. For instance, A23 treatments not only reduced the labeling of endomembrane compartments by AtPIP constructs but provoked a reduced recovery of fluorescence at 30 min and beyond. A similar response was found with NAA and BFA treatments. NAA did not change noticeably the intensity of endosomal labeling in these experiments, suggesting that the site of NAA inhibition may be downstream of endocytotic uptake and upstream of the TGN. Importantly, this new approach using FRAP experiments provided a higher quantitative resolution of constitutive cycling than standard confocal microscopy.

1.2.3 Stress-Induced Change of PIP Dynamics and Localization

Salt treatments (150 mM NaCl) or exogenous application of salicylic acid (0.5 mM SA) concomitantly inhibits the L_{p_r} and provokes an accumulation of reactive oxygen species (ROS) (Boursiac et al. 2008, 2005). These effects are fast and associated with the internalization of the analyzed AtPIP constructs. Importantly, the internalization could be counteracted by ROS-scavenging catalase treatments, indicating the central role of ROS in the cellular relocalization of aquaporins. ROS, and more specifically hydrogen peroxide (H_2O_2), have well-known cell-signaling functions in plants (Apel and Hirt 2004; Foyer and Noctor 2009). An in-depth cell biology analysis of PIP trafficking under ROS stimulus was described by combining several approaches in root cells of *Arabidopsis* (Wudick et al. 2015). Subcellular fractionation

experiments revealed that H_2O_2 treatments induced an enhanced localization of AtPIPs in intracellular membranes, as early as 15 min. In parallel, a twofold increase in the lateral mobility of AtPIP2;1 particles and a 40 % decrease of their density in the PM were observed by sptVAEM and FCS, respectively. This indicates not only a change in the mobility of AtPIP2;1 at the cell surface but also a change in their subcellular localization. This change was clearly observed as labeling of intracellular spherical bodies of ~1–2 μm, dot-shaped-like structures, and a more diffuse labeling. Co-expression of AtPIP2;1 constructs with endomembrane markers or staining with the lipophilic styryl dye FM4-64 identified the LE/MVB as the potential compartments of relocalization. This result suggested the possibility of a stress-induced degradation process similar to well-described ones occurring for the borate transporter BOR1, the flagellin receptor FLS2, the iron transporter IRT1, or the auxin efflux facilitator AtPIN2 (Takano et al. 2005; Robatzek 2007; Barberon et al. 2011; Laxmi et al. 2008). Furthermore, a sequestration process could be possible as for the K^+-channel AtKAT1, which is reversibly sequestered upon ABA (Sutter et al. 2007). However, a series of experiments including biochemical and microscopy approaches did not support either the degradation or the reversible sequestration process, suggesting a stress-induced sequestration of PM aquaporins in LE/MVB that is strictly disconnected from vacuolar degradation. The labeling of spherical bodies observed in these stress-induced conditions may correspond to enlarged MVBs described in *Arabidopsis* mutants of SAND-CCZ1 complex involved in the trafficking toward lysosome/vacuole (Ebine et al. 2014; Singh et al. 2014).

Milder concentration of salt (100 mM NaCl) not only significantly reduces Lp_r of *Arabidopsis* plants but affects the distributions of PIPs and TIPs (Boursiac et al. 2005). The effects on PIP dynamics under these mild salt stress conditions were analyzed by using approaches to study the lateral diffusion of PIPs in the plane of the PM and their cycling in root epidermal cells of *Arabidopsis* (Li et al. 2011; Luu et al. 2012). sptVAEM revealed an increase by twofold of the diffusion coefficient of AtPIP2;1 particles and by 60 % proportion of particles with a restricted diffusion mode. Furthermore, the density of AtPIP2;1 construct in the PM was reduced by 46 %. This reduction could be prevented by pretreatments with A23 or methyl-β-cyclodextrin (MβCD), a sterol-disrupting reagent. Thus, these data support the hypothesis that under standard conditions, the endocytosis of PIPs occurs via predominantly the clathrin-dependent pathway, but it is enhanced under salt stress and occurs via both clathrin-mediated and membrane microdomain-mediated pathways (Li et al. 2011). A dissection of the cycling properties of AtPIP1;2 and AtPIP2;1 under salt stress was investigated using a FRAP approach. A twofold increase in the amplitude of fluorescence recovery curves was observed, suggesting an enhanced cycling of PIPs. To validate this hypothesis, the authors observed an earlier labeling of BFA bodies by AtPIP constructs under a concomitant treatment with NaCl and BFA. As a complementary experiment, an earlier decrease in the labeling of the BFA bodies was observed in the washout of BFA with NaCl solutions. A combination of FRAP and pharmacological interference approaches showed that PIP cycling under salt stress was blocked by NAA but had become insensitive to A23. The whole set of data suggests that salt stress enhances both endocytosis and exocytosis

of PIPs, and therefore their cycling, and that an endocytotic mechanism independent of clathrin-mediated pathway could occur (Luu et al. 2012). This hypothesis supports the possible involvement of a membrane microdomain-dependent pathway previously uncovered in plant cells (Li et al. 2011).

As previously shown, posttranslational modifications on PIPs such as phosphorylation could alter ER-to-Golgi trafficking in resting conditions. Such a regulatory role has also been uncovered in salt stress conditions (Prak et al. 2008). Salt treatments for 2–4 h caused 30 % decrease in the abundance of phosphorylated S283 form AtPIP2;1. S280A mutation of GFP-AtPIP construct was associated with the labeling of "fuzzy" structures, whereas the S283D mutation allowed a labeling of spherical bodies. An adaptive response of plant root to limit the delivery of PIPs to the PM upon salt stress could be invoked.

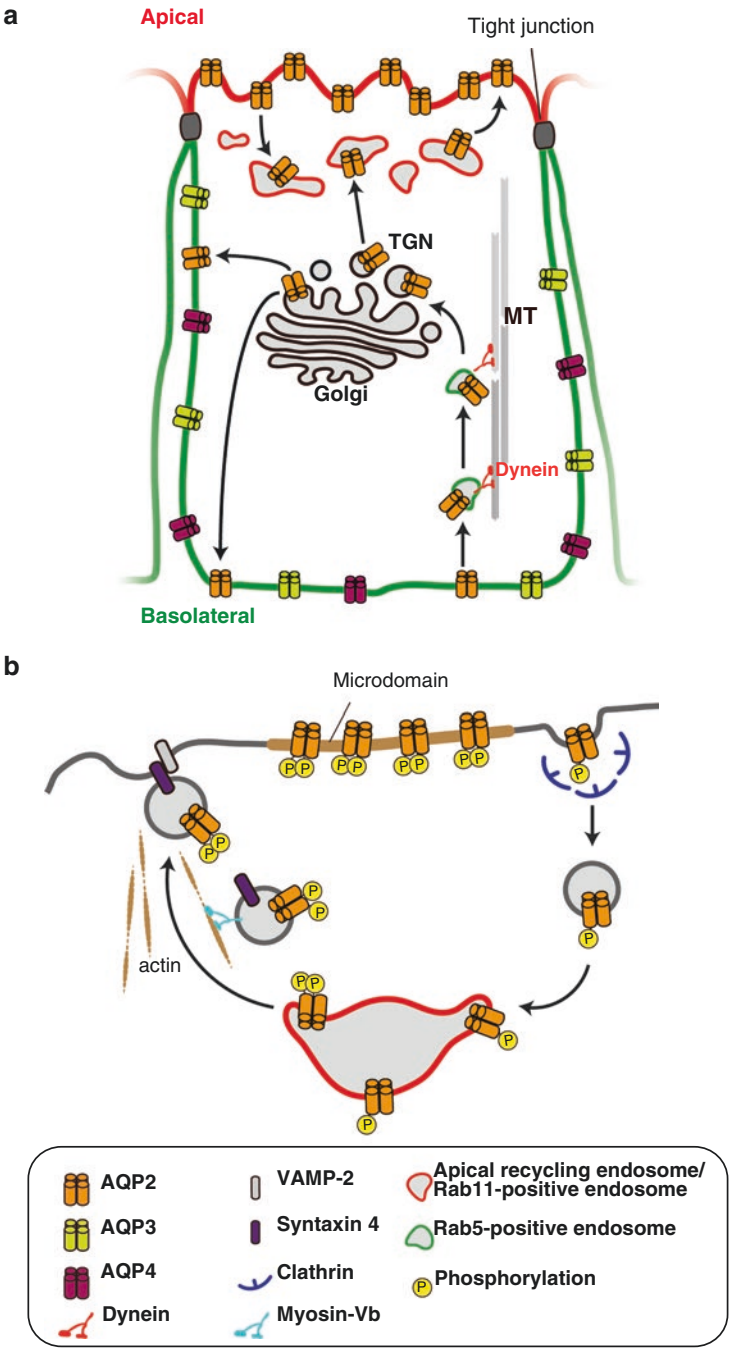
1.3 Polar Localization of Aquaporins in Plant Cells

1.3.1 Polar Localization of Mammalian Aquaporins

Polar localization has been observed on several aquaporins in plant cells, although its physiological role in directional transport of substrates has not been shown. Before summarizing the knowledge on plant aquaporins, we introduce the preceding models of polar localization of mammalian aquaporins. In mammalian epithelial cells, three aquaporins are known to show polarity in their localization in the PM. In the renal collecting duct principal cells, AQP2 is targeted to the apical domain of the PM facing the ducts, while AQP3 and AQP4 are localized to basolateral domain of the PM (Edemir et al. 2011; Fig. 3a).

AQP2 is localized to intracellular vesicles, and vasopressin promotes translocation of AQP2 to the apical PM domain for efficient reabsorption of water from the ducts (Nedvetsky et al. 2007). It has been revealed that phosphorylation at S256 and S269 is crucial for the translocation (Hoffert et al. 2008; Van Balkom et al. 2002). Phosphorylation of S256 promotes translocation to the apical PM (Tamma et al. 2011), while phosphorylation at S269, which depends on the prior phosphorylation at S256, represses the clathrin-mediated endocytosis from the apical PM (Moeller

Fig. 3 Models of mechanisms underlying the polar localization of aquaporins in epithelial cells of mammalian renal collecting duct. (**a**) AQP2 is localized in the apical domain of the PM in the epithelial cell and cycled between the PM and Rab11-positive apical recycling endosomes. Rab5- and dynein-dependent transcytosis from basolateral to apical domain is required for the polar localization. AQP3 and AQP4 are distributed to basolateral domain of the PM. Tight junctions restrict the lateral diffusion of the AQPs. (**b**) In the PM, AQP2 is predominantly accumulated in the microdomains. Phosphorylation at S256 and S269 of AQP2 promotes its apical localization. Phosphorylation at S269 inhibits clathrin-mediated endocytosis and retains the localization of AQP2 in the apical PM domain. AQP2-bearing vesicles are transported along actin filaments and fused with the apical PM in VAMP-2 and syntaxin 4-dependent manner. AQP2-bearing vesicles are trafficked by Myosin-Vb motor protein along actin filaments


a
Apical
Tight junction
TGN
MT
Golgi
Dynein
Basolateral
b
Microdomain
actin
AQP2
AQP3
AQP4
Dynein
VAMP-2
Syntaxin 4
Clathrin
Myosin-Vb
Apical recycling endosome/
Rab11-positive endosome
Rab5-positive endosome
Phosphorylation

et al. 2009, 2014). PM-residing AQP2 is predominantly accumulated in the detergent-resistant membrane (DRM) and thus suggested to be localized in the PM microdomain (Yu et al. 2008). Endocytosis of microdomain-residing proteins is supposed to be through not clathrin- but caveolae-dependent pathway in mammalian cells (Roy and Wrana 2005). The phosphorylation state of AQP2 might alter its distribution between microdomain and non-microdomain to control the endocytic rate of AQP2 cycling (Fig. 3b). This set of data strongly supports the more recent discovery of the membrane microdomain-dependent pathway involved in endocytosis of AtPIP2;1 upon salt stress (Li et al. 2011). However, a caveolae homolog has not been discovered so far in plants. Flotillin endocytosis was also described to be independent from the clathrin-mediated pathway in plant cells and could hypothetically have the same function in plants as caveolae have in mammals (Li et al. 2012).

Another important factor for the polar localization of AQP2 is a PDZ-interacting motif (x-T/S-x-Φ, where x is unspecified amino acid; Φ is hydrophobic amino acid) in its C-terminal tail (Harris and Lim 2001). This motif interacts with SPA-1, a PDZ-domain containing GTPase-activating protein (GAP) for a small GTPase, Ras-related protein 1 (Rap1) (Kurachi et al. 1997; Noda et al. 2004). Rap1 regulates diverse cellular processes including Ras/extracellular signal-regulated kinase (ERK) signaling pathway, cell morphogenesis, and cell growth (Frische and Zwartkruis 2010). Rap1 may be involved in cytoskeletal orientation (Tsukamoto et al. 1999) and vesicular transport (Moskalenko et al. 2002). SPA-1 is localized to the apical surface of the renal collecting ducts and required for the polar localization of AQP2 as a scaffold (Noda et al. 2004; Kim and Sheng 2004). Thus, SPA-1 regulates AQP2 distribution through its GAP activity for Rap1 and/or scaffolding at the apical surface.

In addition, the apical polar localization of AQP2 requires Rab11-dependent apical vesicular trafficking by myosin Vb along actin filaments (Nedvetsky et al. 2007; Fig. 3b) and Rab5-dependent transcytosis by dynein along microtubules (Yui et al. 2012; Fig. 3a). AQP2 colocalizes with a v-SNARE VAMP-2 at the apical vesicles (Gouraud et al. 2002), suggesting that VAMP-2 determines apical sorting of AQP2-bearing vesicles (Fig. 3b). At the apical domain of the PM, syntaxin 4 was implicated to function as a t-SNARE for the AQP2-bearing vesicles (Gouraud et al. 2002). In summary, small GTPase-dependent vesicular sorting, directional transcytosis along cytoskeletons, SNARE-mediated vesicular fusion, interaction to a PDZ-domain containing protein, and inhibition of endocytosis by phosphorylation are underlying the polar localization of AQP2.

In contrast to AQP2, the isoforms AQP3 and AQP4 are localized to the basolateral domain of the PM (Edemir et al. 2011; Fig. 3a). AQP4 has a tyrosine- and a dileucine-based motif (Madrid et al. 2001), well-characterized signals for clathrin-mediated endocytosis and sorting to the lysosome/vacuole (Traub 2009). The tyrosine motif (YxxΦ; Φ is a bulky hydrophobic amino acid residue) and the dileucine motif ([DE]xxxL[LIM]; [ ] indicates alternative) were shown to be recognized by μ subunits of AP complexes (Ohno et al. 1995) and γ/σ1 and α/σ2 hemicomplexes of AP-1 and AP-2 (Doray et al. 2007), respectively. Interestingly, an AQP4 variant, whose tyrosine motif was mutated, showed ectopic localization to the apical PM domain and reduced interaction with μ subunit of AP-2 (Madrid et al. 2001). This finding implies that the polar localization of AQP4 to the basolateral PM requires clathrin-mediated endocytosis from the apical side.

AQP3 carries a conserved YRLL motif in its N-terminal region. A variant of AQP3 whose tyrosine is substituted to alanine (ARLL) displayed less polarized localization than the wild-type isoform, while a variant with AAAA substitution showed intracellular localization, suggesting the tyrosine and leucine residues function distinctly (Rai et al. 2006).

Together, polar localization of AQP3 and AQP4 targeted to the basolateral domain requires their endocytic motifs. In contrast to the case of apical localization of AQP2, there is no indication of requirement of phosphorylation.

1.3.2 Polar Localization of Plant Aquaporins

To date, several plant aquaporins have been reported to show polar localization in the PM (Table 1). Immunocytochemistry of maize and rice PIP isoforms identified at least four PIPs polarly localized in the PM, while other PIPs show nonpolar

Table 1 Polar or nonpolar distribution of aquaporin proteins

Protein	Function	Localization	Organism	References
AtPIP1;1	Water transport	Uniform	*Arabidopsis thaliana*	Boursiac et al. 2005
AtPIP2;1	Water transport	Uniform	*Arabidopsis thaliana*	Boursiac et al. 2005
OsPIP2;1	Water transport	Stele side	*Oryza sativa*	Sakurai-Ishikawa et al. 2011
ZmPIP2;1/2;2	Water transport	Uniform/stele side	*Zea mays*	Hachez et al. 2008
ZmPIP2;5	Water transport	Uniform/soil side	*Zea mays*	Hachez et al. 2006
OsPIP2;5	Water transport	Stele side	*Oryza sativa*	Sakurai-Ishikawa et al. 2011
OsLsil	Si transport	Soil side	*Oryza sativa*	Ma et al. 2006
HvLsil	Si transport	Soil side	*Hordeum vulgare*	Chiba et al. 2009
ZmLsil	Si transport	Soil side	*Zea mays*	Mitani et al. 2009
CmLsil	Si transport	Soil side	*Cucurbita moschata*	Mitani et al. 2011
OsLsi6	Si transport	Soil side (root)	*Oryza sativa*	Yamaji et al. 2008; Yamaji and Ma 2009
		Xylem side (xylem parenchyma in leaf)		
		Xylem side (enlarged vascular bundles in node I)		
HvLsi6	Si transport	Soil side (root)	*Hordeum vulgare*	Yamaji et al. 2012
		Xylem side? (xylem parenchyma in leaf)		
		Xylem side (enlarged vascular bundles in node I)		
AtNIP5;1	B transport	Soil side	*Arabidopsis thaliana*	Takano et al. 2010

distribution. In rice root, OsPIP2;1 and OsPIP2;5 were localized in the stele-side domain of the PM (Sakurai-Ishikawa et al. 2011). In maize root, ZmPIP2;5 was localized to the soil-side (distal) domain of the PM in the epidermal cells (Fig. 4a), whereas it was localized in a nonpolar manner in other cell layers (Hachez et al. 2006). In contrast, ZmPIP2;1/2;2 was localized to the inner side (proximal) domain of the PM in leaf epidermal cells (Fig. 4b; Hachez et al. 2006, 2008). These findings suggested that distribution of PIPs in the PM is regulated in cell type-specific manners. Similar to the case of mammalian AQP2, proteomic analyses have identified PIP2 isoforms in DRM in *Arabidopsis thaliana* (Minami et al. 2009; Shahollari et al. 2004) and *Medicago truncatula* (Lefebvre et al. 2007). It is possible that localization to the microdomain is related to the polarized distribution of ZmPIP2;1/2;2/2;5. So far physiological significance and mechanisms of polar localization of aquaporins remain uninvestigated.

Besides PIPs, some members of NIPs were shown to be localized in the PM in a polar manner. In exodermal and endodermal cells of rice roots, OsLsi1 (NIP2;1), a silicic acid channel, and OsLsi2, an anion exporter, are localized in the soil-side and the stele-side domains of the PM, respectively, and cooperatively play a role in translocation of silicon (Si) into the xylem (Ma et al. 2006, 2007; Ma and Yamaji 2015). Si is a beneficial element for plants and highly accumulated in monocotyledonous plants including rice, maize, barley, and wheat (Ma and Yamaji 2006). Si enhances resistance against abiotic and biotic stresses, and Si deficiency causes agricultural problems such as reduced productivity (Ma and Yamaji 2006). Recently, the physiological importance of the polar localization of OsLsi1 and OsLsi2 in rice root was examined in silico by a mathematical model of root cell layers with parameters such as cellular and subcellular distribution of Lsi1 and Lsi2 and location of the Casparian strips (Sakurai et al. 2015). The model suggested that wild-type pattern of polar localization of Lsi1 and Lsi2 and exodermal and endodermal Casparian strips are all required for efficient Si transport in rice (Sakurai et al. 2015).

Lsi6, a homolog of Lsi1, functions as a silicic acid channel in rice, barley, and maize. Interestingly, patterns of expression and subcellular localization of Lsi6 are distinct between tissues. In rice roots, OsLsi6 (OsNIP2;2) is expressed in the epidermis and cortex and localized to the soil-side domain of the PM. However, in leaves and nodes, OsLsi6 is expressed in xylem parenchyma and xylem-transfer cells, respectively, and localized in the polar PM domains toward the side of xylem, for efficient xylem unloading (Yamaji et al. 2008; Yamaji and Ma 2009). Similarly, in the barley node I, HvLsi6 is expressed in the xylem-transfer cells and localized to the PM domain facing xylem of enlarged vascular bundle (X_E, Fig. 5a, b; Yamaji et al. 2012). The other isoform HvLsi2 is expressed in the xylem parenchyma cells adjacent to the xylem-transfer cells and localized to the PM domain at the side of diffuse vascular bundle (DV, Fig. 5c, d). HvLsi6 and HvLsi2 play important roles for reloading Si to the xylem of diffuse vascular bundle from the xylem of enlarged vascular bundle (Yamaji et al. 2012; Ma and Yamaji 2015).

Rice exodermis and endodermis have Casparian strips, which restrict not only apoplastic flow of water and solutes but also lateral diffusion of PM-residing proteins like the case of tight junctions in mammalian epithelial cells (Geldner 2013).

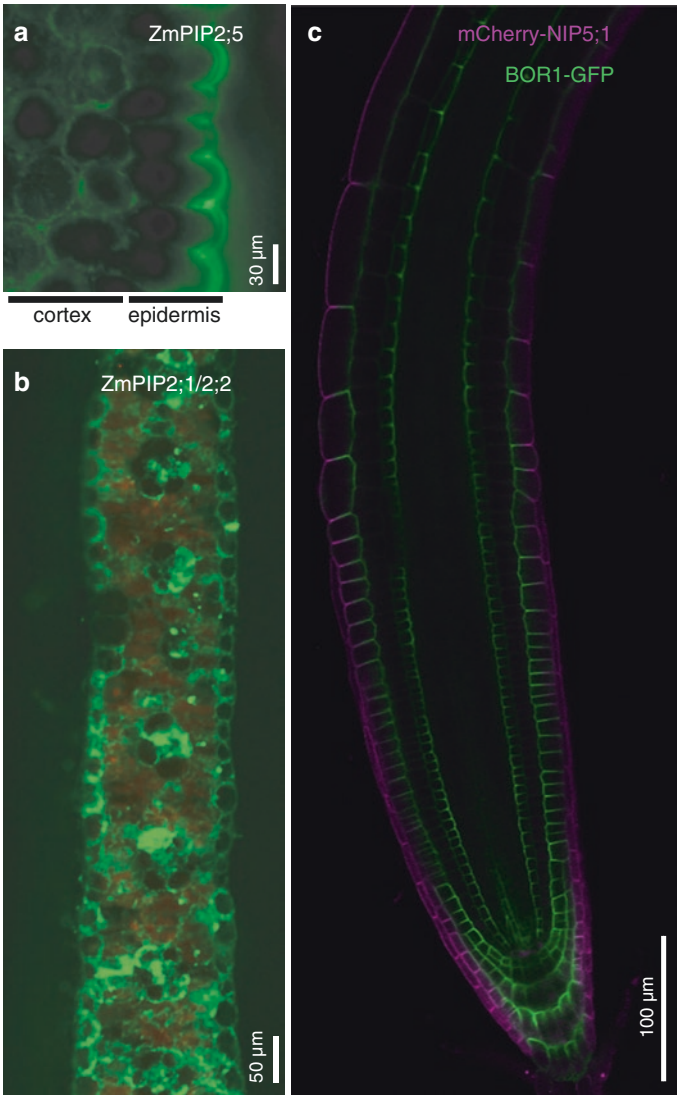


Fig. 4 Polar localization of ZmPIP2;5 in roots, ZmPIP2;1/2;2 in leaves, and AtNIP5;1 in roots. (**a**) Immunofluorescence of ZmPIP2;5 in a maize root. ZmPIP2;5 shows polar localization in the soil-side domain of the PM in the epidermal cells. (**b**) Immunofluorescence of ZmPIP2;1/2;2 in a maize leaf blade. ZmPIP2;1/2;2 shows polar localization in proximal domain of the PM in the epidermal cells. Scale bar = 30 µm (**a**), 50 µm (**b**). Figures are reproduced from Hachez et al. 2006 (**a**) and Hachez et al. 2008 (**b**) with permission. (**c**) A confocal image of a primary root of the transgenic plant carrying *ProAtNIP5;1-mCherry-AtNIP5;1genomic* and *ProAtBOR1,AtBOR1-GFP* grown on low-B media. AtNIP5;1 is expressed which shows polar localization in soil-side domain of the PM in the outermost cell layers, whereas AtBOR1 shows polar localization in stele-side domain in various cells in the root tip (Takano et al. 2010). Scale bar = 100 µm

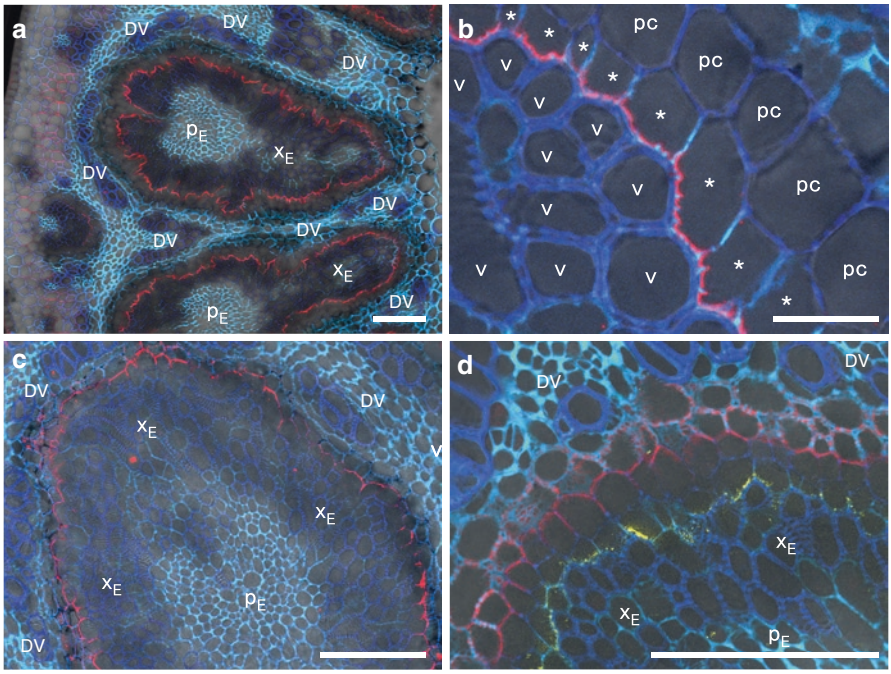


Fig. 5 Localization of HvLsi6 and HvLsi2 in the node I. Immunofluorescence in node I at the flowering stage with anti-HvLsi6 antibody (*red* color in **a**, **b**), anti-HvLsi2 antibody (*red* color in **c**), or double staining with anti-HvLsi6 (*yellow*) and anti-HvLsi2 (*red*) antibodies (**d**). *Blue* and *cyan* colors are UV autofluorescence from the cell wall. x_E, p_E: xylem, and phloem region of enlarged vascular bundle, respectively, *DV* diffuse vascular bundle, *v* xylem vessel, * xylem-transfer cell, *pc* outer parenchyma cell layer next to xylem-transfer cell. Scale bars = 100 μm (**a**, **c**, **d**), 20 μm (**b**) (Reproduced from Yamaji et al. 2012 with permission)

Therefore, one can imagine that the polar localization of OsLsi1 in these cell types is dependent on the presence of the Casparian strip. However, Lsi1 and Lsi6 also showed polar localization in cell types without the Casparian strips (Chiba et al. 2009; Mitani et al. 2009; Yamaji et al. 2012), indicating that the Casparian strip is not necessary for polar localization.

Another example of polar-localized plant aquaporin is NIP5;1, a boric acid channel in *Arabidopsis* (Takano et al. 2006, 2010; see chapter "Plant Aquaporins and Metalloids"). Boron (B) is an essential micronutrient, while excess B is toxic for plants (Marschner 1995). Imaging of GFP fusion revealed that NIP5;1 is localized to the soil-side domain of the PM in outermost cell layers of roots, while a borate efflux transporter BOR1 is localized to the stele-side domain in various cell types, under low-B conditions (Takano et al. 2002, 2010) (Fig. 4c). The opposite polar localization of NIP5;1 and BOR1 is considered to be beneficial for B uptake into root and subsequent translocation to the vascular cylinder under low-B conditions.

Currently, molecular mechanisms underlying polar localization of a subset of PIPs and NIPs are not well understood. As discussed above, a membrane diffusion

barrier such as tight junctions in animal epithelial cells is not necessary for maintaining the polar localization in the plant PM. Limited lateral diffusion of the PM-localized proteins (discussed above) is apparently a basis for the maintenance of the polar localization by vesicle trafficking. Although no canonical endocytic motifs have been identified within their cytosolic sequences, other endocytic signals or posttranslational modification such as phosphorylation might regulate the polar localization of plant aquaporins as it does for some mammalian AQP proteins.

1.4 Trafficking of TIPs

1.4.1 Localization of TIPs to the Lytic Vacuole and the Protein Storage Vacuole

TIPs have functions in transport of diverse solutes including water, glycerol, urea, ammonia, or hydrogen peroxide (H_2O_2) across the vacuolar membrane (tonoplast) (Wudick et al. 2009). TIPs are further classified into five groups. Among them, γ-TIP (renamed AtTIP1;1 in *Arabidopsis*) has been used as a marker for the lytic vacuole, and α- and δ-TIP (renamed AtTIP3;1 and AtTIP2;1 in *Arabidopsis*) have been used as markers for the protein storage vacuole (PSV) (Frigerio et al. 2008; Xiang et al. 2013). The lytic vacuole has acidic pH, occurs in mature tissues, and functions similarly to the lysosome in animals. The PSV has less acidic pH and functions as the main protein storage compartment in the storage tissues. There remains a contradiction whether the different TIPs target specifically toward the lytic vacuoles or the PSVs. In *Arabidopsis* mesophyll protoplasts, AtTIP2;1-GFP was colocalized with the isoform *b* of a rice two-pore K^+ channel construct in small PSVs, whereas the construct with the isoform *a* is localized in the tonoplast of the central vacuole (Isayenkov et al. 2011). However, in transgenic *Arabidopsis* plants, different TIPs (AtTIP1;1, AtTIP2;1, and AtTIP3;1) fused to fluorescent proteins coexisted in the same tonoplast of the central vacuole in roots and leaves (Hunter et al. 2007; Gattolin et al. 2009) and in the same tonoplast of PSVs in embryos (Hunter et al. 2007). The differential localization of TIP isoforms to the lytic vacuoles or the PSVs seems to be dependent on tissues and developmental stages.

Interestingly, specifically in early seed maturation and early germination stages, AtTIP3;1 and AtTIP3;2 were shown to localize to the tonoplast of PSVs and also to the PM (Gattolin et al. 2011). This is apparently not an artifact since both N- and C-terminal fluorescent protein fusions of AtTIP3;1 and AtTIP3;2, but not YFP-AtTIP1;1 expressed under the control of the At*TIP3;2* promoter, showed the dual localization. Although the mechanisms of dual targeting are unknown, this finding suggests the distinct trafficking mechanisms for different TIPs and possible importance of the PM localization of TIPs to compensate the absence or low expression of PIPs in these developmental stages.

1.4.2 Trafficking of TIPs in Golgi-Dependent and Golgi-Independent Pathways

The tonoplast targeting of membrane proteins is apparently more complex than the PM targeting via the ER, Golgi apparatus, and TGN. There exist at least two pathways to the tonoplast: Golgi-dependent and Golgi-independent pathways (Pedrazzini et al. 2013; Rojas-Pierce 2013; De Marchis et al. 2013; Viotti 2014). These pathways have been distinguished by sensitivities to the fungal toxin BFA. BFA sensitivity in a cell type is dependent on the sensitivity/insensitivity of ARF-GEF isoforms responsible for each trafficking step (Robinson et al. 2008). For example, in leaf epidermis of tobacco, hypocotyls, and leaves but not roots of *Arabidopsis*, BFA inhibits the retrograde traffic from the Golgi to the ER and induces uncontrolled fusion of Golgi and ER membranes (Brandizzi et al. 2002; Robinson et al. 2008; Rivera-Serrano et al. 2012). In these cells, BFA inhibits the trafficking of soluble cargoes to the vacuole or the apoplast, and some, but not all, tonoplast proteins are investigated (Pedrazzini et al. 2013; Rojas-Pierce 2013; De Marchis et al. 2013). In an early study, using protoplasts from transgenic tobacco leaf cells, the vacuolar traffic of the soluble protein phytohemagglutinin, but not the tonoplast TIP3;1, was inhibited by BFA treatment (Gomez and Chrispeels 1993). In protoplasts of *Arabidopsis*, the Golgi-independent trafficking of AtTIP3;1 was further supported by the finding that Golgi-specific glycosylation was not detected in a modified TIP3;1 variant that contained a potential glycosylation residue (Park et al. 2004). A recent study showed that the tonoplast targeting of AtTIP1;1-YFP but not AtTIP3;1-YFP and GFP-AtTIP2;1 was sensitive to BFA in *Arabidopsis* hypocotyls (Rivera-Serrano et al. 2012). Furthermore, a screen of chemical inhibitors of TIP trafficking in the same study identified a compound, C834, which retained GFP-AtTIP2;1 and AtTIP3;1-YFP in the ER, but did not affect the tonoplast targeting of AtTIP1;1-YFP. Therefore, BFA and C834 treatments are means to differentiate the Golgi-dependent and Golgi-independent pathways as distinct mechanisms (Fig. 6).

1.4.3 Golgi-Dependent Trafficking to the Tonoplast

Although little is known for the Golgi-dependent trafficking of TIPs, studies on various tonoplast proteins revealed multiple pathways to the tonoplast through the Golgi apparatus (Fig. 6). Studies on some tonoplast proteins in plants identified the requirement of dileucine-based motifs and a tyrosine-based motif located in the cytosolic region for tonoplast trafficking (Pedrazzini et al. 2013). As introduced above, dileucine-based motifs (typically [D/E]xxxL[L/I]) and the tyrosine-based signal YxxΦ, where Φ is any bulky hydrophobic residue, are well-established signals for endocytosis and sorting to lysosomes or vacuoles by binding to AP complexes in mammals and yeast (Bonifacino and Traub 2003). Intriguingly, mutations in the dileucine motifs (not limited to the typical ones) of tonoplast proteins, such as a monosaccharide transporter ESL1, a molybdate transporter MoT2, an inositol

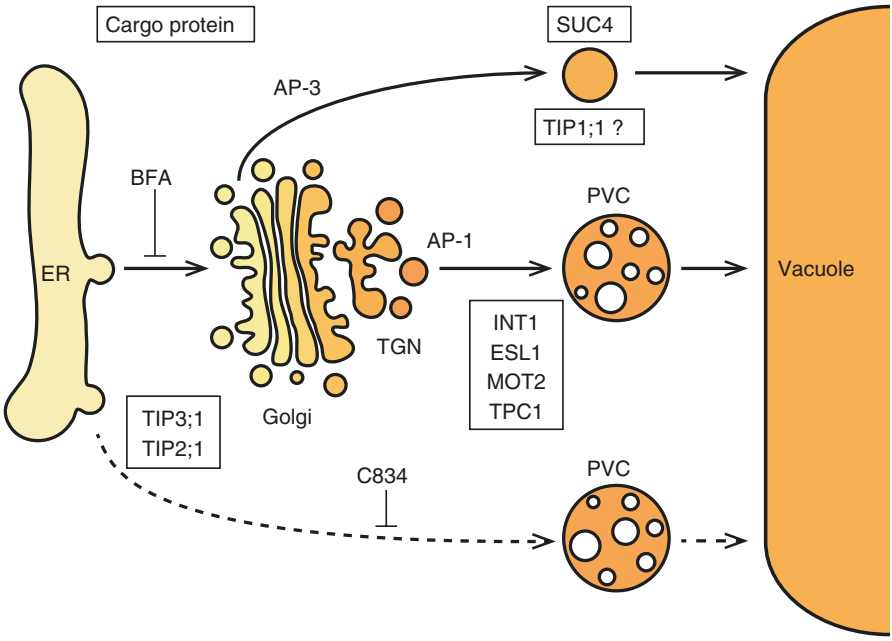


Fig. 6 A model of multiple trafficking pathways to the tonoplast. The trafficking pathway of AtTIP1;1 but not of AtTIP2;1 and AtTIP3;1 is through Golgi. These routes can be differentiated by sensitivities to BFA or C834. The BFA-sensitive, Golgi-dependent pathway is further divided to two pathways, dependent on AP-1 and AP-3 complexes. TIP1;1 does not contain dileucine motifs, which are recognized by the AP-1 complex and found in INT1, PTR2/4/6, ESL1, MOT2, TPC1, and VIT1

transporter INT1, a two-pore channel TPC1, and an iron transporter VIT1, redirected them to the PM (Yamada et al. 2010; Gasber et al. 2011; Wolfenstetter et al. 2012; Larisch et al. 2012; Wang et al. 2014). Furthermore, short amino acid fragments containing the dileucine motif of INT1, PTR2/4/6, or VIT1 were sufficient to redirect PM-targeted proteins to the tonoplast (Wolfenstetter et al. 2012; Komarova et al. 2012; Wang et al. 2014). These results suggest that the tonoplast-targeting mechanism dependent on the dileucine motif is dominant over the PM-targeting mechanism, at least for a subset of membrane proteins. Importantly, the dileucine motif from VIT1 was shown to interact with AP-1 subunits in vitro, and the knockdown of the AP-1 γ-adaptins caused relocation of VIT1 and INT1 to the PM (Wang et al. 2014). Since AP-1 γ-adaptins are mainly localized in the TGN, it was proposed that its complex mediates the targeting of membrane proteins carrying a dileucine motif from the TGN to the tonoplast (Fig. 6).

To add further complexity, there exists another Golgi-dependent vacuolar-sorting pathway, which is dependent on AP-3. The AP-3 complex in *Arabidopsis* is important for biogenesis of the vacuole (Feraru et al. 2010; Zwiewka et al. 2011). The tonoplast trafficking of SUC4 but not of INT1, TPC1, and ESL1 was affected in the AP-3 β-subunit mutant (Larisch et al. 2012; Wolfenstetter et al. 2012). In the AP-3

β-subunit mutant, GFP-SUC4 colocalized with *cis*-Golgi markers (Wolfenstetter et al. 2012). By analogy to the apparent function of yeast AP-3 (Odorizzi et al. 1998; Dell'Angelica 2009), it was proposed that AP-3 is involved in direct trafficking from the Golgi apparatus to the tonoplast (Wolfenstetter et al. 2012; Fig. 6). Since AtTIP1;1, as well as SUC4, does not contain conserved trafficking signals in the cytosolic region, involvement of AP-3 for AtTIP1;1 trafficking needs to be tested.

1.4.4 Golgi-Independent Trafficking to the Tonoplast

What kind of mechanism is conceivable for the Golgi-independent pathway? A recent study carefully investigated the Golgi-independent trafficking of two proton pumps to the lytic vacuoles in the root meristematic cells of *Arabidopsis* (Viotti et al. 2013). The localization of a VHA-a3-mRFP to the tonoplast was not disturbed by concanamycin A treatment, which causes aggregates derived from TGN. This contrasts to the case of INT1 fused to GFP, which was trapped in the aggregates labeled by the endocytic tracer FM4-64. VHA-a3-mRFP was introduced into the BFA-sensitive GNL1/*gnl 1* line (Richter et al. 2007) to block the ER-to-Golgi transport and the post-Golgi transport by BFA treatment. Upon BFA treatment, the tonoplast localization of VHA-a3-mRFA was not affected. These results supported the hypothesis that VHA-a3 trafficking to the tonoplast is Golgi independent. Furthermore, the immunoelectron microscopy analysis of the VHA-a3-GFP and V-PPase AVP1/VHP1 detected both proteins at the tonoplast and double-membrane structures designated as provacuoles, but not in the TGN/EE, Golgi stacks, or ER (Viotti et al. 2013). The formation of the provacuoles was not prevented by complete abolition of Golgi and post-Golgi trafficking in the *gnl* mutant with BFA treatment. Importantly, although at rare incidences, a direct connection between the provacuole and the ER was observed. These results suggested that the biogenesis of the lytic vacuole and trafficking of a subset of tonoplast proteins occur directly from the ER independent of Golgi function. It is tempting to speculate that the tonoplast trafficking of AtTIP3;1 and AtTIP2;1, which is insensitive to BFA, occurs in the similar direct pathway. This hypothesis needs to be tested in the tissue containing PSVs, where native AtTIP3;1 and AtTIP2;1 are expressed. The precursor-accumulating (PAC) vesicles carrying precursors of storage proteins directly from the ER to the PSVs in pumpkin cotyledons (Hara-Nishimura et al. 1998, 2004) or the KDEL vesicles carrying a proform of a KDEL-tailed cysteine protease directly from the ER to the PSVs (Toyooka et al. 2000) might be involved.

1.4.5 Stress-Induced Changes of TIP Localization

The response of aquaporin trafficking to stress conditions was first described for McTIP1;2 in suspension cells and leaves of ice plant (*Mesembryanthemum crystallinum;* Vera-Estrella et al. 2004). Membrane fractionation followed by Western blotting by an anti-McTIP1;2 antibody revealed the changes of localization after

mannitol-induced osmotic stress. An immunofluorescence analysis showed localization of McTIP1;2 in the tonoplast under a control condition and its change to unique intracellular spherical structures after the mannitol treatment. The spherical structure labeled by McTIP1;2 was not labeled with tonoplast markers V-PPase, V-ATPase, and AtTIP2;1, PM markers, and LE/MVB markers. The change was accompanied with increased amount of the protein and glycosylation and blocked by the glycosylation inhibitor tunicamycin. These results suggested that osmotic stress induces glycosylation of McTIP1;2 to change the trafficking pathway to spherical structures, possibly to maintain water balance.

Another example of the changes of TIP localization was in *Arabidopsis* root cells after salt treatment (Boursiac et al. 2005). Under control conditions, both isoforms AtTIP1;1-GFP and AtTIP2;1-GFP labeled the tonoplast of the central vacuole, while after the salt treatments, AtTIP1;1-GFP, but not AtTIP2;1-GFP, appeared in intravacuolar spherical structures tentatively identified as tonoplast invaginations. The spherical intravacuolar structures need to be interpreted with caution since this kind of structures can be artificially induced by dimerization of GFP fused with tonoplast proteins when they are abundantly expressed (Segami et al. 2014). However, the spherical structures labeled by AtTIP1;1-GFP were observed only after salt stress (Boursiac et al. 2005), suggesting that at least a change in localization of AtTIP1;1 or vacuole structure occurred. Since AtTIP1 abundance measured by anti-TIP1 antibody was decreased by approximately 20 % within 6 h after salt treatment (Boursiac et al. 2005), this labeling might reflect protein degradation. Alternatively, it might be accompanied by a reshaping of vacuole structure under salt stress.

2 Aquaporins as Markers for Membrane Dynamics

PIPs and TIPs have been used as PM and tonoplast markers for various studies on organelle and membrane dynamics in plant cells. TIPs were also used as protein markers for identification of new small molecule inhibitors for membrane trafficking (Rivera-Serrano et al. 2012). Recently, Arabidopsis AtNIP5;1, a boric acid channel, was utilized as a PM marker for a forward genetics study. Using chemically mutagenized GFP-AtNIP5;1 lines, a fluorescence imaging-based screening isolated mutants in which AtNIP5;1 localized abnormally in root epidermal cells (Uehara et al. 2014). An allelic group of mutants contained intracellular aggregates co-labeled with GFP-AtNIP5;1, endocytic tracer FM4-64, and TGN markers. A positional cloning identified the responsible gene as *UDP-glucose 4-epimerase*, which is important for synthesis and channeling of D-galactose into the Golgi apparatus (Uehara et al. 2014; Seifert et al. 2002). Further analysis of ultrastructure in the *uge4* mutant cells revealed accumulation of high-electron-density vesicles derived from TGN (Wang et al. 2015). It is most likely that accumulation of galactose-depleted xyloglucan caused abnormal function and structure of TGN and caused a “traffic jam” in the membrane traffic system (Kong et al. 2015). The same

screen identified an allelic group of mutants in which GFP-AtNIP5;1 is accumulated in the ER in addition to the PM (unpublished). Analysis of the mutant has a potential to identify a novel mechanism of ER exit specific to AtNIP5;1 or related aquaporins.

3 Conclusions and Perspectives

Recent studies have greatly improved knowledge on the localization of plant aquaporins. The localization and trafficking of aquaporins were revealed to be different among isoforms even in the same subfamily or in different cell types. PIP trafficking exhibits similarities and originalities with other PM proteins in plant cells. The PM targeting of PIP1s requires heteromerization with PIP2s. They use the secretory pathway to be targeted to the cell surface, and although fairly immobile in the plane of the PM, they are subjected to the constitutive cycling. Some PIPs and NIPs show polar localization in the PM. Comparison with preceding models in mammal aquaporins will be a key to understand the mechanism of polar trafficking of plant aquaporins. It would be also important to examine the physiological significance of the polar localization by substitution of polar aquaporins with their nonpolar variants. It is still controversial whether different TIP isoforms are differentially targeted to the lytic vacuoles or the PSVs. Systematic studies not only on TIPs but also with other tonoplast and vacuolar proteins using recently identified mutants for membrane trafficking will clarify the mechanisms. The trafficking mechanisms underlying localization of NIPs in the peribacteroid membrane of nitrogen-fixing nodules (Fortin et al. 1987) remain unknown. A change of destination of TIPs from the tonoplast to the symbiosome membrane in nodules of *Medicago truncatula* suggests a dynamic change of the membrane-trafficking system during nodulation (Gavrin et al. 2014). The mechanisms underlying dual localization of a PIP1 homolog (NtAQP1) in the PM and the chloroplast inner membrane in leaf mesophyll cells (Uehlein et al. 2008) await investigation. Aquaporins of the PM and the tonoplast exhibit extraordinary dynamic properties. When challenged to stress stimuli, and in order to adjust water status, plant cells respond by a fast membrane redistribution involving aquaporin relocalization. It would be intriguing to reveal the mechanisms not only on the trafficking but also stress sensing which cause post-translational modifications of aquaporins to change their destinations. Clearly, aquaporin trafficking will continue to be a significant model of plant membrane proteins.

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