Multifaceted role and regulation of aquaporins for efficient stomatal movements

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Summary statement

Stomatal movements require the activity of aquaporins in guard cells and, when present, in subsidiary cells. This review explores several regulation mechanisms that control aquaporin expression, trafficking and activity and, consequently stomatal dynamics.

Abstract

Stomata are micropores on the leaf epidermis that allow carbon dioxide uptake for photosynthesis at the expense of water loss through transpiration. Stomata coordinate the plant gas exchange of carbon and water with the atmosphere through their opening and closing dynamics. In the context of global climate change, it is essential to better understand the mechanism of stomatal movements under different environmental stimuli. Aquaporins (AQPs) are considered important regulators of stomatal movements by contributing to membrane diffusion of water, carbon dioxide and hydrogen peroxide. This review compiles the most recent findings and discusses future directions to update our knowledge of the role of AQPs in stomatal movements. After highlighting the role of subsidiary cells (SCs), which contribute to the high water use efficiency of grass stomata, we explore the expression of *AQP* genes in guard cells (GCs) and SCs. We then focus on the cellular regulation of AQP activity at the protein level in stomata. After introducing their post-translational modifications, we detail their trafficking as well as their physical interaction with various partners that regulate AQP subcellular dynamics towards and within specific regions of the cell membranes, such as microdomains and membrane contact sites.

Keywords

Aquaporins, stomatal movement, cellular regulation, water, hydrogen peroxide, carbon dioxide, phosphorylation, ER-PM contact sites.

1. Introduction

Stomata are micropores in the leaf epidermis, allowing carbon dioxide (CO_2) to be absorbed for photosynthesis at the expense of water loss through transpiration. To maximize water use and carbon fixation efficiency, the stomatal pore dynamics are regulated by various environmental stimuli (drought, vapor pressure deficit (VPD), light, CO_2, etc.) (Merilo et al., 2018; Nguyen et al., 2023; Sussmilch et al., 2019; Zhang et al., 2018). Whereas eudicot stomata are formed by two kidney-shaped guard cells (GCs), the grass stomata are made up of two dumbbell-shaped GCs flanked by two subsidiary cells (SCs). Interestingly, in grass stomata, an inverse behavior of the solute movement in SCs versus GCs controls stomatal opening and closing (Cai et al., 2017; Lim et al., 2015; Nunes et al., 2020).

Stomatal opening and closing depend on variations in the turgor pressure of the GCs surrounding the pore and, consequently, on water flux through their plasma membrane (PM). In short, stomatal opening is induced by an increase in cell turgor pressure, resulting from an accumulation of solutes, such as potassium (K^+) and sugars, into the cytosol and the vacuole, followed by an influx of water. Conversely, stomatal closure is the consequence of a decrease in solute accumulation in GCs (Jezek & Blatt, 2017). Events modulating the stomatal opening and closing involve the functional regulation of H^+-ATPases, which modulate other the activity of transporters or channels, such as K^+ inward and outward channels, S- and R-type anion channels, and the regulation of aquaporins (AQPs). All these transporters are activated by complex signaling networks comprising mainly light, CO_2, hydrogen peroxide (H_2O_2) and abscisic acid (ABA) signaling pathways (Grondin et al., 2015; Hsu et al., 2021; Jezek & Blatt, 2017; Rodrigues & Shan, 2022; Takahashi et al., 2022). In addition, the trafficking of above-mentioned transporters may also be affected and further regulate stomatal movement by different stimuli (for detail, see sections 3.2 and 3.3).

AQPs are small membrane proteins (21-34 kD). In vascular plants, they constitute a large and diverse protein family, grouped into five subfamilies, namely plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nodulin 26-like intrinsic proteins

(NIPs) found in the PM and endoplasmic reticulum (ER), small basic intrinsic proteins (SIPs) localized in the ER, and X intrinsic proteins (XIPs) present in the PM (Chaumont et al., 2001; Groszmann et al., 2021; Johanson et al., 2001). AQPs play important roles not only in maintaining cellular water homeostasis, but also in nutrient uptake and transport, signal transduction and metabolism (Chaumont & Tyerman, 2014; Maurel et al., 2008; M. Wang et al., 2016). The multiple isoforms exhibit a wide variety of cellular localization, transport selectivity and regulation to support plant growth and development (Chaumont & Tyerman, 2014; Maurel et al., 2015). As water channels, AQPs affect hydraulic conductivity in roots and leaves (Sack & Holbrook, 2006; Steudle, 2000), as well as cellular water homeostasis particularly in root cortical cells (Ding et al., 2020), leaf bundle sheath cells (BSCs) (Shatil-Cohen et al., 2011), GCs (Grondin et al., 2015), and pollen (Soto et al., 2008). As small solute channels, AQPs facilitate the transport of H_2O_2, CO_2, urea, ammonia, glycerol, as well as metalloids of boric acid, silicic acid and arsenite (for details see in Chaumont and Tyerman (2017)). Interestingly, new evidence shows that plant AQPs can also serve as non-selective cation and anion channels (Tyerman et al., 2021).

Depending on the channel specificity, AQPs, mainly PIPs and TIPs, can affect stomatal movements by regulating the transmembrane diffusion of H_2O and other solutes in GCs (Grondin et al., 2015; Jezek & Blatt, 2017), including CO_2 (C. Wang et al., 2016), H_2O_2 (Rodrigues et al., 2017), as well as K^+ and Na^+ (Qiu et al., 2020) and NO_3^- (S. Liu et al., 2020). Furthermore, AQPs also indirectly affect stomatal movements through the regulation of tissue water relations in roots and leaves (Gambetta et al., 2017; Shatil-Cohen et al., 2011), by triggering short and long-distance hydraulic and chemical signals.

A few reviews describe the contribution of AQPs in regulating H_2O, H_2O_2 and CO_2 facilitated diffusion in GCs and how it affects stomatal dynamics (Ding & Chaumont, 2020; Hachez et al., 2017; Maurel et al., 2016; Rodrigues & Shan, 2022). Here, we first highlight the importance of SCs in grass stomatal function (for more details, see T-H Nguyen & M R Blatt 'Surrounded by luxury: The necessities of subsidiary cells' in this issue) and compile recently

published data on *AQP* expression in GCs and SCs. We also focus on post-translational modifications (PTMs) and the physical interaction between PIPs and various partners that regulate AQP subcellular dynamics towards and within the PM.

2. Aquaporins, subsidiary cells and stomatal movements

2.1. Roles of subsidiary cells

SCs are defined as any cell associated with GCs that has a distinct identity from neighboring cells (Gray et al., 2020). With different arrangements and ontogenies, SCs are present in grasses and succulents of the Crassulaceae family (Chen et al., 2017; Cheng & Raissig, 2023; Gray et al., 2020). Grasses dominate in many ecosystems, with the most successful evolution of four-celled stomatal morphology improving water-use-efficiency due partly to specific morphology/geometry of the stomatal complexes that provides mechanical advantages (Chen et al., 2017; Nunes et al., 2020). The function of grass stomata is indeed improved by the presence of SCs (Chen et al., 2017). Over the past decade, SCs have attracted numerous scientists to decipher their roles in the rapid movement of grass stomata (Chen et al., 2017; Cheng & Raissig, 2023; Hepworth et al., 2018; Lawson & Matthews, 2020; McKown & Bergmann, 2020). Recently, Durney et al. (2023) explained the advantage of four-celled complexes in respect of geometry by finite element method model. They demonstrated that GCs and SCs formed a reciprocal pressure system, and SCs functioned as springs to restrain lateral GC movement. The pressurized SCs have a mechanical influence on the system by restraining the size of the stomatal pore when GC pressure is low. A similar constraint–relaxation–recovery mechanism for stomatal dynamics was proposed in Arabidopsis (Jezek et al., 2019), which highlights the constraining effect of surrounding pavement cells (PCs) on GC volume and stomatal aperture. During stomatal opening, the turgor pressure decreases in the surrounding PCs and this generates a negative pressure, which force the separate of two GCs and increase stomatal pore size. When stomatal fully open, turgor pressure in the

surrounding cells recovers. This regulation mechanism compensates for the missing component in predicting the kinetics of stomatal opening by OnGuard models (Hills et al., 2012; Y. Wang et al., 2017).

Regulation of GC and SC turgor during stomata opening involves inflow and outflow of water in GCs and SCs, respectively, while the opposite behavior occurs when stomata close. No symplastic connection between GCs and SCs is found, meaning that water flow between SCs and GCs must diffuse through the PM of both cells. The presence of PIP AQPs in SCs (and PCs) (Hachez et al., 2008; Sun et al., 2022), known to increase the membrane osmotic water permeability coefficient and therefore the speed of water diffusion, suggest a role of AQPs in the regulation turgor pressure, but their exact contribution to has yet to be characterized. Accordingly, Arabidopsis *pip2;4* mutant exhibits increased stomatal aperture or conductance (Israel et al., 2021), which could be due to a decrease in PC turgor pressure. However, this hypothesis remains to be validated. In addition, it is speculated that AQPs contribute to the communication between GCs and SCs by mediating the transport of additional molecules than H_2O, such as H_2O_2 and CO_2 during stomatal opening and closure (Nunes et al., 2020).

The important contribution of SCs in stomatal movement was demonstrated in *Brachypodium distachyon* by studying different mutants (*bdmute, bdpolar and bdpan*) affected in the formation of SCs (Raissig et al., 2017). Plants with defects in SC formation displayed severely impaired stomatal dynamics, with slower movements. Similarly, maize lines mutated *PAN1* and *PAN2* genes encoding the leucine-rich repeat receptor like kinases (LRR-RLKs) receptors, have part of their stomata with one or two defective SCs, that were not able to properly close induced upon dark and high CO_2 treatment (L. Liu et al., 2023). Finally, maize *lost subsidiary cells* (*lsc*) mutant, also lacking one or two SCs, exhibit a high leaf temperature and a slower leaf water loss rate in comparison with WTs, indicating an impaired stomatal opening (Cui et al., 2023).

2.2. Aquaporin expression profiles in stomata

As early as in 1995, three years after the discovery of first aquaporin CHIP28 (AQP1 in human) (Preston et al., 1992), Kaldenhoff et al. (1995) reported that *AtPIP1;2* is highly expressed in GCs of *Arabidopsis thaliana* and its expression is induced by blue light. Subsequently, more and more results showed that *AQP* genes, including *PIPs* and *TIPs*, were expressed in GCs (Cui et al., 2021; Durand et al., 2020; Sarda et al., 1997) and stomatal complexes of grasses (Heinen et al., 2014). Over the past two decades, technological advances in next generation sequencing have enabled a shift from microarray to RNA-seq and finally single-cell/nucleus RNA-seq (Seyfferth et al., 2021). This is enabling aquaporin scientists to gain new insights into *AQP* expression and roles from the whole organ to cellular resolution.

Using microarray expression analysis of Arabidopsis GCs, Leonhardt et al. (2004) identified 1309 GC-expressed genes, including several *AQP* genes, i.e., *AtPIP1;1*, *AtPIP1;3*, *AtPIP1;4*, *AtPIP2;1* and *AtTIP1;2*. Bulk RNA-seq of pooled GC protoplasts (Lee et al., 2019) or laser microdissected cells (Berkowitz et al., 2021) identified the *PIP* and *TIP* genes expressed in GCs as well as in other leaf cell types (Figure 2). In GCs, *AtPIP1;2*, *AtPIP2;3* and *AtTIP1;1* are the most expressed *AQP* genes, with a low expression of *AtPIP2;3* in the other leaf tissues, pointing to specific expression of *AtPIP2;3* in GCs. Single-cell RNA-seq has also shown that several *AtPIPs* and *AtTIPs* are highly expressed in GCs compared with other cell types (Kim et al., 2023; Z. Liu et al., 2020; Lopez-Anido et al., 2021). In agreement with the work of Berkowitz et al. (2021), *AtTIP1;1* appears to encode a TIP isoform important for GCs, being two-fold more expressed in mature GCs than in other cells (Kim et al., 2023). Furthermore, the relative expression levels of the different *AQP* genes change during the stomatal development. In young GCs, the transcript levels of *AtPIP2;3*, *AtPIP2;5*, *AtPIP2;6* and *AtTIP1;1* are more abundant than in other cells, and the expression of *AtPIP2;5* and *AtPIP2;7* is higher in guard mother cells than in other cells. However, the role of PIPs and TIPs expressed in young stomatal cells is unclear. They may be involved in regulating cell turgor pressure, cell growth, development and signaling.

In grass, maize stomatal complexes were laser microdissected and RT-qPCR results showed that *PIPs* are expressed at different levels (Heinen et al., 2014). The expression of seven *PIP* genes (*ZmPIP1;1*, *ZmPIP1;2*, *ZmPIP1;3*, *ZmPIP1;5*, *ZmPIP1;6*, *ZmPIP2;1* and *ZmPIP2;2*) accounted for over 98% of the total *PIP* transcripts in the stomatal complexes, with *PIP1s* contributing to more than 80%. Interestingly, *ZmPIP1;6* is the only *PIP* isoform that is specifically expressed in stomatal complexes when compared with the whole mature leaves. Expression of most *PIPs* was higher during the day than at night. While these interesting data already suggest the important role of *PIPs* in stomatal movement, it remains unclear whether *PIPs* and *TIPs* are differentially expressed in SCs and GCs. The bottleneck of single cell sequencing with grass stomata is the isolation of protoplasts from GCs, which is very tricky. Recently, however, single-nuclei RNA-seq in maize reveals the secret of the transcriptome in SCs and GCs (Sun et al., 2022). *ZmPIP1;2* and *ZmPIP2;2* are more expressed in SCs than in GCs, while *ZmTIP1;1* are more expressed in GCs than in SCs (Figure 2). Moreover, *ZmPIP1;6* and *ZmPIP2;5* are specifically expressed in SCs and GCs, respectively.

Overall, transcriptomic studies reveal common features between Arabidopsis and maize GCs. For both species, there is one PIP1 isoform in GCs whose expression level is the highest compared to all other PIPs. Furthermore, Arabidopsis and maize transcriptomes show that not all the TIP subfamilies are expressed in stomata cells. Only *TIP1* and *TIP2* genes are expressed in GCs, with one isoform of the TIP1 group being the most highly expressed. Interestingly, proteomic data were also obtained from *Arabidopsis* GCs (Geilfus et al., 2018; H. Wang et al., 2023) and several *PIPs* and *TIPs* were detected. However, it is difficult from different studies to correlate transcript and protein abundance. Finally, it remains unclear why so many *PIP* genes are simultaneously expressed in the GCs or SCs. As discussed previously in the context of the whole plant (Fox et al., 2017), this could be related to different substrate specificities, PTMs and specific protein-protein interactions leading to different trafficking dynamics, localization and activity (see below). Therefore, transcriptomic and proteomic data

only give us information about the relative expression/abundance of each AQP isoform and we cannot conclude about their channel activity in the membrane.

2.3. The expression of aquaporin fluctuates with stomatal movement

The expression of several *AQPs* in stomata varies diurnally and in response to water stress. The mRNA level of sunflower *SunTIP7*, encoding a TIP2 isoform, is enriched in GCs and increases during stomatal closure at the end of the light period, suggesting that SunTIP7 facilitates water efflux from the vacuole and GCs during this process (Sarda et al., 1997). As previously mentioned, most of maize *ZmPIPs* are more expressed in open stomata during the day than in closed stomata during the night (four hours after light off) (Heinen et al., 2014). In poplars, mRNA levels of *PIP1;2*, *PIP2;1*, *PIP2;5* and *TIP1;3* are higher in the afternoon than in the morning (Durand et al., 2020). In addition, *PIP* and *TIP* expression was analyzed under water stress conditions in different poplar genotypes. *PIP2;1* expression is higher under water stress than under control conditions, while *PIP1;2* and *TIP1;3* mRNA levels vary between genotypes (Durand et al., 2020). In *Sorghum bicolor*, *SbTIP1;1* expression is positively correlated with g_s during drought stress and recovery (Schley et al., 2022). These results indicate that *PIP* and *TIP* genes are under the control of a transcriptional machinery which is sufficiently flexible to modify their expression according to the circadian clock or under different stresses that affect stomatal movement. To date, little is known about the transcription factors and enhancers that modulate *AQP* gene expression.

The fluctuating expression of *AQ*Ps highlights the need to control their abundance under stress or day-and-night cycles for proper stomatal movements. Changes in AQP expression certainly contribute to a fine-tuning of H_2O, CO_2, and H_2O_2 diffusion through the membranes and, thus, of the stomatal movements (Ding & Chaumont., 2020). Besides, *AQP* expression also changes in other leaf cells, such as bundle sheath cells, which play a key role in controlling leaf hydraulic conductance and stomatal movements (Shatil-Cohen et al., 2011; Yaaran et al., 2023).

2.4. Deregulation of aquaporins and stomatal movement

Using reverse genetic approaches, the stomatal movement was checked in plants deregulated in *PIP* gene expression, either overexpression (OE) or knockout/knockdown (KO/KD), respectively) (for reviews, see Ding & Chaumont (2020) and Hachez et al. (2017)). The first direct evidence of a role of PIPs in stomatal movement came from analysis of Arabidopsis *Atpip2;1* KO plants (Grondin et al., 2015) that displayed impaired ABA-induced stomatal closure, a phenotype restored by complementation with the wild-type *AtPIP2;1* allele. In *Atpip2;1* mutant, stomatal closure is also impaired upon flagellin (flg22) treatment, which mimics pathogen attack. Actually, AtPIP2;1 contributes to ABA-induced stomatal closure, through both water and H_2O_2 membrane diffusion (Rodrigues et al., 2017; Rodrigues & Shan, 2022). However, no difference in ABA-induced stomatal closure between WT and *pip* quadruple (*pip1;1*, *pip1;2*, *pip2;1* and *pip2;2*) mutant in Arabidopsis was reported in a different study, probably due to different experimental conditions (Ceciliato et al., 2019). These contradictory results draw the attention of plant aquaporin scientists to the fact that more evidence is needed to elucidate the role of AQPs in stomatal movements in different species and under different environmental stimuli. Recently, we demonstrated that *Zm*PIP2;5 affected maize stomatal closure under water deficit conditions (Ding et al., 2022). At whole plant level, the stomatal conductance (g_s) and transpiration decreases more and less in *ZmPIP2;5* OE and *Zmpip2;5* knock down plants, respectively, under mild water deficit or low ABA concentration treatments. At stomatal level, ABA-induced stomatal closure is more sensitive in the OE plants and insensitive in the KD plants when compared with WT plants. Interestingly, *Zm*PIP2;5 is also involved in high VPD-induced stomatal closure (Ding et al., 2022). These phenotypes are associated with H_2O_2 accumulation and hydraulic regulation in GCs/stomatal complexes.

The role of *At*PIP2;1 in light-induced stomatal opening was also investigated (Huang et al., 2020). Stomata open more and less in *Atpip2;1* KO and *AtPIP2;1* OE plants, respectively, compared with the WT, indicating that stomatal opening is negatively regulated

by *At*PIP2;1 through phosphorylation (Huang et al., 2020). Further studies are needed to integrate the responses to different stimuli. g_s was also analyzed in other species overexpressing aquaporins. The stomatal closure is faster in tomato *MaPIP1;3* OE (L. Wang et al., 2017) compared with WT. In contrast, stomata close slower in soybean *GmPIP1;6* OE under salt stress (Zhou et al., 2014), indicating that the effect of AQPs on stomatal dynamics is isoform dependent and needs to be specifically investigated.

To date, there is no direct genetic evidence showing that TIPs expressed in stomata affect the stomatal movements. However, stomatal movement is accompanied by a significant rearrangement of the vacuole morphology in GCs. In closed stomata, GC vacuoles have a shrunken, convoluted structures (Andrés et al., 2014), or are divided into a large number of small vacuoles (Gao et al., 2005), whereas in open stomata most of the cell volume is occupied by one large vacuole (Andrés et al., 2014; Gao et al., 2005). Recently, 3-D imaging has shown that the vacuole is responsible for the increase in GC volume during stomatal opening induced by fusicoccin, a fungal toxin activating the PM proton pumps and stimulating ion fluxes directed to GCs (Mirasole et al., 2023). This is consistent with the observation that osmolyte uptake/efflux into the cytosol represents only a transient step to/from the vacuole (MacRobbie, 1998). Indeed, tonoplast-localized K^+/H^+ exchangers mediate vacuolar accumulation of K^+ in GCs (Andrés et al., 2014). Single-cell transcriptomes of Arabidopsis and maize concordantly showed that mRNA of a single *TIP1* gene and a single *TIP2* gene is detected in GCs (Figure 2), with *TIP1* being the most highly expressed in both species. TIP1s and TIP2s facilitate the diffusion of different solutes including water and H_2O_2 (Fox et al., 2017). However, it remains unknown whether TIPs play a role in stomatal vacuole remodeling by facilitating the diffusion of these molecules.

3. Posttranslational regulation of aquaporins applied to stomata

Integrating transcriptomic data with the AQP channeling capabilities provides clues as to which AQPs are involved in stomatal dynamics and their roles. To complete our understanding of

the mechanisms modulating AQP abundance and activity at their target membranes, we need to obtain additional information on their posttranslational regulation. Indeed, AQPs are also highly regulated at post-translational level, indicating that the control of AQP transcription and translation alone is not sufficient to allow an adequate and dynamic response within cells, tissues and the whole plant. This was illustrated by Pou et al. (2016), in the case of Arabidopsis roots exposed to salt stress. High salt exposure triggers rapid transcriptional repression of *AtPIP2;7* as well as internalization of the channel in intracellular structures to decrease the water conductivity of the cortex cell and the root. Both transcriptional and post-transcriptional regulations act in concert to a fine-tune the AQP abundance and activity in the membrane. We also believe that the variety of regulation mechanisms is also important for integrated responses to environmental cues at different temporal levels (minutes, hours, day/night). In this section, we will review several aspects related to AQP phosphorylation and AQP trafficking and dynamics in membrane contact sites (MCSs), and discuss their relevance to the regulation of stomatal dynamics. An overview of AQP posttranslational regulations that may be relevant for stomatal dynamics is discussed in this section and illustrated in Figure 3.

3.1. Aquaporin phosphorylation in gating and trafficking

Environmental signals trigger multiple PTMs of AQPs (Di Pietro et al., 2013). These include methylation, acetylation, and deamidation of the channels, but how these PTMs affect AQP activity still unknown. In contrast, phosphorylation and ubiquitination of PIPs are better understood. PIP phosphorylation influences their trafficking (Prak et al., 2008) and gating (Törnroth-Horsefield et al., 2006). PIP ubiquitination targets their degradation (Lee et al., 2009). Here, we will focus on PIP phosphorylation dynamics due to the key role of phosphorylation cascades during stomatal movement (Chen, 2023; Zhang et al., 2014).

Phosphorylation of PIPs occurs at the N- and C-termini as well as at the cytosolic loops B and D, and most of the phosphorylation sites appear to be conserved in land plants (Prak et al., 2008). Phosphorylation of the N-terminus is observed for PIP1s and PIP2s, while phosphorylation of the C-terminus was only detected for PIP2s (Prak et al., 2008; Van Wilder

et al., 2008). Interestingly, in contrast to PIPs cellular abundance, modulation of PIP1 and PIP2 phosphorylation is a predominant factor controlling root hydraulic conductivity (Di Pietro et al., 2013). The C-terminal phosphorylation of *At*PIP2;1 is required for various responses, such as the leaf hydraulic conductance enhancement in the dark (Prado et al., 2013), its internalization induced by NaCl in roots (Prak et al., 2008), and water movement in leaves induced by ethylene (Qing et al., 2016). There are two phosphorylation sites at the C-terminus of *At*PIP2;1 and *Zm*PIP2;1, one associated with their gating and the other with their trafficking (Prak et al., 2008; Van Wilder et al., 2008). Both trafficking directions, from ER to PM and from PM to endosomes appear to require phosphorylation of S283 in *At*PIP2;1. Moreover, upon salt treatment, a reduction in the abundance of the *At*PIP2;1 phosphorylated at S283 is observed, suggesting that PIP abundance in the PM is reduced by preventing PIPs from leaving the ER (unphosphorylated PIPs) and internalizing PM-localized channels (phosphorylated). Two other phosphorylation sites in PIP cytosolic loops are also important for efficient water channeling: one on a serine in loop B, also conserved in several TIPs, and the other in the loop D (Johansson et al., 1998; Maurel et al., 1995; Van Wilder et al., 2008). These two phosphorylation sites have been directly associated with pore gating, and hence channel activity (Mom et al., 2023; Törnroth-Horsefield et al., 2006).

The described PIP phosphorylation sites also play role in modulating stomatal movements. Specific phosphorylation at the S121 in loop B of *At*PIP2;1 by Open Stomata 1 (OST1) contributes to ABA- and flg22-induced stomatal closure by facilitating the efflux of water and influx of H_2O_2 in GCs (Grondin et al., 2015; Rodrigues et al., 2017). The C-terminal phosphorylated form of *At*PIP2;1 also plays a negative role in light-induced stomatal opening (Huang et al., 2020). The mechanism behind the latter phenotype has yet to be elucidated. *At*PIP2;1 is the most studied PIP with regard to phosphorylation variations. Whether other PIP2 isoforms are similarly regulated or whether other specific PTMs occur within the PIP family needs to be investigated.

3.2 Trafficking: tetramer composition and aquaporin partners

The variables that influence the fine-tuning of AQP abundance and activity are to be found right at the start of the trafficking pathway, in the ER, where AQPs oligomerize into tetramers. Indeed, PIPs and TIPs homo- and hetero-oligomerize (Fetter et al., 2004; Harvengt et al., 2000), and, at least for PIPs, oligomer composition modifies the cell water membrane permeability and the solute transport selectivity (Fetter et al., 2004; Otto et al., 2010). Moreover, for PIP1s to reach the PM, hetero-oligomerization with PIP2 isoforms is required (Zelazny et al., 2007). Transiently expressed *Zm*PIP1s localize to the ER, while when co-expressed with *Zm*PIP2s, PIP1s reach the PM by interaction with *Zm*PIP2s. Consistently, PM trafficking motifs are found in PIP2s but not in PIP1s (Chevalier & Chaumont, 2015). In stomatal complexes, PIP1s and PIP2s are co-expressed (Figure 2), suggesting modulation of PIP activity by hetero-oligomerization mechanisms. Yet, there is an unbalance between the mRNA level of *PIP1*s and *PIP2*s in maize stomatal complexes, with PIP1s accounting for over 80% of total *PIP* mRNA. Consequently, it would be useful to know how PIP1s reach the PM of GCs/SCs, whether all PIP1s are in the PM, and what the stoichiometry organization of the tetramers is. Interestingly, tetramers have a flexible stoichiometry that depends on the amount of PIP1 and PIP2 molecules available (Jozefkowicz et al., 2016) and the heteromerization affects the intrinsic permeability of the channels (Yaneff et al., 2014). Whether the kinetic of oligomer trafficking to the PM and internalization are affected by the tetramer composition is not yet known, but may be an additional mechanism for regulating AQP activity.

PIPs physically interact with proteins of the SNARE (soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor) superfamily. SNAREs integrate complexes that facilitate the fusion of vesicles with target membranes (recently reviewed in Luo et al., 2022). At the trans-Golgi network, PIPs interact with SNARESs of the Syntaxin of Plant (SYP) 1 family, also known as Qa-SNAREs (Besserer et al., 2012; Hachez et al., 2014a, Laloux et al., 2021; Baena et al., 2024). SYP1s drive vesicles fusion with the PM, leading to cargo secretion and delivery of PM proteins to their final destination (Uemura et al., 2004). Interestingly, in GCs, the SYP1 isoform SYP121 is implicated in modulating of K^+, Cl^-, and

Ca^{2+} channel activity in response to ABA (Eisenach et al., 2012; Leyman et al., 1999; Sokolovski et al., 2008). SYP121 physically interacts with the voltage sensor domain of the K^+ channels KC1 and KAT1 (Grefen et al., 2015). ABA treatments trigger endocytosis of AtKAT1 K^+ channel in epidermal cells and GCs (Sutter et al., 2007). The authors proposed that both water and K^+ channel activity be activated to decrease GC turgor pressure and close stomata, followed by channel endocytosis to decrease their density in the PM. A recycling of the proteins to the PM would occur, leading to the reopening of the stomata. Interestingly, *Zm*PIP2;5 and *At*PIP2;7 also physically interact with *Zm*/*At*SYP121 (Besserer et al., 2012; Hachez et al., 2014a), pointing to an evolutionary conserved mechanism event. This interaction is necessary for proper targeting of the AQPs to the PM (Besserer et al., 2012; Hachez et al., 2014a), but a dominant negative form of SYP121 also affects PIP channel activity, when co-expressed in Xenopus oocytes. Other results showed that *At*PIP2;7 interacts with other SYP1s and, also that most of the PIPs interact with SYP121 (Laloux et al., 2021). Whether these various interactions are redundant or linked to different cellular processes need to be addressed. Indeed, even if there is some redundancy between SYP1s, there is evidence of isoform-specific roles. For example, the close homologs SY121 and SYP122 mediate the secretion of distinct cargo subsets during plant growth (Waghmare et al., 2018). Consistently, the Arabidopsis *syp121* mutant plants, but not *syp122* mutants, displayed delayed stomatal opening in light and following Ca^{2+}-evoked closure compared with WT plants (Eisenach et al., 2012). A tripartite interaction between K^+ channels, SYP121 and PIP may be an elegant way to coordinate the regulation of turgor pressure in GCs and, thus, stomatal movement. Very recently, Baena et al. (2024) report that the interaction of *At*SYP132, homologous of *At*SYP121, with *AtP*IP2;1 and *At*AHA1 (H^+-ATPase) induces endocytosis of these transporters. This work presents a nice example of how SYP1s coordinate the differential abundance of PM transporters in response to stress. Upon osmotic or salt stress, the *At*SYP132-*At*PIP2;1 interaction increases and the *At*SYP132-*At*AHA1 interaction decreases, differentially modulating the PM abundance of these proteins. As discussed above, there is a close relation between the abundance PIPs in PM and the interaction with various SYP1s. Future work is

388 needed to understand whether there is a specific regulation of PIPs-SYP1s interactions in
389 stomata cells.

390 Internalization of PM-localized PIPs is regulated by two main mechanisms, namely
391 clathrin-dependent pathway, which appears to be the predominant recycling pathway under
392 steady state conditions, and a membrane microdomain-mediated pathway that occurs under
393 stress conditions (Li et al., 2011). Furthermore, under abiotic stress conditions, the abundance
394 of PM-localized PIP2s decreases via proteasomal degradation (Lee et al., 2009) and the
395 autophagic pathway (Hachez et al., 2014b). For *At*PIP2;7, the autophagic pathway depends
396 on its physical interaction with the multi-stress regulator of tryptophan-rich sensory
397 protein/translocator (TSPO) (Hachez et al., 2014b). Arabidopsis TSPO binds the
398 phosphoinositide PI(4,5)P2, which is a requirement for its interaction with AQP located in PM
399 (Jurkiewicz et al., 2020). In addition, TSPO expression remodels the phosphoinositide
400 composition of the PM through depletion of PI(4,5)P2 from the PM and its enrichment in the
401 Golgi membrane (Jurkiewicz et al., 2020). Interestingly, in TSPO overexpressing plants, YFP-
402 TSPO does not accumulate in most of the tissues analyzed, but is detected in cell types known
403 to be enriched in the expression of ABA biosynthetic genes, such as in GCs and vein cells
404 (Hachez et al., 2014b). Furthermore, the overexpressing plants show reduced g_s compared
405 with WT plants, while *tspo* knock out plants display higher g_s (Jurkiewicz et al., 2020).
406 Altogether, these results suggest that TSPO negatively affects stomatal opening. Exogenously
407 administration of PI(4,5)P2 induces stomatal opening and increases swelling of GC
408 protoplasts, a phenotype associated with the inhibition of anion channels by this
409 phosphoinositide (Lee et al., 2009). Further work will be necessary to explore in depth the
410 effects of TSPO effects on (i) the PM phosphoinositide pool with the modulation of PIP
411 abundance at the PM and (ii) the PM density of the other channels involves in stomatal
412 movements.

413 Regarding TIP trafficking, few data are available, and there is no evidence of regulation
414 of TIP permeability or oligomer trafficking by heteromerization. To date, two different trafficking

pathways to the vacuole have been described for TIPs, one Golgi-dependent and the other Golgi-independent (Rojas-Pierce, 2013). The trafficking of *At*TIP1;1 to the tonoplast takes place via a Golgi-dependent pathway whereas *At*TIP2;1 trafficking is considered to be Golgi independent (Rojas-Pierce, 2013). Targeting of *At*TIP1;1 is sensitive to the overexpression of the SNARE *At*SYP21. In cells expressing this form of *At*SYP21, TIP1;1 accumulates in a pre-vacuolar compartment instead of the tonoplast (Tyrrell et al., 2007). It would be interesting to study whether blocking TIP1 trafficking to the vacuole has any effect on the morphology of the stomatal vacuole and ultimately on stomatal dynamics.

3.3. Channel activity modulation by its lateral diffusion

Significant progress has been made in describing PIP dynamics during stomatal closure (Cui et al. (2021). By combining confocal microscopy and variable-angle total internal reflection fluorescence microscopy, GFP-*At*PIP2;1 particles were tracked in GCs and surrounding cells during flg22-induced stomatal closure induced. Treatment with flg22 induces endocytosis of GFP-*At*PIP2;1 in these cells (Cui et al., 2021). This internalization of *At*PIP2;1 in GCs was not expected considering that it facilitates the diffusion of H_2O_2 and water in response to flg22 treatment (Rodrigues et al., 2017). But an additional process is associated with *At*PIP2;1 within the PM: the velocity and lateral diffusion of GFP-AtPIP2;1 particles increase in GCs upon flg22 treatment, while these parameters do not change in the neighboring cells (Cui et al., 2021).

The lateral diffusion of PIPs is restricted by the cytoskeleton (Hosy et al., 2015) and cytoskeleton reorganization is essential during stomatal movement (Eisinger et al., 2012; Hosy et al., 2015; Hwang & Lee, 2001). Recently, *At*OST1 was found to mediate microtubule disassembly in GCs required for the induction of stomatal closure by ABA (Pan Wang et al., 2023). Interestingly, disruption of the cytoskeleton organization in both GCs and neighboring cells increases the diffusion of *At*PIP2;1 within the PM (Cui et al., 2021). Moreover, while microtubules play a crucial role in regulating the *At*PIP2;1 dynamics in GCs, the actin filaments appear to play a primary role in the neighboring cells. Therefore, this scenario in neighboring cells resembles the one observed in root epidermis, where actin microfilaments rather than

microtubules are involved in restricting the lateral diffusion of *At*PIP2;1 particles (Hosy et al., 2015). Taken together, these results indicate a specific modulation of the GFP-*At*PIP2;1 channel activity as a function of its lateral mobility. However, the molecular mechanism behind this modulation remains unknown. Given the coexistence of different diffusion modes for *At*PIP2;1 within the PM, with channels moving into and out of membrane microdomains (Li et al., 2011), we can speculate that specific stimuli distribute PIPs to specific lipid environments within the PM to modulate their channel activity.

3.4. Potential roles for membrane contact sites-located aquaporins in stomata

MCSs are defined as areas of close apposition between the membranes of two organelles (for more details, see in Scorrano et al. (2019)). MCS are relevant for lipid biosynthesis, metabolite channeling, cell signaling and regulation of membrane dynamics (Prinz et al., 2020, Pengwei Wang et al., 2023). Tethers and spacers are structural proteins of MCSs that maintain both membranes connected at a given distance. In plants, two families of structural proteins have been described to be present at ER-PM contact sites (EPCSs): synaptotagmins (SYT) and vesicle associated protein 27 (VAP27s). Some tethers, such as VAPs, have multiple interaction partners on the opposing membrane, which could underline different functions (Prinz et al., 2020). Indeed, VAP27s are found in EPCS as well as ER-mitochondria and ER-chloroplast contact sites (Pengwei Wang et al., 2023a).

The first evidence of a role of MCS on stomata physiology arises from the work done by Ho et al. (2016). The authors showed that VAP-RELATED SUPPRESSOR OF TMM (VST) family was required to inhibit stomatal development. VST1-3, also named VAP27-8-10 (Pengwei Wang et al., 2016), share the major sperm domain (MSD) domain that characterizes VAP27s, but lack the ER anchoring domain. VST are present on EPCSs through their interaction with the ER residents SYT1 and SCR2 (Ho et al., 2016). The Arabidopsis triple mutant *vst1vst2vst3* exhibits larger MCSs, which could explain why the ERECTA-mediated signaling is abolished in this mutant, leading to up-regulation of stomatal number (Ho et al.,

2016). This evidence highlights the importance of a defined distance on MCS for cell signaling in stomata. The maize genome also encodes three VST isoforms, one of which displays higher expression levels in GCs than in other leaf cell types (Figure 4), indicating a role in stomatal development that needs to be elucidated.

It has been proposed that, in specialized cells that require rapid ion exchange such as the GCs, the EPCSs can regulate the ion channel activity (Pengwei Wang et al., 2023). PIPs are present at EPCSs (Fox et al., 2020) and we propose that this presence is relevant for stomatal movement. Interestingly, in maize SCs and GCs, different VAP isoforms are expressed, while SYT appears to be specifically expressed in GCs (Figure 4). Arabidopsis mutants lacking SYT1 exhibit decreased PM integrity under osmotic stress (Ruiz-Lopez et al., 2021) and increased stomatal closure under salt stress (Krausko et al., 2022). We have shown that *Zm*PIP2;5 interacts with VAP27s of different clades and that *Zm*VAP27-1 positively modifies the osmotic membrane water permeability of *Zm*PIP2;5 expressing oocytes (Fox et al., 2020). The physiological relevance of PIP-VAP interaction remains to be investigated in stomata. Furthermore, *At*VAP27-1 binds a PIEPPPHH peptide present in the C-term of *At*TIP3;1 and these proteins colocalize in the protein storage vacuole (PSV) (Oufattole et al., 2005), suggesting that VAP27-TIP interactions may also play a role in stomatal regulation.

4. Conclusion and future directions

Knowledge on PIP expression, function and regulation in the context of stomatal dynamics has progressed in recent years. Overexpression and KO of *PIP* genes affect stomatal movement upon ABA and/or water deficit treatments. In addition, numerous regulatory mechanisms have been identified in different cell types including GCs, but their relevance in controlling stomatal dynamics under different environmental conditions remains to be elucidated. Below, we propose several specific perspectives to better understand the function, regulation and integration of AQPs in the highly controlled stomatal cells.

(1) *The roles of AQPs in grass stomata, given their relevance in the agricultural systems*. Maize stomata are a powerful model for the investigation of stomatal movement in grasses. Single nuclei RNA-seq data show that AQPs are expressed in both maize GCs and SCs, with some isoforms preferably expressed in a single cell type. Tissue-specific silencing technique, i.e., CRISPR-TSKO (Decaestecker et al., 2019), could be applied to investigate the role of differentially expressed *PIPs* in GCs and SCs in maize stomatal movement. This approach applied to *TIPs* would be an elegant way to assess the role of the tonoplast AQPs, for which no genetic evidence about their role in stomatal complexes is available.

(2) *The substrate specificity of AQPs in relation to stomatal movement*. AQPs have been demonstrated to channel many different small solutes. PIPs might affect CO_2-induced stomatal closure by facilitating CO_2 diffusion across the PM (Zhang et al., 2018), but additional studies are, however, needed to further explore the exact role of PIPs in CO_2-induced stomatal movement. PIPs have been shown to contribute to the transport of cations, such as Na^+ and K^+ (Tyerman et al., 2021; Ahmed et al., 2024). A possible coupling between water and ion flow has been proposed in relation to water pumping and energy generation (Tyerman et al., 2021). However, as the cation fluxes associated with aquaporins are several orders of magnitude below those needed (Tyerman et al., 2021), and provided via other transporters, the relevance of this activity in stomatal complexes might be questioned. Further research is required to mechanistically elucidate this paradigm at the protein level and determine whether or not this transport capacity plays a role in stomatal function.

(3) *Cellular regulation of AQPs and stomatal movements*. Oligomer composition, PTMs, trafficking, PM lipid environment, and the cytoskeleton are all variables that influence PIP channeling activity and are known to occur in stomatal cells. The relevance of each of these variables and how they integrate in response to different environmental signals influencing stomatal aperture dynamics need to be better addressed in the future. Furthermore, the presence of AQPs in EPCSs is hypothesized to be relevant for regulating stomatal movement.

The physiological role of VAP27-PIP/TIP interaction in these cells in relation to AQP fate and cell structural organization still need to be investigated.

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Conflict of interest statement

The authors declare no conflict of interest.

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Figure legends

Figure 1 Expression of *PIPs* and *TIPs* in *Arabidopsis* (a) and maize (b) leaves from RNA-seq analysis. (a) The expression data in GC protoplasts was obtained from Lee et al. (2019). The expression data in epidermis, mesophyll, vasculature and whole leaf was obtained from Berkowitz *et al*. (2021). Epidermis, mesophyll and vasculature cells were collected by laser microdissection. (b) The expression data was obtained from single-nucleus RNA-seq (Sun *et al*., 2022, http://songlab.henu.edu.cn:3838/2021maizestomata/). TPM, transcript per million reads. GCs, guard cells. ECs, epidermal cells. MCs, mesophyll cells. VCs, vasculature cells. WL, whole leaf. PCs, pavement cells. BSCs, bundle sheath cells. SCs, subsidiary cells.

Figure 2 Schematic model of PIP-mediated stomatal closure induced by ABA and CO_2 in *Arabidopsis* and maize. In *Arabidopsis*, ABA enters GCs and OST1 is activated. OST1 then phosphorylates *At*PIP2;1 and activates its H_2O and H_2O_2 channel activity (Grondin *et al*., 2015; Rodrigues *et al*., 2017). Downstream ion channels are activated and water exits GCs to decrease the turgor pressure and stomatal closure. When expressed in Xenopus oocytes *At*PIP2;1 facilitates the diffusion of CO_2 and interacts with carbonic anhydrase (CA) to further activate the SLAC1 anion channel (Wang *et al*., 2016a). It is therefore proposed that *At*PIP2;1 affects high CO_2-induced stomatal closure by regulating its transport into GCs (Zhang *et al*., 2018). In maize, *ZmPIP2;5* is specifically expressed in GCs, and *Zm*PIP2;5 controls stomatal closure induced by water deficit or ABA by facilitating the diffusion of H_2O and H_2O_2 through GCs (Ding *et al*., 2022). In maize GCs, the ABA-induced signaling pathway activating *Zm*PIP2;5 activity and stomatal closure may be similar to that acting on *At*PIP2;1 in Arabidopsis. In SCs, ions and water accumulate during ABA-induced stomatal closure. PIPs expressed in SCs, also affect H_2O and H_2O_2 diffusion. *Zm*PIP1;6 appears to be specifically expressed in SCs, and may be involved in high CO_2-induced maize stomatal closure as it also

953 acts as a CO_2 channel when expressed in yeast (Heinen *et al*., 2014). It remains unclear which
954 PIPs facilitate the transport of CO_2 in maize GCs. RBOHs, respiratory burst oxidase homologs.
955 OST1, open stomata 1 (SnRK2 kinase). CA, carbonic anhydrase. P_{Turgor}, turgor pressure. GC,
956 guard cell. SC, subsidiary cell.

957

958 **Figure 3 A diversity of mechanisms controls PIP and TIP dynamic in stomatal cells.**
959 After their synthesis in the endoplasmic reticulum (ER), PIPs and TIPs assemble in homo- and
960 hetero-oligomers. All PIPs follow the conventional vesicular trafficking pathway, which
961 involves COPII vesicle-mediated ER export and subsequent transport to the Golgi and trans-
962 Golgi network (TGN). TIP1s follow the same ER-Golgi-TGN route while TIP2s follow a Golgi
963 independent pathway to the vacuole. At the TGN, PIPs load in secretory vesicles to reach the
964 plasma membrane (PM). Proteins of the SYP1 family drive PIP targeting to the PM. SYP121
965 plays a relevant role in GCs in response to ABA. Upon an osmotic or salt stress, SYP132
966 interacts with PIP and induces the endocytosis of the channel. TIP1 loading on pre-vacuolar
967 compartments (PVC) is mediated by SYP21 before reaching the vacuolar membrane.
968 Internalization of PM-localized PIPs occurs via a clathrin-dependent pathway (represented by
969 clathrin coated vesicles, CCV), and via a membrane microdomain-mediated pathway. This
970 last pathway is enhanced under stress conditions. Once internalized, PIPs are delivered to
971 the TGN/Golgi, from where they can be recycled to the PM or follow a degradation pathway.
972 The autophagic degradation of PIPs mediated by TSPO requires the binding of PI(4,5)P2 by
973 TSPO and was suggested to happen in GCs. The light-induced phosphorylation at the C-
974 terminus of PIP2s is associated to the inhibition of stomata opening by a mechanism still
975 unclear. The flg22 treatment induces PIP endocytosis, increases the lateral mobility of the
976 channels at the PM and, as ABA treatment, induces the phosphorylation of the PIP loop B
977 mediated by OST1 resulting in stomata closure. At the same time OST1 mediates the ABA-
978 induced disassembly of microtubules. Microtubules also play a crucial role in regulating the
979 dynamics of PIPs in GCs, whereas actin appears to regulate the PIP dynamic in neighboring

cells. PIPs are present at ER-PM contact sites (EPCSs) in interaction with the ER resident VAP27s. The relevance of this interaction on stomatal dynamics needs to be evaluated. Another EPCS structural protein, SYT, is also expressed in GCs, and is involved in stomatal closure even though its interaction with PIPs remains unknown. VAP27s are found in contact sites between the ER and different organelles. VAP27s and TIPs can interact in ER-Vacuole contact sites and play a still unknown role in stomatal regulation. Compartment names are indicated in bold black font. Question marks indicate possible pathways not yet supported by experimental evidence, at least in stomatal cells. Abbreviations: PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; SYP121, syntaxin 121; SYP132, syntaxin 132; SYP21, syntaxin 21; TSPO, tryptophan-rich sensory protein/translocator; OST1, open stomata 1; VAP27, vesicle associated protein 27, SYT, synaptotagmin, ABA, abscisic acid; flg22, flagellin 22.

Figure 4 Expression of genes relevant for stomatal development and dynamics, encoding proteins that interact or are potential interactants of PIPs. Expression data from single-nucleus RNA-seq of maize leaves (Sun *et al*., 2022, http://songlab.henu.edu.cn:3838/2021maizestomata/). Abbreviations: SYP121, syntaxin 121; SYT, synaptotagmin; VAP27, vesicle associated protein 27. VST, VAP-related suppressor of TMM.

1000 Figure 1

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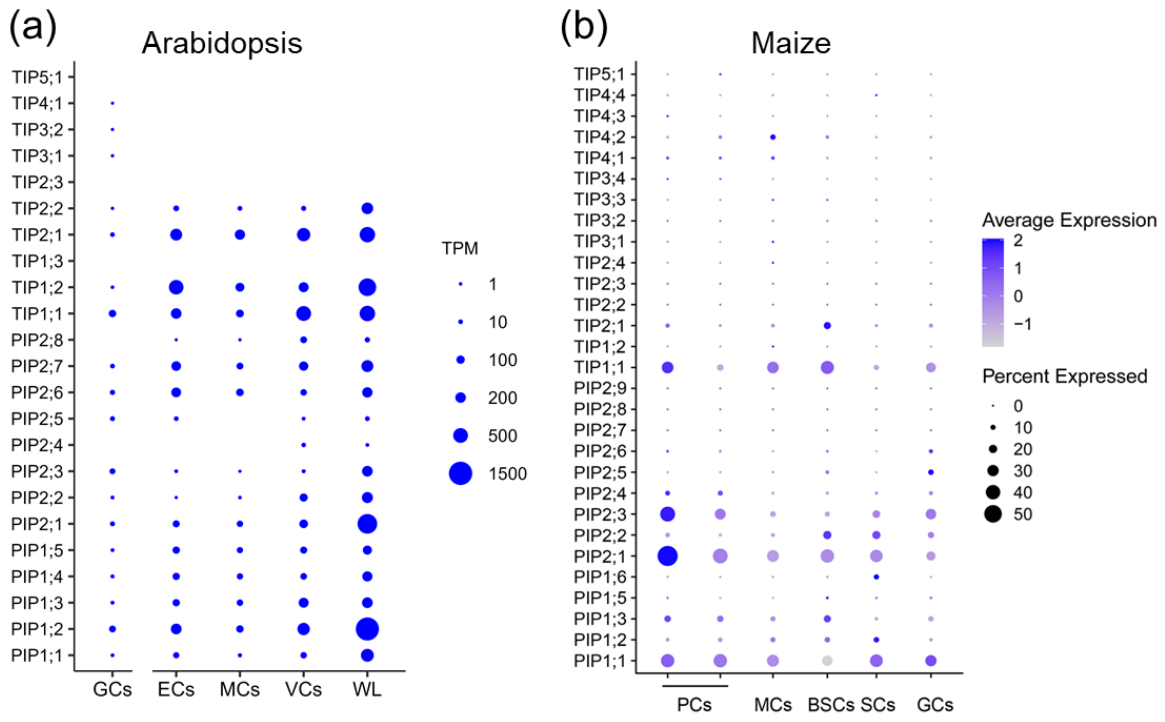

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1005 Figure 2

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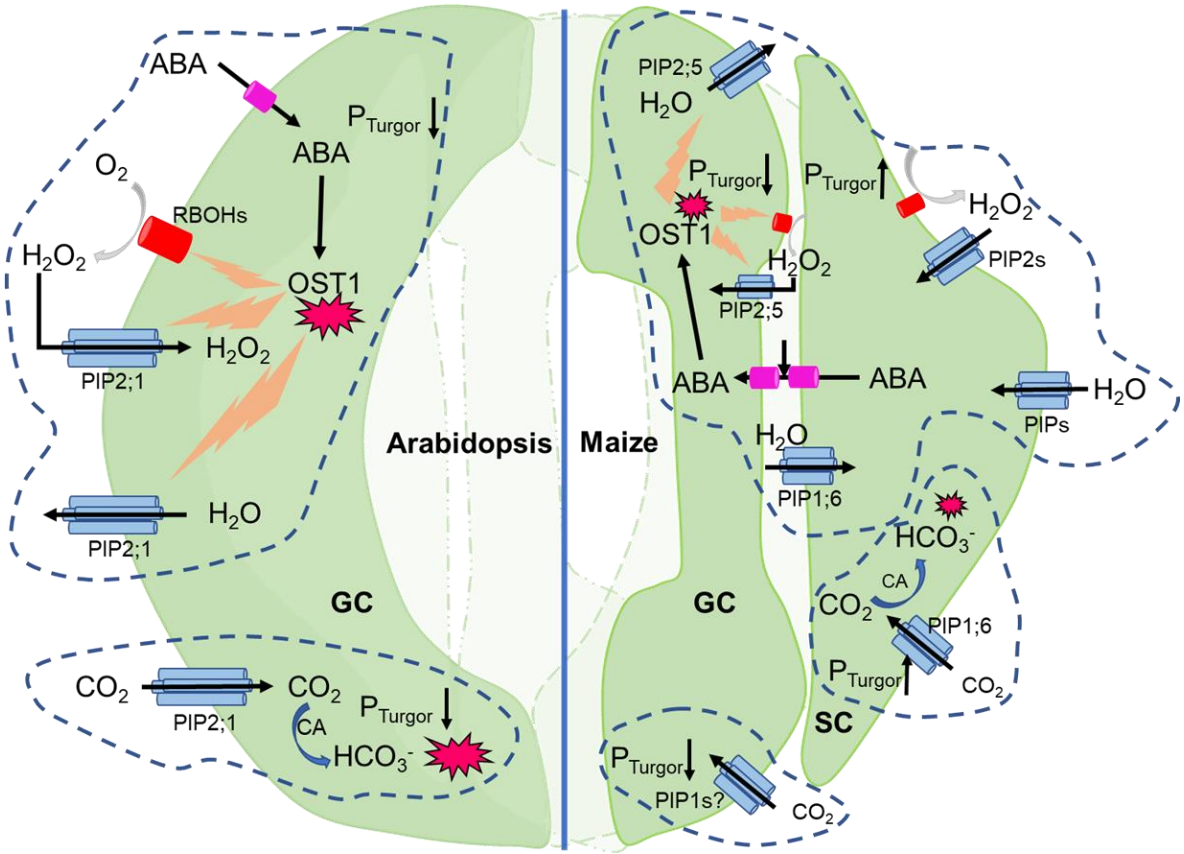

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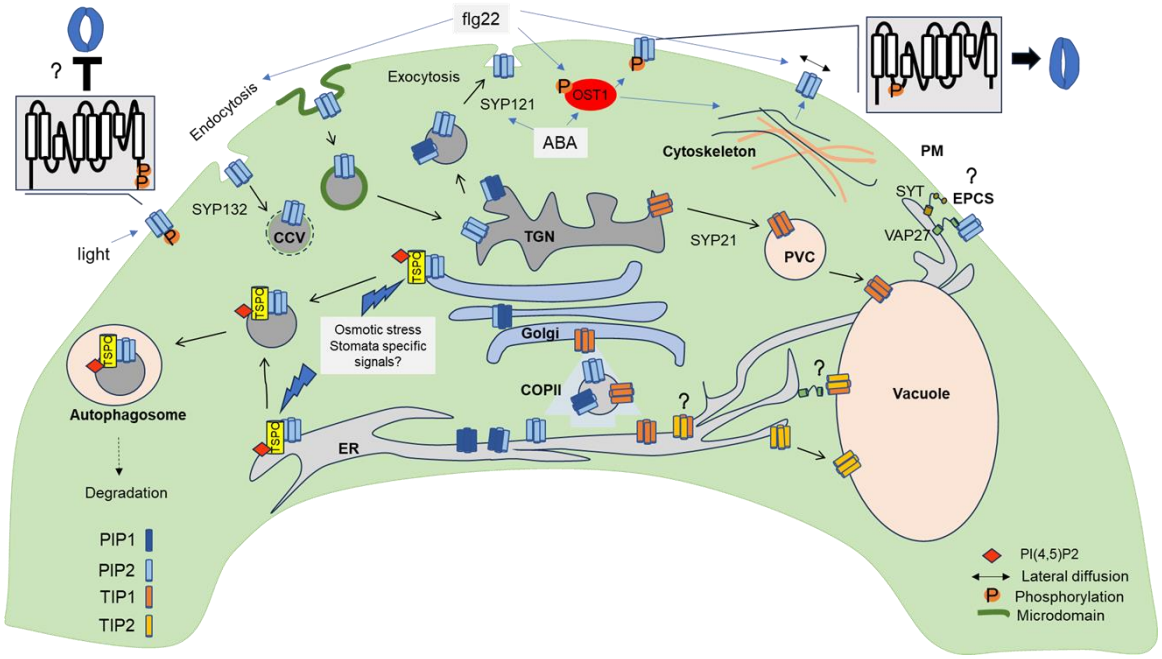

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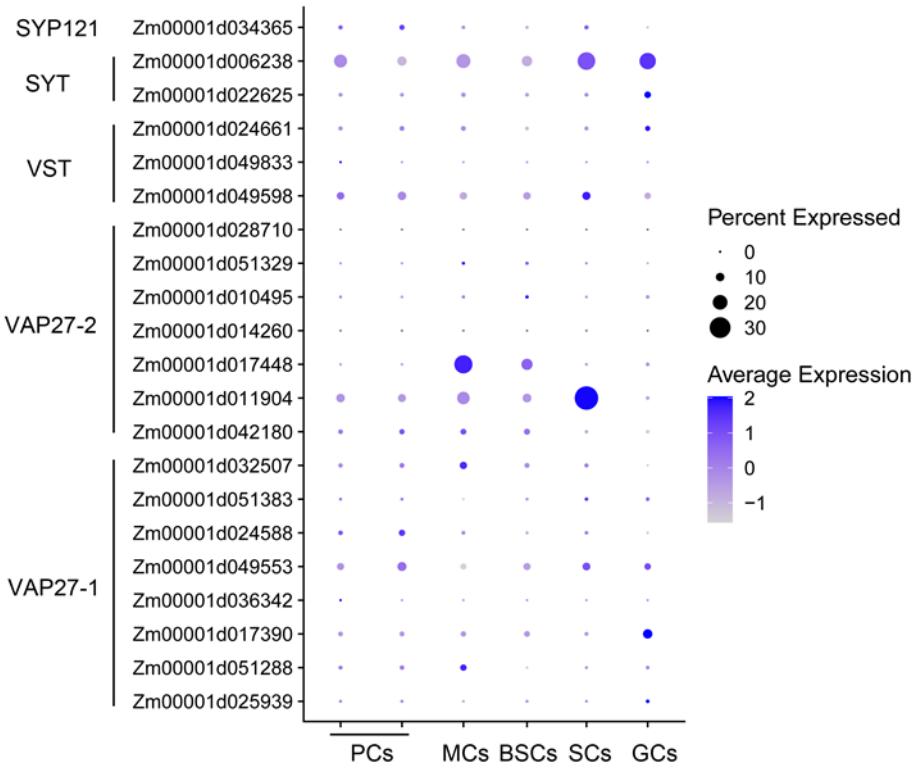

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