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Review

Toward understanding of the high number of plant aquaporin isoforms and multiple regulation mechanisms

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ABSTRACT

Since the discovery of the first plant aquaporin (AQP) in 1993, our conception of the way plants control cell water homeostasis as well as their global water balance has been revisited. Plant AQPs constitute a large family of evolutionarily related channels that, in addition to water, can also facilitate the membrane diffusion of a number of small solutes, such as urea, CO_2, H_2O_2, ammonia, metalloids, and even ions, indicating a wide range of cellular functions. At the cellular level, AQPs are subject to various regulation mechanisms leading to active/inactive channels in their target membranes. In this review, we discuss several specific questions that need to be addressed in future research. Why are so many different AQPs simultaneously expressed in specific cellular types? How is their selectivity to different solutes controlled (in particular in the case of multiple permeation properties)? What does the molecular interaction between AQPs and other molecules tell us about their regulation and their involvement in specific cellular and physiological processes? Resolving these questions will definitely help us better understand the physiological advantages that plants have to express and regulate so many AQP isoforms.

1. Introduction

Plant growth and development occur under ever-fluctuating environmental conditions, and their ability to continuously sense and respond to these changes guarantees their survival and reproduction. During their lifespan, plants have to adjust the abundance of different transporters and channels in their membranes depending on their own developmental requirements and on the environmental availability of water and nutrients. Aquaporins (AQPs) are proteinaceous channels, first described in the early 1990's as water transporters [1]. Since then, huge progress has been made in the characterization of this family, allowing insights to be gained into their role in the control of plant-water relations [2].

The plant AQP family is a large family of evolutionarily related channels with a generally conserved hourglass pore structure, and includes not only water channels, but also channels that allow the membrane diffusion of other solutes, in addition or instead of water. Therefore, the physiological roles of AQPs expand to more than water channels, being involved in a diversity of functions such as the transport of micronutrients (boron, silicon…), signaling molecules (H_2O_2…) or photosynthetic substrates (CO_2) [3].

Based on sequence identity, five AQP subfamilies have been identified in vascular plants: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin-26 like intrinsic proteins (NIPs) (found in the symbiotic membranes of legumes but also in the plasma membrane and endoplasmic reticulum (ER)), the small basic intrinsic proteins (SIPs) (located in the ER and the plasma membrane) and, finally, the X-intrinsic proteins (XIPs) found in the plasma membrane [4–8]. Whereas PIPs, TIPs, NIPs, and SIPs have been described for most land plant lineages, XIPs have not been found in Brassicaceae and monocots [6]. The expansion of these subfamilies by gene duplications and horizontal gene transfer events during the course of the evolution of higher plants has resulted in AQP families, including between 30 and 70 AQPs isoforms [9].

Many excellent reviews on plant AQP regulation have been published [2,3,10–12]. Here, we will discuss several specific questions that would need to be addressed in future research. Why are so many different AQPs simultaneously expressed in specific cellular types? How is the selectivity to different solutes controlled, particularly those that appear to have multiple permeation properties? What does the molecular interaction between AQPs and other proteins and lipids tell us about their regulation and their involvement in specific cellular and

Abbreviations: AQP, aquaporin; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; NIP, nodulin-26 intrinsic protein; SIP, small basic intrinsic protein; XIP, X-intrinsic protein; ER, endoplasmic reticulum; PTM, post-translational modification; TM, transmembrane domain

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physiological processes?

2. Why are so many different AQPs simultaneously expressed in specific cellular types?

2.1. Evolution and diversity of membrane intrinsic proteins

Since the discovery of the first AQPs in the early 90s, a vast number of AQP sequences have been identified in the three kingdoms of life, potentiated mainly by genome and transcriptome sequencing initiatives. This collection of data prompted different studies intending to understand the coexistence of a great diversity of membrane intrinsic proteins on an evolutionary framework. In this regard, phylogenetic analyses depicted scenarios of evolution where an early gene duplication event gave origin to water channels and glycerol channel families [13–15]. Whereas water channels are present in all eukaryotes sequenced so far, glycerol channels (also named aquaglyceroporins) are present in most eukaryotes including green algae and mosses, but not in vascular plants. Interestingly, in vascular plants, the AQP family present a great expansion, even in terms of AQP subfamilies (*i.e.* PIPs, TIPs, NIPs, XIPs, and SIPs) as well as members within each subfamily [9]. This multiplicity of AQP isoforms raises the following questions: does such gene redundancy imply a diversification of functions, or is there only a high functional overlap between duplicated genes? Closely related plant AQPs evolved under purifying selective pressure which means that, between them, a limited functional divergence occurred in the coding region [16,17]. In this regard, the study of the impact of *Glycine max* whole-genome duplication on gene expression revealed that, generally, paralogs evolve under purifying selection and 50% of them undergo tissue expression sub-functionalization [18]. Accordingly, in *Populus trichocarpa*, most of the pairs of duplicated AQP genes show divergent patterns of expression, even if there are cases where the functional redundancy cannot be excluded [16]. In addition to that, a certain degree of redundancy between paralogs is supported by the absence of the obvious phenotype of different single AQP mutants [2,19,20]. Besides the spatio-temporal sub-functionalization, neo-functionalization may have evolved, particularly in the case of intracellular AQPs [9], as the ancestral membrane intrinsic protein was only exposed to the extracellular medium. Neo-functionalization can originate from the acquisition or loss of different solute selectivity, as water transport is the only ancestral feature shared by the PIP, TIP, and SIP subfamilies [14]. The acquisition by horizontal gene transfer of the NIP subfamily from bacteria also contributes to the diversification of land plant AQPs (reviewed in [21]).

2.2. Plant AQP expression

Nowadays, RNA-seq technology produces high coverage of transcriptomes and allows a more complete profiling of AQP expression previously circumscribed by the high sequence similarity between *AQP* genes from the same subfamily. While the mRNA level of a gene is not necessarily strictly related to the abundance and activity of a protein in a cell or tissue, changes in the mRNA expression level often reflect the protein abundance. Developmental transcriptome profiles of different angiosperms, such as Arabidopsis and maize have been obtained in recent years [22,23]. The RNA-seq databases constitute interesting tools to analyze how the different AQP subfamilies/isoforms are regulated, and to deduce their putative physiological role in cell water or solute homeostasis. We organized the RNA-seq developmental data of maize [23] to better depict the complex expression profile of AQPs (Fig. 1). As expected from previous qPCR or protein expression studies performed on maize *PIP* genes [24–26], AQP isoforms have different patterns of expression according to the organs and the developmental stages. *PIP* genes are generally highly expressed (absolute values – circle size), especially in roots, and show a large amplitude of variation in expression (relative values – circle color). TIPs are also highly expressed in roots, especially TIP1s and TIP2s, whereas TIP3s are mostly expressed in seeds. SIPs show quite a constant and low expression level. Similarly, a globally low expression is observed for NIPs, that however display a larger amplitude of variation than SIPs. This dynamic is very similar to the one reported for Arabidopsis [27], highlighting the existence of similar patterns across monocot and dicot species estimated to diverge 150–300 million years ago [28]. This suggests that the physiological diversification of AQPs is likely conserved between distant plant species.

Transcriptomic studies also have an immense potential to help understand the functional contribution of AQPs in response to different stresses. Changes in the expression pattern of closely related AQPs in plants exposed to stress point to differential roles of AQP paralogs under stress conditions (reviewed in [12,29]). Transcriptomic studies now offer the possibility to assess a potential correlation between the expression of specific AQPs and other cellular transporters, a topic that has been poorly studied in the past. However, the disadvantage of these high throughput studies performed from different tissues is the loss of information about individual cell types. The application of recent advances in single-cell type isolation protocols and single cell profiling in plants [30,31] provide a unique opportunity for detailed studies of AQP paralogs. For instance, laser micro-dissection of maize stomatal complexes allowed us to identify the PIPs specifically expressed in these cells during the day or during the night [32]. Surprisingly, in these stomatal complexes like in all other cell types or tissues analyzed so far, members of the PIP1 and PIP2 subfamilies are always co-expressed. Interestingly, the ratio between the PIP1 and PIP2 isoforms can differ significantly between the cell types/tissues, but we wonder why a single cell needs to express several paralogs at the same time. A more complete understanding of the *in vivo* transport activity of these AQPs is definitely required to discern between diversification and redundancy among paralogs, and to obtain a deeper understanding of the adaptive advantage conferred by the expression of several AQPs in a specific cell type.

2.3. Substrates

Plant AQPs, first discovered as water channels, also facilitate the membrane diffusion of an increasing number of small solutes, such as urea, CO_2, H_2O_2, ammonia, metalloids and, as recently reported, ions, O_2, and Al-Malate [33–35]. This large list of solutes suggests a wide range of putative physiological roles that have been recently reviewed (for metalloids transport [36], H_2O_2 [37], CO_2 [38], or more general reviews [3,39,40]). Currently, the solute transport description of AQPs is far from exhaustive, even for model species that have been extensively studied. Nevertheless, the channel substrate specificity is generally conserved within a given family, even if exceptions are reported (Figs. 1 and 2). For instance, most of the characterized PIPs facilitate water diffusion; the TIPs facilitate the diffusion of water, urea, ammonia, and H_2O_2, and the NIPs the diffusion of metalloids (boric acid and arsenite) in addition to glycerol and water. In addition, some AQPs exhibit specific channel activities, such as, for instance, the ability to transport CO_2, which is restricted to some PIP isoforms [41]. However, it has to be mentioned that the transport specificity of AQPs is generally tested after heterologous expression in Xenopus oocytes or in the yeast *Saccharomyces cerevisae*. While the water channel activity of AQPs in the plant cell membrane can be deduced from protoplast swelling assays or the use of a cell pressure probe and treatments with AQP inhibitors (such as mercury, silver, or cytoplasmic acidification), demonstrating the facilitated diffusion of other solutes through AQPs in a plant cell is more complex. In heterologous expression systems, the functional assays may detect a substrate specificity that might not be relevant in plant cells due to specific regulation events or to the absence of substrate. To overcome this issue, several studies analyzed the physiological effects resulting from the deregulation of AQP expression (knockout, down- or over-expression). For example, knockout mutants

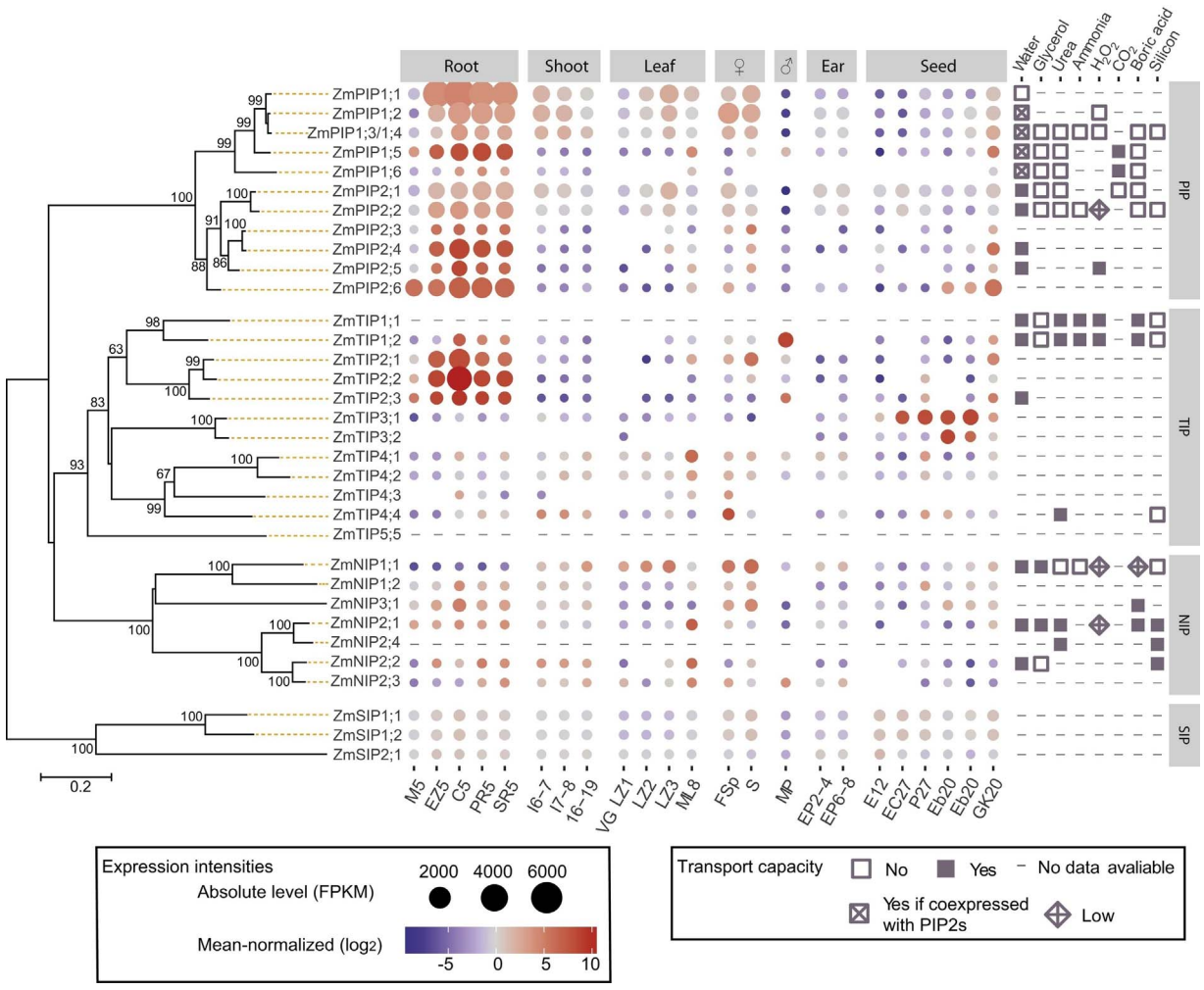

Fig. 1. Diversity and sub-functionalization of *Z. mays* AQPs. On the left, phylogeny of the 36 AQP paralogs of *Z. mays*. The distance tree was built using the neighbor-joining method using MEGA7 software. Bootstrap (3000 iterations) are expressed as percentages above the branches. The central heatmap shows mRNA gene expression intensities as absolute levels (circle size) and normalized across the tissues for each isoform (colors). The organs are root meristem, elongation zone, cortex at day 5, and primary root at day 5 (M5, E5, C5, and PR5), secondary root at day 7–8 (SR7-8), internode at day 6–7 (I6-7) and at day 7–8 (I7-8), vegetative meristem at day 16–19 (VM16-19), leaf zone 1 (symmetrical) (LZ1), 2 (stomatal) (LZ2), 3 (growth) (LZ3), mature leaf at day 8 (ML8), female spikelets (FSp), silk (S), mature pollen (MP), ear primordium 2–4 mm and 6–8 mm (EP2-4 and EP6-8), endosperm at 12 DAP (E12), endosperm crown and pericarp/aleurone at 27 DAP (EC27 & P27), embryo at 20 and 38 DAP (Eb20 & Eb38) and germination kernel at 2 DAJ (GK2). RNA-seq data from [23]. The right part of the figure compiles the current knowledge about the substrate specificity [32,36,37,60,97–100].

of *NIP* genes facilitating boron diffusion (*ZmNIP3;1, AtNIP5;1*, *and AtNIP6;1*) have defects in vegetative and inflorescence development, and reduce the accumulation of borate [42–44], revealing that their physiological roles cannot be substituted by other boron transporters or paralogue genes. Yet, it is often more complicated to establish a direct link between one AQP isoform, its substrate, and its physiological role due to compensation mechanisms by paralogs. For instance, the mutation of a single *PIP* or *TIP* gene does not necessarily result in a particular phenotype at the plant level, while the combined mutations of several paralogs can ([20]; reviewed in [2]).

2.4. Post-translational modifications and sub-functionalization

In addition to the control of the expression of AQP isoforms in a specific cell type/tissue that could be linked to different substrate specificities (Fig. 1), there are examples of the co-expression of AQP paralogs (for instance PIP isoforms) with similar localization and putative transport abilities. In this case, sub-functionalization might occur at the post-translational level by post-translational modifications (PTMs).

PTMs such as deamidation, phosphorylation, methylation, ubiquitination, and acetylation have been detected in AQPs by biochemical approaches and the use of mass spectrometry [45]. To date, most of these modifications have been reported for PIPs (reviewed in [46]). Phosphorylation has been shown for PIPs, and also for TIPs, NIPs, and XIPs [46–48]. Regarding PTMs impact, phosphorylation of PIP and NIP specific residues has been implicated in the gating of the pore and/or the regulation of the protein subcellular localization [48–50], whereas PIP ubiquitination is involved in the retention of the protein in the ER and its degradation [50]. However, the significance of PTMs like methylation, acetylation, and deamidation is still unknown [46].

While PTMs affecting PIPs specifically modulate their trafficking and activity, data regarding PTMs of other AQP subfamilies are still scarce. It is striking that several changes in the PTMs of PIPs are related to environmental changes, whereas no changes in the PTMs of TIPs are reported [45]. Two possible explanations are that this complex pattern of PTMs evolved only on the PIP family or, taking into account the sub-stoichiometric abundance of PTMs, the mass spectrometry techniques have not been sensitive enough to detect low abundant but still important PTMs on TIPs. So, as differential patterns of PTMs may occur within AQP subfamilies, this can undoubtedly be a way in which closely related paralogs diversified.

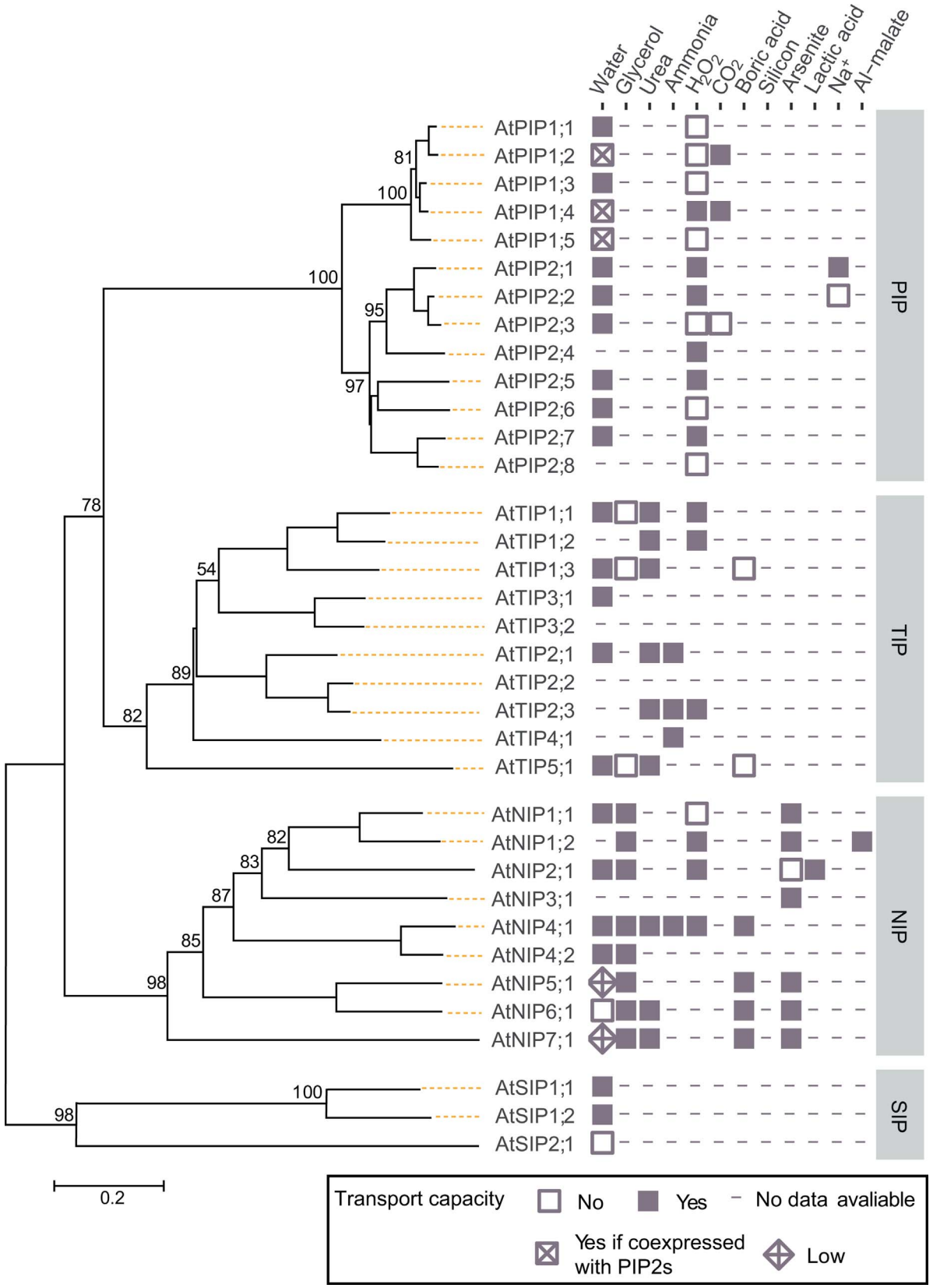

Fig. 2. Substrate specificity of Arabidopsis AQPs. On the left, the phylogeny of the 35 AQP paralogs of *A. thaliana*. The distance tree was built using the neighbor-joining method using MEGA7 software. Bootstrap (3000 iterations) are expressed as percentages above the branches. On the right, the current knowledge about the substrate specificity of the different paralogs (reviewed in [101] and [33,48,75,102–110]).

3. How is the selectivity to different solutes controlled, particularly those that appear to have multiple permeation properties?

3.1. The selectivity filters

AQP structure consists of six transmembrane helices (TM1-TM6) connected by five loops (A-E), the N and C-termini facing the cytosol. The loops B (cytosolic) and E (non-cytosolic) hold short α-helices containing the conserved Asn-Pro-Ala (NPA) motifs. These short α-helices, dipping halfway into the membrane from opposite sides, form a first filter region of the pore, at the position where the pair of NPA motifs meet. A second filter region is the aromatic/Arg (ar/R) constriction located at the non-cytosolic end of the pore. Both filters constitute barriers for the passage of inorganic cations (such as Na^+ and K^+) and, importantly, of protons [51]. The ar/R filter also forms the narrowest part of the pore providing the substrate specificity to the channel. At this constriction region, strict water transporters, such as mammal AQP1 or spinach *So*PIP2;1, have a narrower pore diameter than the aquagyceroporin GLPs or the plant NIPs that also conduct solutes like urea, glycerol, and/or metalloids [52]. An extensive review about plant AQP tridimensional structure discussed the molecular biology studies determining the solute selectivity and the recently resolved TIP crystal structure [53].

Modifying the ar/R filter has been one of the strategies used to better understand how the selectivity of the pore works. Point mutations in the ar/R filter of *Rn*AQP1 increase the diameter of the pore and allow the passage of urea, glycerol, ammonia, and even protons through its channel [54]. Also, substitution of the residues of the ar/R filter of *Hs*AQP1 with those from the ar/R filter of *At*TIP2;1, converts AQP1 into an ammonia transporter [55]. However, the explanation of the selectivity is not so simple. Substituting residues in the constriction regions of *At*PIP2;1 with those found in the ammonia-transporting TIPs did not turn the *At*PIP2;1 into an ammonium-permeable channel [56]. Neither was it possible to turn *Nb*XIP1;1α, a tobacco AQP facilitating the diffusion of different solutes, metalloids, but not water, into a water channel by mimicking the *At*TIP2;1 ar/R filter, without mutating additional residues [47]. Finally, another example is the failure to provide *At*NIP5;1 with silicic acid transport capacity by modifying its NPA motifs and ar/R filter with residues of *Os*NIP2;1 that naturally transport this solute [57]. Altogether, these data indicate that the substrate selectivity of AQPs is not only controlled by the amino acid residues of the NPA and ar/R filters, and point to a more complex mechanism of selectivity involving other parts of the channel.

Obtaining structural data of different plant AQP isoforms will be essential to discover new properties and understand the specificity of these channels. This is exemplified by the recent crystal structure of the ammonia and water permeable *At*TIP2;1 that has revealed new features that were not predicted by homology modeling using previously crystallized AQPs as template (*i.e.* water-specific channels or aquaglyceroporins) [55]. *At*TIP2;1 pore diameter is around 3 Å throughout the pore and the arginine residue of the ar/R constriction is at an unusual position, interacting with a histidine (His) residue from the loop C, previously implicated in the H^+-dependent inhibition of the TIP water permeability [58]. This highlights for the first time a fifth residue of the ar/R filter contributing to the selectivity filter. The structure also suggests that ammonium might be deprotonated by the interaction with this His, ammonia then moving through the main pore and protons through a side pore to the vacuolar surface [55]. Considering the acid pH of the vacuole, this His residing in the loop C might be constantly protonated, and therefore, inhibit the water permeability of TIPs. Further investigations are still needed to better understand the pH-dependent activity of TIPs and whether the vacuolar acid pH may favor the transport of ammonia against that of water. The crystal structure of TIP provides novel unpredicted information regarding its transport capacities and proves the importance of promoting further efforts to obtain the crystal structure of other plant isoforms. In this respect, the structural data of NIPs and XIPs, which are permeable to a diverse array of solutes including metalloids, will be very useful to better understand their pore selectivity.

3.2. Hetero-oligomerization and selectivity

AQPs assemble as tetramers in the membranes but monomers are considered as the active unit. To our knowledge, there are no reports proving the existence of free wild-type AQP monomers in the cell membranes. AQPs have always been found as homo- and/or hetero-tetramers [59–61] (for a review about heteromerization read [62]). In the following paragraphs, we will discuss several discoveries and the putative function regarding the heterotetramerization of plant and mammal AQPs.

While the hetero-oligomerization of plant AQPs was first reported for TIPs in lentil seeds [63], this protein assembly has been much more thoroughly studied for PIPs, since the discovery that the maize PIP1 and PIP2 isoforms physically interact to modify the cell membrane water permeability [60]. Since then, the formation of PIP heterotetramers has been described in many plant species [62]. When transiently expressed in maize cells, *Zm*PIP1s are retained in the ER, whereas *Zm*PIP2s localize in the plasma membrane. However, when *Zm*PIP1s and *Zm*PIP2s are co-expressed, *Zm*PIP1s are re-localized from the ER to the plasma membrane due to their physical interaction with *Zm*PIP2s [60]. Different plasma membrane trafficking motifs have been found in PIP2s but not in PIP1s (reviewed in [64]). In plants, PIP1s and PIP2s are always co-expressed in the same tissues (Fig. 1), meaning that PIP1s could be targeted to the plasma membrane thanks to this heterotetramerization mechanism. However, as previously mentioned, the proportion of PIP1s and PIP2s can vary significantly according to the tissues or cell types. For instance, 85% of the PIPs expressed at the mRNA level in maize stomatal complexes belong to the PIP1 subfamily [32]. How do all these PIP1 proteins, if translated, reach the plasma membrane? Do they also have a role in the ER? As heterotetrameric complexes can have different compositions [65], and different ratios of PIP1/PIP2 might result in different regulation or function according to the cell type and/or environmental stimulus, how is the stoichiometry of the heterotetramers composed of the PIP1 and PIP2 isoforms regulated? This could be physiologically relevant as the tetramer composition also might modulate the intrinsic water permeability of the monomers [60,66], although this still needs to be demonstrated *in planta*.

The interaction between transmembrane domains within each monomer and between monomers affects the oligomerization status of PIP2s [67], and also the water transport activity of PIP monomers [65]. An interesting illustration of the complexity behind the solute transport capacity is the case of a phenylalanine in a well-conserved region (APLPIG**F**AVF) of the TM5 of PIPs. This Phe is involved in the TM interaction between monomers (TM5-TM2) (F210 on *Zm*PIP2;5, F220 on *Zm*PIP1;2), and is crucial for the water transport activity and plasma membrane localization of *Zm*PIP2;5. Wild-type *Zm*PIP1;2 only translocates to the plasma membrane and facilitates water diffusion when interacting with *Zm*PIP2;5 [60]. However, the mutation of F210 in *Zm*PIP1;2 allows the protein, when expressed alone, to be addressed to the plasma membrane, where it acts as an active water channel [65]. Interestingly, this mutated *Zm*PIP1;2 inactivates the water channel activity of *Zm*PIP2;5 within a heterotetramer, indicating that one amino acid residue mutation in a TM of one monomer can affect not only its own behavior, but also the activity of an adjacent monomer, probably through conformational changes [65].

Another interesting question to be addressed is how the tetramer composition affects the selectivity to other solutes, like CO_2 or cations in the case of PIPs. The structural organization of AQPs in tetramers results in the presence of a central or fifth pore located at the fourfold symmetry axis of the four monomers. Current data suggests that this

pore could be involved in the transport of gases and ions. Molecular dynamic simulations of the permeation of CO_2 and ions through the human *Hs*AQP1 suggest that, whereas the monomer pores are not permeable to these solutes, the fifth pore may permeate them [68,69]. Interestingly, the tetrameric composition of tobacco PIPs was demonstrated to be important for facilitating the membrane CO_2 diffusion through *Nt*AQP1 (belonging to the PIP1 subfamily) [70]. Indeed, analysis of artificial heterotetramers with a defined proportion of *Nt*AQP1 to *Nt*PIP2;1 demonstrates that, while a single *Nt*PIP2;1 protein in a tetramer is sufficient to significantly increase the water permeability, the maximum CO_2 diffusion rate is observed when the tetramer consisted of NtAQP1 only [70]. Similarly, the recently described ionic conductance of *At*PIP2;1 [33] is abolished when the latter is co-expressed with *At*PIP1;2, even though it increases the water permeability of the cell [33]. So, as in the case of CO_2, the heterotetramerization state influences the transport of these solutes, possibly affecting the central pore conformation. These results are highly interesting and molecular dynamic simulations of CO_2 and cation diffusion through these plant oligomers might offer new insights into how this pore functions, and would be the basis for the generation of mutants to test their functionality as previously reported, to identify the residues involved in Na^+ transport by *Hs*AQP1 [69].

Other data on the role of oligomerization in plant and mammal AQP localization have been recently obtained. The mutation of residues in TM5 of *At*PIP2;1, predicted to be involved in the tetramerization, affects the trafficking of the protein that remains blocked at the ER [67]. Moreover, these mutations induce the formation of oligomers larger than tetramers [67] or, maybe, monomer aggregation in the ER membrane, even if they seem to be correctly inserted by the translocon machinery. On the other hand, the mutation of TM5 residues, predicted to be involved in the tetramerization of *Hs*AQP4, does not affect the tetramer assembly, contrary to specific mutations in the loop D, which prevent it, resulting in the accumulation of free monomers in the cell [71]. These monomers are able to reach the plasma membrane and act as active water channels, but they do not relocalize after an osmotic stress event, pinpointing a role for loop D for controlling oligomerization and stability [71]. Further studies are needed to determine whether common patterns of oligomerization exist between the mammalian and plant AQP subfamilies. Are there free monomers of plant AQPs in the plasma membrane? How crucial are tetramerization and tetramer composition for the recycling of plant AQPs from the plasma membrane?

Both homo- and heterotetramer functional units probably co-exist in plant membranes but, to date, the relative proportions of the different oligomers in the membranes according to the isoforms, cell types, tissues, developmental stages, and environmental conditions are unknown. Tracking AQP oligomer formation and dynamics in the different membranes is therefore an important aspect to be investigated. In this regard, following individual particles in the plasma membrane is now becoming possible using microscopy tools and fluorescence tags [72]. Therefore, in the near future, one would probably be able to track how the oligomerization state of specific AQPs changes under different stimuli to respond to the cell requirements. However, determining how cells sense and transmit the need to modify AQP oligomerization in the membranes to control water and/or solute homeostasis will probably need much more time to be resolved.

4. What does the molecular interaction between AQPs and other proteins tell us about their regulation and their contribution in specific cellular and physiological process?

4.1. AQP interacting proteins

Beyond the physical interaction between different AQP isoforms within heterotetramers which have significant impacts on their subcellular trafficking and channel activities, AQPs also transiently interact with other proteins resulting in PTMs or regulation affecting a diversity of processes, such as the gating of the monomers and their subcellular localization [11,46,64]. The list of putative AQP interacting proteins is currently increasing due to the large number of interactomic and biochemical studies, and the increasing sensitivity of the mass spectrometers. Accordingly, it has been recently reported that almost 400 proteins may directly or indirectly interact with *At*PIP2;1 and *At*PIP1;2 [73]. It is interesting to observe that 80% of the interactants are shared between the isoforms. However, the interaction with these proteins still has to be validated by other experimental approaches, such as *in vivo* or *in vitro* pull-down assay, bimolecular fluorescence complementation, Förster resonance energy transfer, split-ubiquitin assay *etc*. For instance, regarding the regulation of the channel trafficking, we demonstrated that PIPs interact with SNARE syntaxins from the trans Golgi network and the plasma membrane to regulate their abundance and, possibly, their activity, resulting in modification of the cell membrane water permeability [74,75]. In addition, *At*PIP2;7 also interacts with TSPO, a multi-stress regulator transiently induced by abiotic stresses, to regulate the cell-surface abundance of PIP2;7 during abiotic stress conditions, through the autophagic pathway [76]. As exemplified by the latter example, it will be essential to decipher the physiological consequences of key AQP-protein interactions *in planta*.

4.2. Is the lipid environment of AQPs conditioning their transport activity?

A high level of organization characterizes cell membranes: across the lipid bilayer (the asymmetric distribution of lipids between the leaflet) and, laterally, along the bilayer (the lipid microdomains). Changes in the physical properties of a membrane (fluidity, charges, thickness, *etc*.) affect the transport characteristics of the resident proteins, probably by inducing conformational changes in the proteins [77]. Accordingly, *in vitro* experiments have shown that the water permeability of *Bt*AQP0, *Rn*AQP4, and *Nt*PIP2;1 is modulated by the lipid bilayer composition [78–81]. In plants, the lipid composition of the plasma membrane changes in response to different environmental conditions that are already known to influence AQP activity, including drought and salt stress [82,83]. Besides, in several plant species, PIPs have been found in detergent-resistant membranes enriched in sphingolipids and sterols [83–86]. The study of detergent-resistant membranes gave the first evidence of laterally heterogeneous bilayers and of the existence of lipid microdomains, even if these results needed to be confirmed by other approaches (reviewed in [87]). In this regard, the diffusion of *At*PIP2;1 in the plasma membrane by variable-angle evanescent wave microscopy and fluorescence correlation spectroscopy has been studied [88]. The authors observed that drugs affecting the biosynthesis of sterols and sphingolipids modified the plasma membrane distribution and trafficking of *At*PIP2;1. Additionally, single-particle tracking using photoactivated localization microscopy revealed that *At*PIP2;1 is relatively immobile in the plasma membrane, whereas the tonoplast *At*TIP1;1 diffuses faster, at a similar velocity as that observed for vacuolar lipids [89]. In this context, it is possible that modification of the properties of the bilayer by environmental stresses generates a rearrangement of the AQPs exposed in the membranes: some isoforms might be internalized or relocalized, while others might be exposed in these new remodeled bilayers displaying appropriate physicochemical properties. Nevertheless, how lipids can influence the tridimensional structure of the AQPs or modulate their activity and motility is still far from completely clear.

The crystal structures of *So*PIP2;1, in the open and closed conformations, have been a valuable tool for understanding the conformational changes linked to its water-channel activity [90]. They also serve as templates for the homology modeling of other plant AQPs and molecular dynamic (MD) simulation experiments. However, the crystal environment is hardly similar to the cellular lipid environment, where the lipid head groups might interact with and change the packing of the helices [91]. In general, MD simulations of membrane proteins

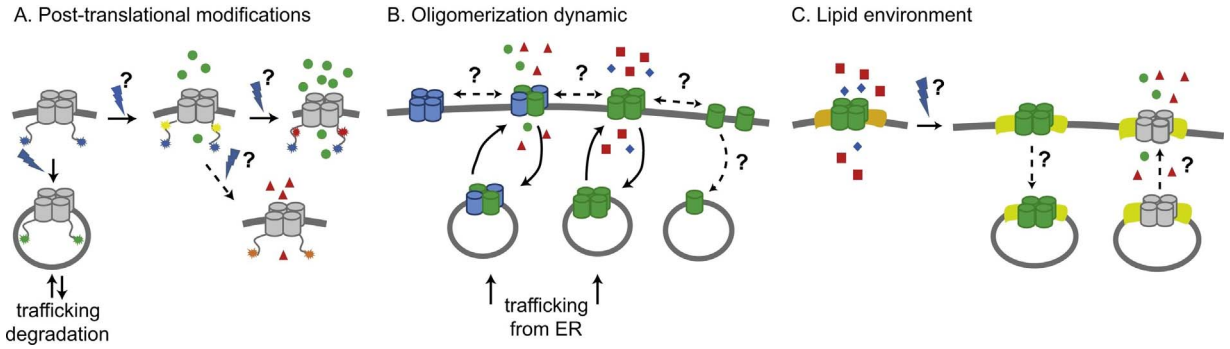

Fig. 3. AQP activity modulation. Schematic representation of still open questions regarding AQP activity modulation by interactions that might affect its structure and, consequently, its activity. A) Post-translational modifications of AQPs in response to different stimuli lead to responses such as gating of the pore, trafficking, and degradation of the protein. A single AQP could be the target of different PTMs (deamidation, phosphorylation, methylation, ubiquitination, and acetylation). There are no reports of modifications of the solute selectivity by PTMs (dotted lines), and still a big lack of information about the nature of the stimuli that induce the PTMs and the consequences of the different PTMs. B) The selectivity of AQPs can be modified by the tetramer composition. Homo- and hetero-tetramers assembled at the ER reach the target membranes and their density could be regulated by membrane recycling. A dynamic change in the oligomer composition and free monomers could occur in the target membrane (dotted lines) but has not been reported yet. C) The lipid environment modulates AQPs activity. In addition, stimuli that modify the lipid arrangement on the membrane might induce the internalization of some isoforms and favor the presence of other isoforms (dotted lines). This hypothetical regulation has not been proved yet.

integrated on lipid bilayers have been simplified to a maximum of three lipid types [92] and, for AQPs, generally only one lipid type has been used. Thus, it needs to be considered that the exclusion of specific lipids might negatively affect the concordance between the MD simulation results and what happens in the cell. In this regard, the simulation of more complex lipid bilayers is continuously under development, and it is expected that future MD simulations of AQPs will consider different bilayer compositions. For instance, the behavior of gangliosides, which are special ceramides enriched in lipid rafts of mammal cells, has recently been studied by MD simulation [93]. Interestingly, the authors proposed that these ceramides might interact with AQP1 by hydrogen bond formations. This report could be useful to support future wet experiments regarding the biological meaning of the interaction, and to promote the study of AQPs in more complex models of lipid membranes. As lipid rafts still elude direct microscopic detection, *in silico* modeling that can be considered as “computational microscope” [87] are a nice tool to study AQPs in lipid microdomains. In the long-term, we can expect that the integration of lipid membrane models with AQP structures could help to understand better the residue differences between isoforms, and could provide the possibility of testing different hypotheses such as the possible involvement of lipids in the transport selectivity of AQPs.

The improvement of already available tools and the development of new ones to study the interaction of membrane lipids with membrane proteins *in vivo*, for instance to measure the strength of the deformation that lipids exert over a protein and its influence on its activity, will be crucial for going forward in the understanding of AQPs in their cellular environment. We previously mentioned the importance of resolving the crystal structures of new plant AQPs, including NIPs and XIPs, in the context of solute selectivity. Here, in the context of lipids-AQPs interaction, we believe that interesting information might arise from the crystal structure of SIPs. Indeed, SIPs are evolutionarily very distant from PIPs and TIPs, and obtaining a reliable structure of SIPs by homology modeling is actually not easy [94]. Moreover, SIPs are residents of the ER membranes, whose lipid composition is extremely different from that of the tonoplast and plasma membrane bilayers [95,96].

5. Conclusions

The expansion of AQP families and isoforms is a common feature among vascular plants. It is remarkable to observe how similar AQP expression profiles occur in evolutionarily distant plant species, in which genomes underwent different rounds of duplications, deletions, and rearrangement of their chromosomes [28]. This reinforces the idea that the conservation of a high number of expressed AQP paralogs, especially PIPs, TIPs, and NIPs, might provide the plant with some selective advantages. The redundancy between close paralogs in certain environmental conditions does not mean that they will be redundant under other environmental conditions (for example under stress [12]). As discussed in this review, numerous aspects have to be considered when trying to understand the differential role and regulation of AQPs, and, to date, there are still open key questions regarding the AQP activity modulation (Fig. 3). To understand the physiological importance of AQP diversification, it will be necessary, for each AQP or class of AQPs, to systematically get insights into: (i) their single-cell expression profile, (ii) their differential transport solute capacity, (iii) their PTMs and interactants, (iv) their oligomerization status and structures, (iv) how the composition of the oligomers is defined *in vivo* and can be modified in the target membrane, and (v) how changes in the lipid bilayer composition affect AQP functionality.

Altogether, further advances in the areas discussed herein will undoubtedly help better understand the selective advantage of keeping such a high number of AQPs in vascular plants. Finally, on the other hand, one could also wonder whether higher plants have been keeping this high number of AQPs and regulation mechanisms as a consequence of the ever-changing evolutionary constraints that have, inevitably and irreversibly, multiplied them but, at the end, plants would be able to face developmental and environmental cues with fewer AQP isoforms and regulation mechanisms.

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