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Review

PIP1 aquaporins: Intrinsic water channels or PIP2 aquaporin modulators?

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ABSTRACT

The highly conserved plant aquaporins, known as Plasma membrane Intrinsic Proteins (PIPs), are the main gateways for cell membrane water exchange. Years of research have described in detail the properties of the PIP2 subfamily. However, characterizing the PIP1 subfamily has been difficult due to the failure to localize to the plasma membrane. In addition, the discovery of the PIP1–PIP2 interaction suggested that PIP1 aquaporins could be regulated by a complex posttranslational mechanism that involves trafficking, heteromerization and fine-tuning of channel activity. This review not only considers the evidence and findings but also discusses the complexity of PIP aquaporins. To establish a new benchmark in PIP regulation, we propose to consider PIP1–PIP2 pairs as functional units for the purpose of future research into their physiological roles.

1. Introduction

Aquaporins are multifunctional channels that facilitate the passage of water and/or other small solutes across cell membranes. They are organized in highly conserved tetrameric structures in cell membranes that are abundant in all kingdoms. There have been 13 different aquaporin genes identified in mammals, while data from flowering plants revealed the presence of 20–60 different loci [1–3]. The large number of aquaporin genes in flowering plants made them an interesting target of study. Aquaporins with a high capacity for water transport govern membrane osmotic permeability (P_f) and thus, the rate of water transfer turns in a putative key component of physiological processes [4]. Plant aquaporins are currently characterized based on their sequence composition as PIPs (Plasma membrane Intrinsic Proteins), TIPs (Tonoplast Intrinsic Proteins), NIPs (NOD26-like Intrinsic Proteins), SIPs (Small basic Intrinsic Proteins), XIPs (X Intrinsic Proteins), HIPs (Hybrid Intrinsic Proteins) or GIPs (GlpF-like Intrinsic Proteins) [5]. Recently, through phylogenetic analysis, PIPs, TIPs, NIPs and SIPs were subdivided into 19 orthologous clusters that show congruence among aquaporins and organism trees [6,7]. PIPs, NIPs and XIPs primarily localize to the plasma membrane (PM). PIPs were proposed to be the main gateways controlling water permeability as NIPs and XIPs have been widely described as solute transporters [8–10]. Traditionally, PIPs have been clustered into two groups, PIP1 and PIP2; however, a subsequent phylogenetic analysis revealed three groups: one cluster for the PIP1 group and two clusters for the PIP2 group [6]. Although these proteins have a highly conserved amino acid sequence, the main structural difference between PIP1 and PIP2 aquaporins is the length of their N and C terminal ends. Interestingly, the ratio of PIP1/PIP genes is approximately 40% which is relatively constant among plant species [11]. However, in terms of function, their behavior is drastically different. Functional experiments on PIP1 aquaporins in Xenopus oocytes or other heterologous systems suggested that PIP1s were non-functional; however, it was later shown that some PIP1s do not localize to the PM when expressed alone [12]. The discovery that PIP1 trafficking to the PM depends on a functional interaction with PIP2s provided new insight into the study of PIP aquaporins [12,13].

This review suggests that PIP1 must be studied within the contexts of its previous history and the current evidence. Therefore, the intrinsic water and solute permeability of PIP1s, and their interaction with PIP2s, are vital for understanding their roles. Particularly, heterotetramerization with PIP2 is discussed as an emerging regulatory mechanism for PIP1 due to its influence on the intrinsic permeability and trafficking dynamics of PIPs. Finally, this review outlines the proposal that PIP1–PIP2 pairs are relevant to plant cell physiology and consequently may warrant further investigation.

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2. PIP1 and PIP2: different paths to functional characterization using heterologous systems

2.1. The functionality and structure of PIPs: a progression of PIP2 discoveries

PIP aquaporins have been recently considered to be the main water gateways at the level of the plant plasma membrane. Since the cloning and identification of the first PIP2 [14] and the first characterization of their water transport activity [15], much progress has been made in understanding the properties of PIP2 aquaporins. Most of these findings have been obtained from studies performed in heterologous systems, such as Xenopus oocytes, that do not express endogenous aquaporins [16,17]. Previous studies that were focused on the process of gating used the X-ray structure of SoPIP2;1 in both the closed and open conformations [18]. Subsequent studies examined the regulation of trafficking and the interaction between aquaporins and proteins of the plant secretory pathway [19,20]. Currently, it is understood that PIP2s are organized in homotetramers that translocate to the PM via interactions with SNARE proteins [19]. It has been demonstrated that PIP2 trafficking and sorting depend on the diacidic N-terminal motif, the TM-based signal on TM3 and the phosphorylation of C-terminal serine residues [20,21]. The closure of PIP2 channels [18] occurs as a consequence of various mechanisms, including intracellular acidification [22–25], increased divalent cation concentration [22] and specific serine dephosphorylation [26–28]. Several highly conserved residues that are responsible for pH and Ca^{2+} gating were unequivocally identified [22,23].

3. Why was it difficult to study PIP1?

In contrast to PIP2-related advancements, research conducted on PIP1 aquaporins followed an extremely complex path. There is a 10-year gap between the first report on the expression of a PIP1 in Xenopus oocytes [15] and the demonstration that at least some PIP1 aquaporins do not reach the PM when expressed alone in this system [12]. During this 10-year gap, there have been many reports showing that several PIP1s did not increase the P_f of the PM when expressed in Xenopus oocytes [23,29], but unfortunately, no localization studies within the oocytes were performed. Currently, we can assume that the lack of a P_f enhancement reflected the fact that many PIP1s were retained in internal cellular structures. Oocytes overexpressing some PIP1s demonstrated a small but significant enhancement of the P_f compared with control oocytes [29–31], whereas subsequent replications of these experiments revealed no P_f enhancement [1,12,32]. Table S1 shows all of the reports of the P_f measurements performed in Xenopus oocytes expressing a PIP1. For each report, a relative permeability to control is informed. In most cases (35 studies), the P_f reported for Xenopus oocytes expressing PIP1 was not significantly different from that reported for non-injected or water-injected oocytes. Additionally, the number of reports associated with high P_f values is extremely low. Initially the assumption was made that PIP1 and PIP2 were functionally distinct in terms of their water transport capacities; it is now understood that PIP1 might display differential activity and/or trafficking to the PM, thereby masking its intrinsic properties.

4. Do PIP1 aquaporins transport only water?

During the time period in which it was proposed that PIP1 aquaporins were not functional in water transport, many efforts focused on testing the abilities of these proteins to transport other solutes. These experiments showed strong evidence that other aquaporins, particularly NIPs and XIPs, have the capacity to transport solutes [8–10]. PIP1 aquaporins showed positive [29,30,33–35] and negative [29,36–38] results regarding solute transport. In interpreting the negative results, one must consider the possibility that PIP1 did not reach the PM; therefore, in the absence of AQP localization, the negative results would be difficult to interpret. Nevertheless, there is solid evidence from heterologous and homologous systems that some PIP1 aquaporins are functional CO_2 channels, which is important for the mesophyll and stomatal CO_2 conductance in leaves to sustain high rates of photosynthesis [21,39–43]. Additionally, while some PIP2s seem to transport H_20_2 [44,45], ZmPIP1;2 does not facilitate peroxide transport when expressed in *Saccharomyces cerevisiae* [44].

5. Was the expression system a limitation?

Several plant PIP1s have been expressed in various heterologous systems. Purified PM vesicles from *S. cerevisiae* were used to analyze PIP1 derived from *Oryza sativa* [46,47] and *Raphanus sativus* [48]. Although RsPIP1;2 and RsPIP1;3 increased the P_f of PM vesicles, RsPIP1;1, OsPIP1;1 and OsPIP1;2 induced little or no increase in the P_f. In some reports, the P_f of yeast protoplasts expressing AtPIP1;2 or NtAQP1 did not increase, although a higher permeability to carbon dioxide was observed [41,49].

Another example of the heterologous expression of a PIP1 was the expression of OsPIP1;1 in *Escherichia coli* [50]. Liposomes reconstituted using purified OsPIP1;1 showed a high P_f that was inhibited with Hg^{2+} and reversed with β-mercaptoethanol. The fact that several PIP1s have shown varying results depending on the heterologous expression system used might reflect either the influence of the PM lipid composition or the failure of PIP1s to reach the PM in some systems [23,33,41,46,49–52]. Both of these explanations are plausible, as it has been demonstrated that at least some PIP1s are functional water channels [11,50]. In addition, protoplasts isolated from plants overexpressing PIP1s showed a higher P_f than those obtained from wild-type plants [53–55] and protoplasts obtained from PIP1 antisense plants showed a lower P_f compared with wild-type plants [56–59].

The use of plants as an expression system not only contributed to the understanding of the functional properties of PIP1 but also provided evidence of the consequence of modulating PIP1 expression. For instance, *Nicotiana tabacum* and/or *Arabidopsis thaliana* transgenic plants highlight the contribution of PIP1 to hydraulic conductivity both at the root [57] and shoot [58] levels. A decrease in PIP1 expression produced a significant reduction in root [57,59] and rosette [58] hydraulic conductivity. It has been observed that plants overexpressing PIP1 exhibited changes in the water use efficiency (WUE) [53,55,57,60,61], photosynthesis rate [39,40,61,62] or growth rate [50,55,60,63], parameters that are all closely associated with plant water status. However, the overexpression (or suppression) of PIPs has equally led to successes and failures in improving stress resistance [64]. The main hypothesis proposed to explain these conflicting results is that plants need to increase (or decrease) the expression of a particular PIP in a particular organ at a specific time to overcome stress. Moreover, PIP1 localization and expression have been correlated with the presence of apoplastic barriers, endodermis and/or exodermis in the roots or cells of suberized sheath beam leaves, suggesting an essential role for PIP1 in the diffusion of transmembranous water [65]. Analyzing PIP1 participation in physiological or pathological processes, including embolism [66], germination [67] and tolerance to the abiotic stresses of salt, drought and low temperatures [46,68–72], has been a common strategy for understanding the role of PIP1s in plant water homeostasis.

6. Analysis of the PIP1–PIP2 functional interaction to understand the role of PIP1

A key turning point in the study of PIP1s was the discovery that when PIP1 aquaporins were co-expressed with a PIP2 aquaporin in the Xenopus oocyte system, they not only reached the PM but also induced an enhancement in the P_f compared with the overexpression of a PIP2 alone [12]. This evidence suggested a functional interaction between PIP1 and PIP2 and supported a role for PIP1 in water transport.

6.1. The importance of PIP1/PIP2 heterooligomers

The oocyte plasma membrane P_f reflects the PIP1–PIP2 functional interaction resulting from the co-injection of PIP1 and PIP2 RNAs into Xenopus oocytes. Interestingly, the co-expression of a PIP1 and PIP2 produces a greater increase in the P_f than that produced by PIP2 alone. Since the first report of this phenomenon [12], many studies have confirmed this effect with several PIP1–PIP2 pairs [11,12,24,32,50,51,72–81]. Some of these studies combined the measurement of the P_f in Xenopus oocytes with additional experiments to confirm the PM localization of PIP1. PIP1 co-expressed with PIP2 re-localizes to the plasma membrane in Xenopus oocytes [11,12,51,77], protoplasts or plant cells [13,50,82].

The observed functional interaction between PIP1 and PIP2 suggested the existence of a physical interaction between them. This has been demonstrated in oocytes by co-purifying ZmPIP1;2-GFP and His-ZmPIP2;1 [12], and in COS cells with *Mimosa pudica* PIPs [73]. Subsequent studies using FRET and co-purification have shown that this interaction occurs *in planta* [13]. The PIP1–PIP2 interaction has been proposed to be a restraint to the evolution of the PIP family [6], as the interacting partners were proposed to evolve at low rates [83].

It was initially suspected that these interactions occurred through the formation of heterotetrameric structures [12] due to the evidence that aquaporins were organized in homotetramers [18,84–86]. However, subsequent studies addressed the hypothesis that these structures could be heterooligomers [13]. In the animal kingdom, a similar example of aquaporin heterotetramerization has been reported for the AQP4 isoforms M1 and M23. In this case, heterotetramer formation does not affect the intrinsic water permeability of each monomer [87], but is important for delimiting the formation of orthogonal arrays of particles (OAPs) [88,89]. OAP formation by AQP4 is of great importance to the pathogenesis of the multiple sclerosis-like disease neuromyelitis optica [90]. Regarding PIPs, it has been demonstrated that NtAQP1 and NtPIP2;1 form heterotetramers when co-expressed in *S. cerevisiae* [49]. In a more recent work, a conserved cysteine residue located in loop A of both PIP1 and PIP2 was suggested to form disulfide bridges between monomers of ZmPIPs [78]. Although the absence of the cysteine residue did not prevent the assembly of the tetramer, the authors proposed that the formation of homodimers could configure the tetramer. One of the strongest pieces of evidence that PIP1 and PIP2 form heterotetramers was collected for *Beta vulgaris* PIPs [77]. The authors observed that although BvPIP2;2 interacts with BvPIP1;1, BvPIP2;1 showed no functional interaction. However, the BvPIP2;1 loop A mutant showed a restored functional interaction with BvPIP1;1. Homology modeling and computer simulations suggested that this loop area is located towards the center of the tetramer; thus, changes in the interaction capacity indicated that this interaction might reflect heterotetramer formation. The stoichiometric arrangement of the tetramer has yet to be elucidated, and the relevance of the tetramer should be contextualized at a functional level. In a recent study, we proposed that all of the stoichiometric arrangements could be formed on the basis of a mathematical model of the results of co-expressing various ratios of the RNAs for FaPIP1;1 and FaPIP2;1 or a FaPIP2;1 non-functional mutant. The random heterotetramerization between these two aquaporins was dependent on the abundance of each protein, which not only determined FaPIP1;1 PM localization but also enhanced FaPIP2;1 activity as part of a heterotetramer [11]. Regarding gating regulation, PIP1 aquaporins induce a shift in pH sensing to less acidic values when co-expressed with a PIP2 [11,24,77]. The closure of PIP1 aquaporins is more sensitive to a decrease in the cytosolic pH [11], and a shift in PIP2 sensitivity could be achieved through an interaction at a more physiological pH [24].

Recent evidence suggests that this interaction is not an intrinsic property of an individual PIP1 or PIP2, but rather a characteristic of each PIP1–PIP2 pair. For example, there are reports of PIP1 that presented different co-expression functional activity, depending on the PIP2 partner [12,74,80]. Additionally, some PIP1–PIP2 pairs did not functionally interact [38,74,77,80]. In this context, it is important to analyze each PIP1–PIP2 pair independently and to further investigate the pairs expressed in the same cell type.

6.2. The PIP1–PIP2 pair hypothesis

Various reasons have been proposed to explain the large number of aquaporins present in plant genomes and the relevance of these proteins to plant physiology: (i) redundancy, (ii) different transport properties, or (iii) different expression patterns [91,92]. Currently, many of these hypotheses are supported by adequate evidence. Interestingly, a redundancy in aquaporins to guarantee water balance in plants has been recently explored in terms of convergent or divergent regulatory mechanisms for Poplar aquaporins [93]. This analysis demonstrates that residual functional redundancy is not conserved at a higher level, particularly in the PIP subfamily. As previously stated, each aquaporin shows different transport properties in terms of permeability not only to water but also to other solutes. Furthermore, the anatomical localization and the expression patterns of PIPs during specific developmental stages and in different plant species are consistent with the idea that the two PIP subfamilies do not act redundantly [94].

To establish a new benchmark in PIP regulation, we propose to consider PIP1–PIP2 pairs as functional units for the purpose of future research into their physiological roles (Fig. 1). As discussed in the previous section, the PIP1–PIP2 interaction and the formation of heterotetramers represent interesting post-translational regulatory mechanisms that influence both the trafficking and the intrinsic permeability of PIPs and determine the plasma membrane P_f. In this review, we analyze the transcriptional profiles of both PIP subfamilies in different physiological stages to investigate if transcriptional regulation could dictate the PIP1–PIP2 association. The transcriptional profiles of PIPs can provide evidence for the PIP1–PIP2 pair hypothesis, such as the joint increase (or decrease) in expression of specific PIP1–PIP2 pairs when a plant is exposed to a certain stress or environment. Thus, the formation of heterotetramers composed of two specific PIP1s and PIP2s could be regulated by transcript abundance (Fig. 1).

Transcriptional studies have been the predominant strategy to explore the physiological roles of these genes in different plant species. The association with the vasculature of many aquaporin genes was not surprising and is consistent with the importance of water distribution for plants [95,96]. Similar to many other membrane proteins, aquaporin genes are temporally and spatially expressed in a specific pattern [47,97–99] not only during ontogeny but also after plants are exposed to environmental challenges. Multiple transcriptional responses have been observed during different stages of development [68,97,100–103], during abiotic

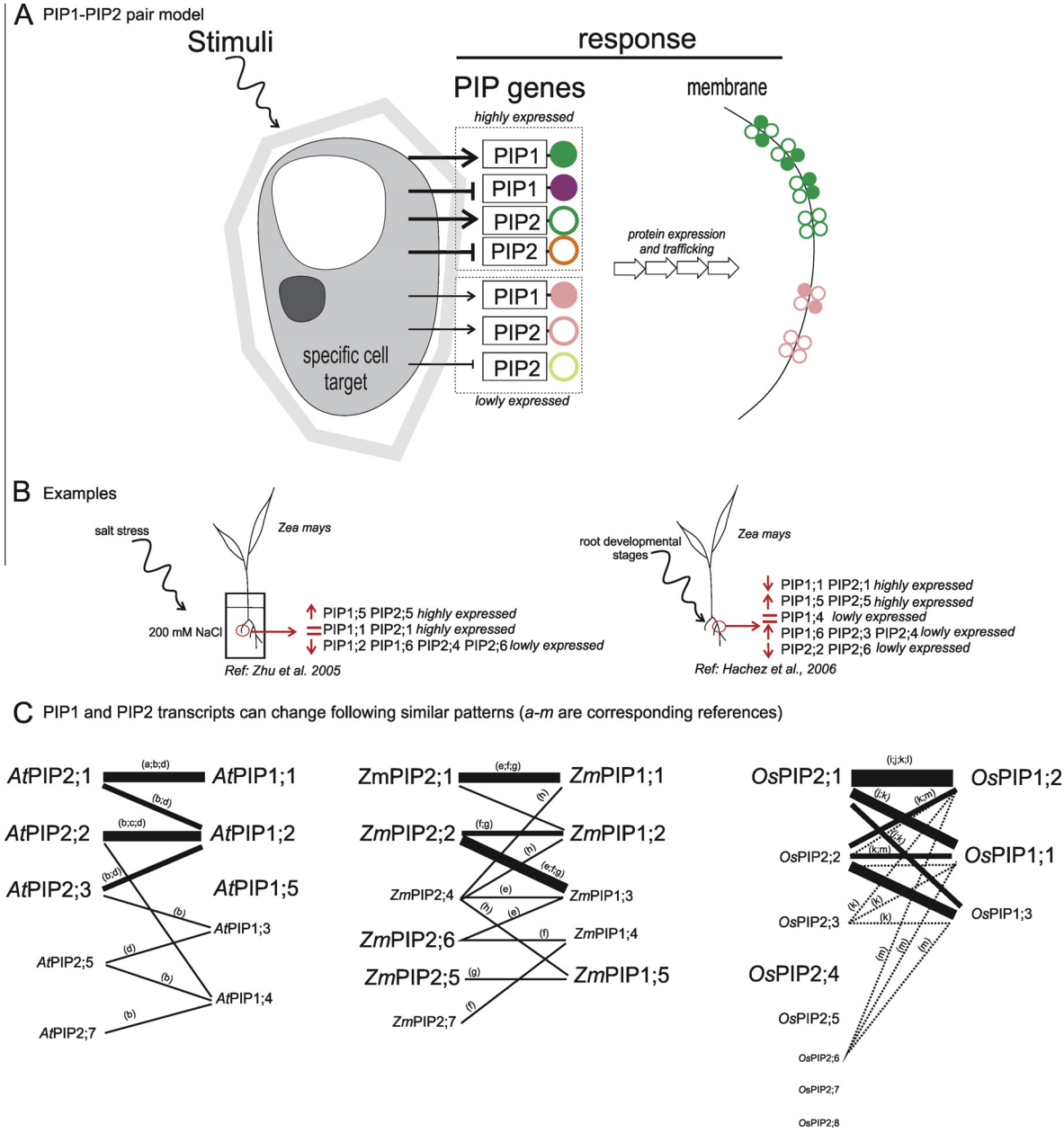

Fig. 1. The PIP1–PIP2 pair hypothesis. (A) A schematic representation of the model. When subjected to stimuli, a specific plant cell regulates the PIP1 and PIP2 transcript abundances. Different stimuli would enhance or diminish different PIP transcripts, thus determining which heterotetramers or homotetramers could be assembled. PIP1 and PIP2 transcripts that varied together are good candidates for the analysis of a subsequent interaction between and the formation of heterotetramers composed of the corresponding proteins. (B) Examples of *Zea Mays* plants subjected to salt stress [68] or the analysis of different stages of root development [65]. In both cases there are pairs of PIP1 and PIP2 transcripts with similar levels of expression (high or low) that show similar regulatory patterns: both increase, decrease or remain static. (C) Transcriptional relationship between PIP1 and PIP2 members of three different plant models (*Arabidopsis thaliana*, *Zea mays* and *Oryza sativa*). The published results of the transcriptional responses to different stimuli were analyzed. In each case, the relative expression level of each PIP was normalized to the lowest of each subfamily. Discriminating PIP isoforms based on their relative transcript abundance, highly and lowly expressed groups were generated and differentiated by the font size indicated in the figure. The response of each transcript (an increase or a decrease) was categorized according to the statistics provided by each work and the method of quantification chosen in each case. Thus, for each transcript we provide information as to whether it increases or decreases in response to different stimuli. We found that there were PIP1–PIP2 pairs that had a similar profile change (increase or decrease) regardless of the treatment applied. The line that connects each pair of PIP transcripts represents a pair of PIPs whose expression jointly changes upon a certain treatment. The line thickness is correlated with the number of published reports that support the association. The dotted lines represent expression of the linked PIPs in roots. The letters in brackets above each line indicate the following references: (a) Vander Willigen et al., (2006) [70]; (b) Alexandersson et al., (2005) [111]; (c) Sutka et al., (2011) [114]; (d) Alexandersson et al., (2010) [112]; (e) Moshelion et al., (2009) [119]; (f) Hachez et al., (2008) [98]; (g) Hachez et al., (2006) [65]; (h) Zhu et al., (2005) [68]; (i) Yooyongwech et al., (2013) [117]; (j) Kuwagata et al., (2012) [116]; (k) Liu et al., (2007) [67]; (l) Sakurai-Ishikawa et al., (2011) [109]; (m) Matsumoto et al., (2009) [32].

stress [46,68,104] or even in response to light regimes [98,105–107]. Interestingly, some studies have reported that changes in PIP1 expression would directly induce functional changes in the hydraulic conductivity throughout the day in *Lotus japonicus* [108], the hydraulic conductivity of the roots in two grapevine cultivars [79] or the recovery of poplars from embolism [66]. Thus, the results of transcriptional profiling highlight the relevance of aquaporins as water channels but do not elucidate their specific roles in the regulation of the plant water status. Therefore, the most recent experimental approaches have focused on integrating both the

gene and protein regulation with the functional responses at various levels, including cell, tissue and organ [79,99,106,107,109,110].

In previous work, the use of Arabidopsis as a tool to build a network of interrelations through the statistical analysis of transcriptional levels and gene profiles has contributed additional, valuable information [111,112]. In light of these observations, we analyzed the transcriptional profiles reported in the literature of PIPs in response to different treatment conditions. We found that certain aquaporin isoforms shared the same variation in their transcriptional profiles even in distinct physiological conditions. Furthermore, certain PIP1–PIP2 pairs formed by specific PIP1 and PIP2 isoforms seem to be correlated with one another (Fig. 1B and C). The transcript profiles of both PIP subfamilies can be classified into two subgroups based on mRNA expression levels: (i) highly expressed transcripts and (ii) lowly expressed transcripts. Interestingly, a correlation between aquaporins is also evidenced by similar relative transcript abundances. For instance, *AtPIP2;5* and *AtPIP2;7* showed a coupled profile with *AtPIP1;4* and *AtPIP1;3*, as well as *AtPIP2;1* and *AtPIP2;2* with *AtPIP1;2* and *AtPIP1;1* [58,69,70,104,111–115]. In rice, transcriptional correlations were corroborated by the *in situ* co-localization of *Os*PIP2;1, *Os*PIP1;2 and *Os*PIP1;1 [109,116]. Interestingly, *Os*PIP2;1 and *Os*PIP1;2 show similar transcriptional patterns in rice mutant lines under water deficit [117]. In maize, the transcriptional regulation of the highly expressed aquaporins such as *ZmPIP1;1*, *ZmPIP1;2* and *ZmPIP1;5* have been associated with those of *ZmPIP2;1* and *ZmPIP2;5* [30,68,97,98,105,118,119]. The pairs *ZmPIP1;5-ZmPIP2;5* and *ZmPIP1;1-ZmPIP2;1* might be important functional units in the *Zea Mays* root based on the responses observed in response to salt stress and during root development (Fig. 1B). Furthermore, the uncorrelated transcriptional responses of *ZmPIP1;1* and *ZmPIP2;5* are in accordance with no functional interaction between these two aquaporins reported in Xenopus oocytes [12].

The traits involved in plant strategies are rather complex; in particular, the understanding of the regulation of water balance and water use efficiency still require further research focused on aquaporins and their role in root–shoot signaling [120]. As an example *Vv*PIP1;1 plays a role in regulating root conductance when co-expressed with a PIP2 partner [79]. On the contrary, in stress conditions, the mRNA levels of *GmPIP1;6* rapidly change, but no significant differences were observed in the PIP2 subfamily or in cortical cell conductivity [121]. Although the genetic relevance of the two different levels of aquaporin expression (high and low) has not yet been uncovered, these observations reinforce the hypothesis that the PIP1–PIP2 pair is a functional unit. The co-localization of aquaporin transcripts and their similar expression patterns in response to various treatments support the hypothesis that the interaction between them is a key mechanism for the modulation of water balance.

7. Conclusions and perspectives

After an unexpected, confusing and serendipitous research path, PIP1s have been identified as water and/or gas channels with the potential to form heterotetramers with PIP2s, thereby modifying the characteristics of both subfamilies in terms of their activity, trafficking and gating. However, much remains to be investigated regarding the role of each PIP1 *in planta*. Our proposal, based on an exhaustive analysis of the literature, is that the functionality of several PIP1s must be linked to their interaction with a PIP2. Furthermore, the interaction between PIP1s and PIP2s must be regulated at the transcriptional and post-translational levels. Variations in PIP transcript levels in plant models that are subjected to environmental changes seem to be not necessarily critical for plant adaptation; rather the majority of evidence currently highlights the importance of post-translational mechanisms. Nevertheless, transcriptional variations modify protein levels, which consequently determine whether PIP1 or PIP2 are present in the membrane, thereby dictating whether PIP homotetramers and/or heterotetramers are formed.

Unlike PIP2, the structure of PIP1 has not yet been defined through X-ray crystallography. In addition, the mechanism of PIP1 trafficking to the plant plasma membrane had not been investigated prior to recent studies confirming the PIP1–PIP2 interaction. Because several PIP1–PIP2 pairs functionally interact when expressed in heterologous systems, more research is needed to address whether heterotetramerization between PIP1 and PIP2 is conserved. Here, we reviewed studies reporting PIP1–PIP2 pairs whose transcriptional levels vary under different stress conditions. Studies of these pairs could provide a new benchmark in the biophysical characterization of PIPs, with the PIP1–PIP2 pair being considered a minimal unit for understanding the physiological role of these proteins. These findings suggest a wide range of options for their activity, pH sensing and versatility in response to environmental changes. Furthermore, the PIP1–PIP2 interaction represents an interesting model for studying protein interaction as a strategy for functional regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.10.018.

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