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Mechanisms Activating Latent Functions of PIP Aquaporin Water Channels via the Interaction between PIP1 and PIP2 Proteins

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Plant plasma membrane-type plasma membrane intrinsic protein (PIP) aquaporins are classified into two groups, PIP1s and PIP2s. In this study, we focused on HvPIP1;2, a PIP1 in barley (*Hordeum vulgare*), to dissect the molecular mechanisms that evoke HvPIP1-mediated water transport. No HvPIP1;2 protein was localized to the plasma membrane when expressed alone in *Xenopus laevis* oocytes. By contrast, a chimeric HvPIP1;2 protein (HvPIP1;2_24NC), in which the N- and C-terminal regions were replaced with the corresponding regions from HvPIP2;4, was found to localize to the plasma membrane of oocytes. However, HvPIP1;2_24NC showed no water transport activity in swelling assays. These results suggested that the terminal regions of PIP2 proteins direct PIP proteins to the plasma membrane, but the relocalization of PIP1 proteins was not sufficient to PIP1s functionality as a water channel in a membrane. A single amino acid replacement of threonine by methionine in HvPIP2;4 (HvPIP2;4T229M) abolished water transport activity. Co-expression of HvPIP1;2_24NC either with HvPIP2;4_12NC or with HvPIP2;4TM_12NC, in which the N- and C-terminal regions were replaced with the corresponding regions of HvPIP1;2, increased the water transport activity in oocytes. These data provided evidence that the HvPIP1;2 molecule has own water transport activity and an interaction with the middle part of the HvPIP2;4 protein (except for the N- and C-termini) is required for HvPIP1;2 functionality as a water channel. This molecular mechanism could be applied to other PIP1s and PIP2s in addition to the known mechanism that the terminal regions of some PIP2s lead some PIP1s to the plasma membrane.

Keywords: Barley • Co-expression • Oocyte • PIP aquaporin • Protein–protein interaction • Water transport.

Accession numbers: The GenBank accession number for each gene is **AB275278** (*Hordeum vulgare HvPIP1;2*) and **AB219525** (*H. vulgare HvPIP2;4*).

Introduction

Adequate water uptake across biological membranes is essential for living cells. Aquaporins, also known as membrane intrinsic proteins, play a pivotal role in this process as water channels and are known to be widespread in bacteria, animals, and plants (Maurel et al. 2015, Chaumont and Tyerman 2017). The membrane intrinsic protein gene family is large and diverse, and many family members have been found to transport small molecules including ions in addition to water (Dordas et al. 2000, Tyerman et al. 2002, Gaspar et al. 2003, Uehlein et al. 2003, Ma et al. 2006, Takano et al. 2006, Bienert et al. 2007, Ma et al. 2008, Mori et al. 2014, Byrt et al. 2017, Rhee et al. 2017). Plasma membrane intrinsic proteins (PIPs) are considered to be primary water channels that mediate water transport in the plasma membrane of plant cells, including water uptake from the outer environment (Javot and Maurel 2002, Katsuhara et al. 2008). Plant PIPs can be divided into two groups, PIP1s and PIP2s, according to their sequence similarity (Tyerman et al. 1999, Chaumont et al. 2001). In the tissues of several plant species, the expression of PIP1s has been reported to be predominant compared with PIP2s (Alexandersson et al. 2005, Matsumoto et al. 2009, Horie et al. 2011). However, PIP1s often showed no water transport activity when expressed in *Xenopus laevis* oocytes and yeast vesicles (Suga and Maeshima 2004, Fetter et al. 2004, Sakurai et al. 2005, Horie et al. 2011), although some PIP1s showed low water transport activity in oocytes (Kammerloher et al. 1994, Yamada et al. 1995, Moshelion et al. 2002, Tournaire-Roux et al. 2003, Li et al. 2009, Horie et al. 2011, Jozefkowicz et al. 2016).

In contrast to PIP1s, all PIP2s exhibit high water transport activity. Fetter et al. (2004) showed that, in *X. laevis* oocytes, co-expression of ZmPIP1;2 and ZmPIP2;1 increased the cell osmotic water permeability coefficient (P_f). Co-expression of PIP1 and PIP2 proteins was also investigated in Mimosa (Temmei et al. 2005), tobacco (Mahdieh et al. 2008), grapevine (Vandeleur et al. 2009), beet (Bellati et al. 2010), rice (Matsumoto et al. 2009), barley (Horie et al. 2011) and strawberry (Yaneff et al. 2014). These data indicated that PIP1s are involved in enhancing water transport across the plasma membrane together with PIP2s. Physical interactions between PIP1 and PIP2 aquaporins were confirmed with FRET-FLIM experiments and bimolecular fluorescence complementation using PIPs from maize (Zelazny et al. 2007), tobacco (Otto et al. 2010) and Arabidopsis (Sorieul et al. 2011). Zelazny et al.

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(2007) demonstrated that interactions between ZmPIP1s and ZmPIP2s occurred in maize mesophyll protoplasts, and the PIP1–PIP2 interaction induced the relocation of ZmPIP1s to the plasma membrane. These results showed that PIP1 proteins are involved in water transport when co-expressed with a PIP2. Fetter et al. (2004) observed that the fusion of ZmPIP1;2 with ZmPIP2;5GW, an inactive mutant of ZmPIP2;5, increased the P_f to a level similar to that of ZmPIP1;2–ZmPIP2;5. More recently, Berny et al. (2016) demonstrated that single-residue substitutions in ZmPIPs could alter ZmPIP1–ZmPIP2 protein interactions and the water transport activity. For instance, the Phe220Ala mutation in ZmPIP1;2 activated ZmPIP1;2 itself but inactivated ZmPIP2;5 when co-expressed.

We reported previously that the co-expression of HvPIP1 and HvPIP2 aquaporins from barley (*Hordeum vulgare*) induces enhancement of the water transport activity in *X. laevis* oocytes, as has been seen in PIP aquaporins from other plant species (Horie et al. 2011). In the present study, we prepare artificial HvPIP mutants and chimeric proteins between HvPIP1;2 and HvPIP2;4 to investigate the water transport activity of HvPIP1;2 of which mRNA is most abundant among HvPIP1s in barley roots (Horie et al. 2011). We discuss the mechanism activating the latent function of PIP1s as water a channel.

Results

Co-expression of HvPIP1;2 and HvPIP2;4 in *X. laevis* oocytes

Co-expression of HvPIP1;2 and HvPIP2;4 showed higher P_f than the expression of HvPIP2;4 alone in *X. laevis* oocytes (Horie et al. 2011). Therefore, we examined this combination in the present study. No increase in P_f values was observed in oocytes expressing HvPIP1;2 alone in contrast to the oocytes expressing HvPIP2;4 alone, which showed high P_f values (**Fig. 1A**, bars 1, 2 and 4). Co-expressing HvPIP1;2 with HvPIP2;4 showed higher P_f values than the sum of those obtained by expressing HvPIP1;2 and HvPIP2;4 separately (**Fig. 1A**, bars 2, 4 and 6).

Replacement of the cysteine residue located three amino acids N-terminally upstream of the second NPA motif with an amino acid containing a large side chain, such as methionine or tyrosine, has been reported to lead to the inactivation of CHIP28 aquaporin (Jung et al. 1994). Because plant PIPs have a threonine residue instead of cysteine at the homologous location, HvPIP2;4T229M was produced by the replacement of threonine 229 with methionine (**Fig. 2A**). HvPIP2;4T229M was detected in the plasma membrane of oocytes by immunostaining as same as HvPIP2;4 wild type (**Fig. 1B**, **Supplementary Fig. S1**), but this replacement abolished the water transport activity of the protein (**Fig. 1A, C**). Bright dots along the region of the plasma membrane in **Fig. 1B**-c are also seen in **Fig. 3B-b**, which may show membrane rafts where PIP aquaporins accumulate (Mongrand et al. 2004).

Similarly, HvPIP1;2T238M was produced by the replacement of threonine 238 with methionine in HvPIP1;2, which did not lead to a significant difference in the water transport activity when expressed alone (**Fig. 1A**, bars 1–3). Of note, the co-expression of HvPIP1;2 and HvPIP2;4T229M resulted in significant water transport activity (**Fig. 1A**, bar 7), suggesting that the T229M replacement mutation did not abolish the ability of HvPIP2;4 to interact with HvPIP1;2 leading to a relocalization of HvPIP1;2 to the plasma membrane and a P_f increase. On the other hand, the co-expression of HvPIP1;2T238M and HvPIP2;4T229M showed similar P_f values to the negative control (**Fig. 1A**, bars 1 and 9), indicating that the HvPIP1;2T238M mutant lost water transport activity. The co-expression of HvPIP1;2T238M and HvPIP2;4 also showed water transport activity, but lower than the co-expression of HvPIP2;4 and HvPIP1;2 (**Fig. 1A**, bars 6 and 8).

Protein targeting to the plasma membrane and water channel function

We produced chimeric HvPIP proteins by swapping N- and/or C-termini of HvPIP1;2 with those of HvPIP2;4 to investigate the role of the parts in the plasma membrane targeting HvPIP proteins. **Fig. 2** shows the schematic structures of chimeric PIPs and amino acid sequences of swapped sections including epitopic regions for antibodies used in the present study.

Both HvPIP1;2_24N and HvPIP1;2_24NC showed no water transport activity in *X. laevis* oocytes (**Fig. 3A**, bars 1–4). However, immunofluorescence analysis indicated that the HvPIP1;2_24NC protein accumulated in the plasma membrane (**Fig. 3B-b**, **Supplementary Fig. S1**). These observations suggested that the HvPIP1;2 water channel may be inactive even when localized in the plasma membrane. By contrast, HvPIP2;4 showed high water transport activity with accumulation in the plasma membrane (**Fig. 3A**, bar 5), but water transport activity was hardly observed in oocytes expressing HvPIP2;4_12N or HvPIP2;4_12NC (**Fig. 3A**, bars 6 and 7). Immuno-staining revealed that HvPIP2;4_12NC was not observed in the plasma membrane (**Fig. 3B-a**, **Supplementary Fig. S1**).

Latent PIP1;2 water transport activity appeared when co-expressed with PIP2;4

HvPIP1;2 could not be detected in the plasma membrane if expressed alone (**Fig. 1B-a**, **Supplementary Fig. S1**). When co-expressed with HvPIP2;4, HvPIP1;2 was localized to the plasma membrane and accumulated there (**Fig. 4A-a**). HvPIP2;4_12NC showed the recovery of accumulation in the plasma membrane when co-expressed with HvPIP1;2_24NC (**Fig. 4A-c**). These results suggested that HvPIP1;2_24NC recruits HvPIP2;4_12NC to the plasma membrane, as seen in general PIP1–PIP2 co-expression. Although neither HvPIP1;2_24NC nor HvPIP2;4_12NC exhibited any water transport activity when expressed alone (**Fig. 4B**, bars 3 and 6), significant water transport activity was detected in oocytes co-expressing both HvPIP1;2_24NC and HvPIP2;4_12NC (**Fig. 4B**, bar 9) as well as in oocytes co-expressing HvPIP1;2 and HvPIP2;4 (**Fig. 4B**, bar 8). When HvPIP2;4TM_12NC (N- and C-termini of HvPIP2;4T229M were swapped with those of HvPIP1;2) was used for co-expression with HvPIP1;2_24NC instead of HvPIP2;4_12NC, the oocytes showed significant

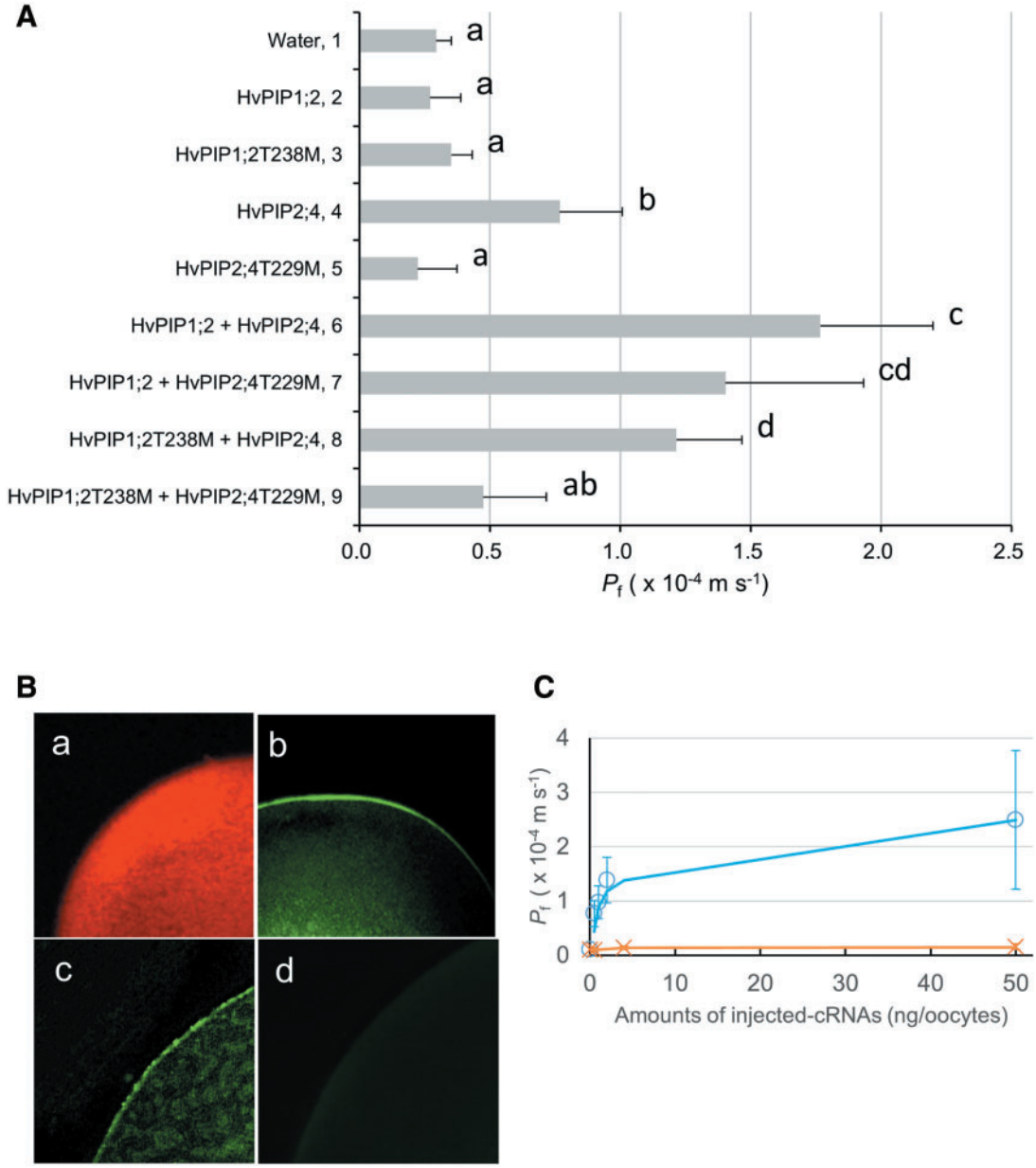


Fig. 1 Osmotic water permeability coefficient (P_f) values of oocytes expressing HvPIP1;2. HvPIP2;4 and their mutants. (A) P_f values of oocytes co-expressing HvPIP mutants with a single amino acid replacement of threonine with methionine. The amounts of cRNAs injected were 0.5 ng for *HvPIP2;4* and *HvPIP2;4T229M* and 2.5 ng for *HvPIP1;2* and *HvPIP1;2T238M*. Each bar shows the mean ($n = 7–10$) ± SD. Different lowercase letters indicate a significant difference at $P < 0.05$. (B) Localization of HvPIP1;2 (a), HvPIP2;4 (b) and HvPIP2;4T229M mutant (c). Immuno-staining was performed using the anti-HvPIP1s antibody (B-a), anti-HvPIP2;4 antibody (B-b to B-d) on sections of oocytes, which were injected with either 50 ng of *HvPIP1;2* (B-a), *HvPIP2;4* (B-b), *HvPIP2;4T229M* (B-c) cRNA or water (B-d) as a negative control. (C) Dependency of P_f of HvPIP2;4 and HvPIP2;4T229M expressed in oocytes on the amount of cRNA. Blue circles and orange crosses represent HvPIP2;4 and HvPIP2;4 T229M, respectively. Each spot shows the mean ($n = 9–10$) ± SD.

water transport activity, but the activity was lower than that of oocytes co-expressing HvPIP1;2_24NC and HvPIP2;4_12NC (**Fig. 4B**, bars 9 and 10). In addition, co-expression of HvPIP1;2TM_24NC with HvPIP2;4_12NC led to significant but lower water transport activity than the combination of HvPIP1;2_24NC and HvPIP2;4_12NC (**Fig. 4B**, bars 9 and 11). Note that a combination of TM mutations on HvPIP1;2_24NC and HvPIP2:4_12NC did not provide significant water transport activity (**Fig. 4B**, bars 9 and 12).

Discussion

Many PIP1 aquaporins show no water transport activity because such PIP1s failed to be targeted to the plasma membrane in oocytes when expressed alone (Fetter et al. 2004). Zelazny et al. (2007) showed that PIP1s expressed alone were also not targeted to the plasma membrane of maize protoplasts. Co-expressing HvPIP1;2 with HvPIP2;4 induced high P_f values in the present study, which was consistent with previous reports

Fig. 2 Schematic structures of HvPIP1;2, HvPIP2;4 and their chimeric forms. (A) Orange parts indicate regions derived from HvPIP1;2, and green parts indicate regions derived from HvPIP2;4. Dark orange and dark green sections represent the epitopic region for the anti-HvPIP1 antibody and anti-HvPIP2;4 antibody, respectively. 'M' in the schematic structures indicates the position where threonine was replaced with methionine. (B) Amino acid sequences of N- and C-termini of HvPIP1;2 and HvPIP2;4. The upper two sequences show the N-termini of HvPIP1;2 and HvPIP2;4. Regions to be swapped are indicated in bold. The epitopic region is shown in red. The lower two sequences show the C-termini of HvPIP1;2 and HvPIP2;4. Regions to be swapped are indicated in bold. The epitopic region is shown in green. The asterisk indicates the position where threonine was replaced with methionine in mutants HvPIP1;2T238M or HvPIP2;4T229M.

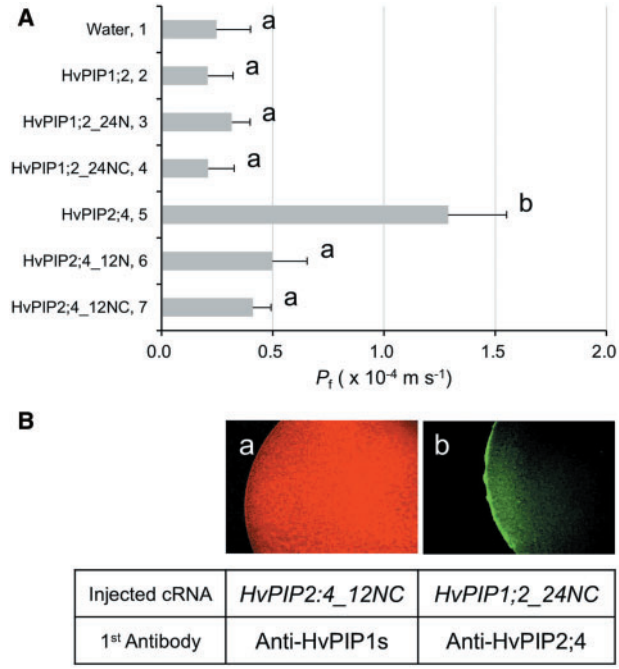
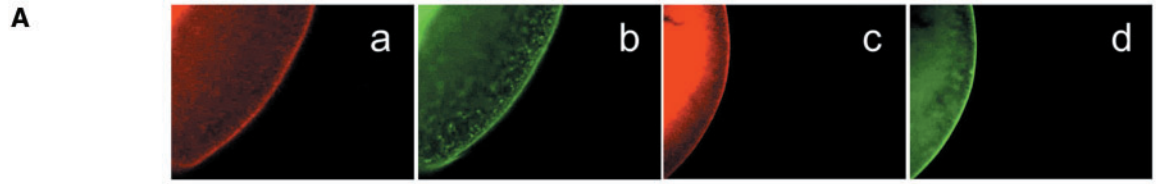


Fig. 3 Osmotic water permeability coefficient (P_f) values of oocytes expressing HvPIP1;2-, HvPIP2;4-, HvPIP1;2- or HvPIP2;4-derived mutants. (A) Oocytes were injected with water, 10 ng of *HvPIP1;2*, or its derivative cRNA, and 2 ng of *HvPIP2;4* or its derivative cRNA to measure P_f (A). Each bar shows the mean ($n = 7$–10) ± SD. Different lowercase letters indicate significant differences at $P < 0.05$ (B). Localization of HvPIP2;4_12NC (a) and HvPIP1;2_24NC (b). For fluorescence microscopic images, oocytes were injected with 50 ng of cRNAs. Oocytes injected with cRNA containing the N-terminus of HvPIP1;2 were fixed and incubated with anti-HvPIP1s antibody, followed by visualization with Alexa Fluor 647-conjugated secondary antibody (a). Similarly, oocytes injected with cRNA containing the C-terminus of HvPIP2;4 were fixed and incubated with anti-HvPIP2;4 antibody, followed by visualization with Alexa Fluor 488-conjugated secondary antibody (b).

(Fetter et al. 2004, Temmei et al. 2005, Mahdieh et al. 2008, Matsumoto et al. 2009, Vandeleur et al. 2009, Bellati et al. 2010, Horie et al. 2011). Aquaporins form tetramers, and heterotetramer formation is thought to be involved in the high P_f upon the co-expression of PIP1s and PIP2s (Zelazny et al. 2007). However, the mechanism of such high P_f is not fully understood.

In the present study, we investigated the molecular mechanisms that activate the latent function of PIP1 aquaporin water channels via interaction with PIP2s. Data indicated that at least two distinct mechanisms should be considered: first, the relocation mechanism of PIP1 proteins from the ER in the cytoplasm (Zelazny et al. 2009) to the plasma membrane and, second, the mechanism of PIP1 functionality as a water channel in the plasma membrane.

As for the relocation of PIP1s, the termini of PIP proteins were essential. Replacement of the only N-terminus of HvPIP2;4 with that of HvPIP1;2 markedly reduced the P_f (**Fig. 3A**, bar 6) as low as the negative control (**Fig. 3A**, bar1) or HvPIP2;4_12NC (**Fig. 3A**, bar 7). This result was consistent with findings in Zelazny et al. (2009) and Sorieul et al. (2011), in which it was demonstrated that the N-terminus is required for the trafficking of maize PIP2s to the plasma membrane. A diacidic motif (DIE) in the N-terminus is required for several PIP2s to exit the endoplasmic reticulum (Zelazny et al. 2009, Sorieul et al., 2011). HvPIP2;4 also contains a DIE motif in the N-terminus (**Fig. 2B**). In maize, however, the N-terminus alone was not enough for PIP1 targeting to the plasma membrane and the C-terminus may also be required (Zelazny et al. 2009). Our preliminary experiments indicated that swapping of only the C-terminus led to a small effect on the water transport activity of HvPIP2;4 (**Supplementary Fig. S2**). Although the effect of the C-termini



Injected cRNA	*HvPIP1;2* + *HvPIP2;4*	*HvPIP1;2* + *HvPIP2;4*	*HvPIP1;2_24NC* + *HvPIP2;4_12NC*	*HvPIP1;2_24NC* + *HvPIP2;4_12NC*
Visualized target	HvPIP1;2	HvPIP2;4	HvPIP2;4_12NC	HvPIP12_24NC
1st antibody	anti-HvPIP1s	anti-HvPIP2;4	anti-HvPIP1s	anti-HvPIP2;4

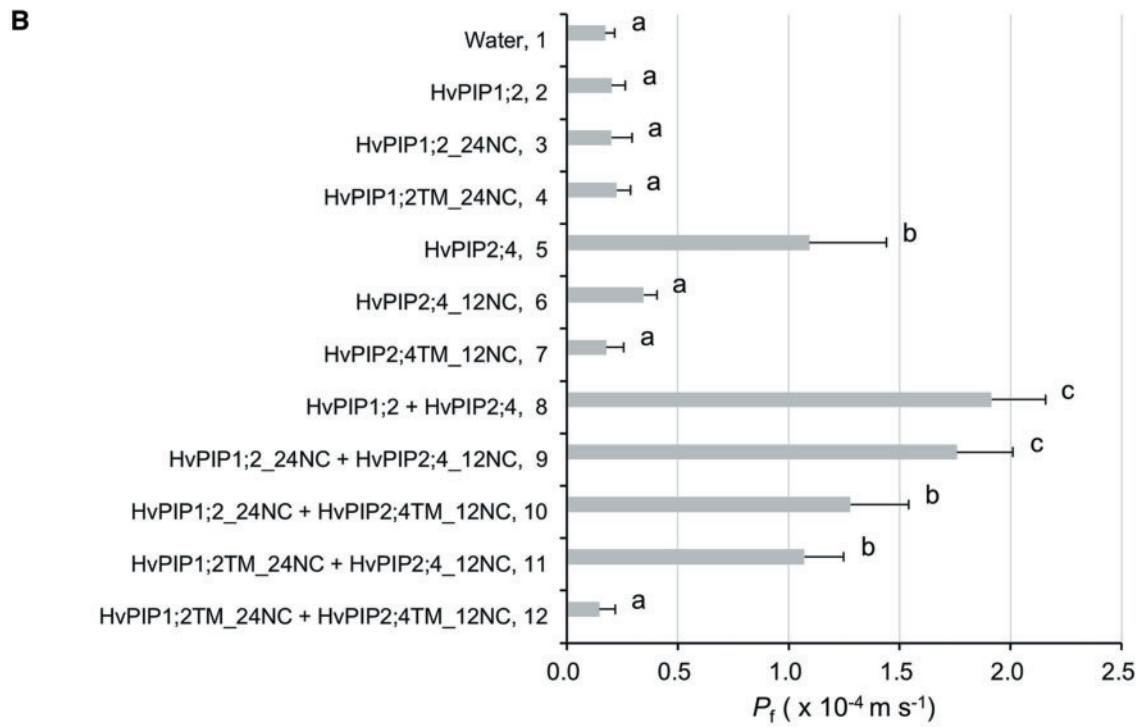


Fig. 4. Plasma membrane localization of PIPs and P_f values of oocytes co-expressing HvPIP1;2- and HvPIP2;4-derived mutants. (A) For fluorescence microscopic images, an oocyte was co-injected with 25 ng of *HvPIP1;2* + 25 ng of *HvPIP2;4* cRNAs (a, b), or with 25 ng of *HvPIP1;2_24NC* + 25 ng of *HvPIP2;4_12NC* cRNAs (c, d). Fixed oocytes were incubated simultaneously with anti-HvPIP1s and anti-HvPIP2;4 antibodies, followed by visualization with Alexa Fluor 647-conjugated secondary antibody (647-SA) and Alexa Fluor 488-conjugated secondary antibody (488-SA), respectively. The green and red images were obtained from one sample for (a) and (b) and another one for (c) and (d). (B) Oocytes were injected with water, 10 ng of *HvPIP1;2* or its derivative cRNA, or 2 ng of *HvPIP2;4* or its derivative cRNA to measure P_f. Each bar shows the mean ($n = 6$–10) ± SD. Different lowercase letters indicate significant differences at $P < 0.05$.

is not completely clear, we focused mainly on HvPIP1;2_24NC, a chimeric HvPIP1;2 harboring both the N- and C-termini of HvPIP2;4. Of note, HvPIP1;2_24NC was detected in the plasma membrane (**Fig. 3B-b**) but showed no water transport activity (**Fig. 3A**, bars 1–4). Co-expression of HvPIP1;2 and HvPIP2;4_12NC showed no increase in P_f (**Supplementary Fig. S3**, bar 7) because both protein lacked HvPIP2's N- and C-termini. It has been reported that the ER export signal in PIP2 N-terminal domains is essential for the plasma membrane localization of PIP proteins. Co-expression of HvPIP1;2_24NC and HvPIP2;4 showed the same P_f to co-expression of HvPIP1;2 and HvPIP2;4 (**Supplementary Fig. S3**, bars 6 and 8). Co-expression of HvPIP1;2_24NC with HvPIP2;4TM_12NC (**Fig. 4B**, bar 10) induced high P_f. This result indicated that the middle part of PIP2 protein (except for the N- and C-termini) was required for PIP1;2 functionality as a water channel.

The T229M mutation in HvPIP2;4 abolished water transport activity (**Fig. 1A**, bars 4 and 5). Although co-expression of ZmPIP1;2 with the inactive ZmPIP2;5GW mutant, where glycine 104 in loopB was replaced with tryptophan, did not increase P_f because of a misfolding and/or misrouting of ZmPIP2;5GW (Fetter et al. 2004), the T229M mutation in loopE of HvPIP2;4 in the present study had no significant effect on the relocation of HvPIP1;2 and the induction of HvPIP1;2 functionality as a

water channel in the plasma membrane (Fig. 1A, bars 6 and 7, and Fig. 4B, bars 9 and 10). This difference between ZmPIP2;5GW and HvPIP2;4T229M may depend on the location of amino acids in loopB or loopE. Data in the present study provided evidence that HvPIP1;2 functions as a water channel in the plasma membrane when it is stimulated by the interaction with HvPIP2;4, at least in the *X. laevis* oocyte-expression system. Furthermore, our results suggested that the plasma membrane targeting of HvPIP1;2 is not the only requirement, but an additional process, such as a conformational change in HvPIP1;2, stimulated by the physical interaction with HvPIP2;4, might also be required for HvPIP1;2 functionality as a water channel in the plasma membrane. Supporting this notion, co-expression of HvPIP1;2T238M and HvPIP2;4 has led to showing higher water transport activity than HvPIP2;4 alone (Fig. 1A, bars 4 and 8), indicating the possibility that HvPIP2;4 functionality as a water channel in the plasma membrane was stimulated by HvPIP1;2T238M, or that protein amount of HvPIP2;4 was increased by HvPIP1;2T238M. Enhancement of water transport activity of HvPIP2;4 by HvPIP1;2 is consistent with the past findings, in which PIP1s affect PIP2 proteins (Yaneff et al. 2014, Berny et al. 2016, Jozefkowicz et al. 2016). Further study is required to conclude whether the same mechanisms underlie the enhancement of the water transport activity upon the co-expression of HvPIP1s and HvPIP2s in general (Horie et al. 2011). In addition, it is important to test whether the same mechanisms can be applied to the co-expression of PIP1s and PIP2s from other plant species.

In this study, we demonstrated that not only plasma membrane targeting but also interaction with the middle part of PIP2 protein (except for the N- and C-termini) are essential for PIP1 functionality as a water channel. Although molecular mechanisms of interaction between PIP1 and PIP2 have been investigated (Berny 2016, Vajpai et al. 2018), further study is needed to determine the more detailed mechanisms of interaction between PIP1 and PIP2 proteins *in planta*.

Materials and Methods

Gene constructs for chimeric HvPIP proteins

Barley *PIP* genes were isolated and analyzed as described previously (Horie et al. 2011). The coding regions of *HvPIP* cDNAs were subcloned into the pXβGev1 oocyte-expression vector between 5′- and 3′-UTR sequences of the *Xenopus* globin gene.

Point mutation constructs, *HvPIP2;4T229M* and *HvPIP1;2T238M*, were achieved using one-step site-directed and site-saturation mutagenesis protocols described previously (Zheng et al. 2004).

For chimeric proteins used in this study, the BamHI site in N-terminus in *HvPIP1;2* or *HvPIP2;4* was introduced by the one-step mutagenesis to generate N-terminus BamHI mutants. Because the pXβGev1 vector has one BamHI site after 3′-UTR of the *Xenopus* globin sequence, digestion of BamHI-introduced *HvPIPs* in pXβGev1 with BamH1 resulted in two DNA fragments. *HvPIP1;2_24N* was obtained as follows: (i) two fragments, *HvPIP1:2* lacking N-terminus and pXβGev1 with N-terminus of *HvPIP2;4*, were isolated and ligated and then (ii) the BamHI mutation in the N-terminus was recovered by the one-step mutagenesis. *HvPIP2:4_12N* was obtained in the same way. *HvPIP1:2_24NC* was generated by the exchanges of a C-terminus of *HvPIP2:4_12N* and a C-terminus of *HvPIP1;2* with BamH1 mutations in the C-terminus. BamHI site in C-terminus was generated as the generation of BamHI site in N-terminus. *HvPIP2:4_12NC* was obtained in the same way. *HvPIP1;2_24N* and *HvPIP2:4_12N* were used as templates to generate *HvPIP1;2TM_24N* and *HvPIP2:4TM_12NC*, respectively, following the same strategy that was applied to the preparation of *HvPIP2;4T229M* and *HvPIP1;2T238M* as mentioned above. Primers used in the present study are listed in Supplementary Table S1.

HvPIP expression in *X. laevis* oocytes

Oocytes were isolated from adult female *X. laevis* frogs and maintained as described previously (Katsuhara et al. 2002). Oocytes were injected with 50 nl of a capped cRNA solution containing the designated amount of cRNA, 0.5–50 ng for water channel assays or 25 or 50 ng for immunochemical methods. As a negative control, water-injected oocytes were used in all experiments. Capped cRNA was synthesized using an mMESSAGE mMACHINE T3 in vitro transcription kit (Ambion, Austin, TX, USA) after the linearization of pXβGev1-based constructs with an appropriate nuclease.

Immunofluorescence microscopy

Oocytes were injected with 50 ng cRNAs, incubated for 24 h at 18°C and then fixed with 4% (w/v) formaldehyde solution (pH 7.4) overnight. Fixed samples were embedded in 4% agarose. Micro-sliced (50 μm thick) samples were treated with blocking solution (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 3% BSA) for 1 h at 25°C. After blocking, anti-HvPIP1s rat antibody (Horie et al. 2011) and/or anti-HvPIP2;4 rabbit antibody (generated against the synthetic peptide TKFGSSASFGSR, Medical & Biological Laboratories, Japan) or anti-HvPIP2;1 rabbit antibody (Katsuhara et al. 2002) were applied to samples for 1 h at 25°C, they were washed three times and, then, secondary antibodies [anti-rat IgG goat antibody conjugated with Alexa 647 (Invitrogen, Carlsbad, CA, USA) and/or anti-rabbit IgG goat antibody conjugated with Alexa 488 (Invitrogen)], were added, with incubation for another hour. After being washed twice with TBS-T (Tris-Buffered Saline with Tween20; 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and once with TBS (Tris-Buffered Saline; 50 mM Tris, pH 8.0, 150 mM NaCl), samples were analyzed under a fluorescence microscope (BZ-8000, Keyence, Japan).

Water transport activity assay in *X. laevis* oocytes

Oocytes were subjected to a swelling assay to measure P_f in accordance with the procedures described previously (Katsuhara et al. 2002). Biological replication included the testing of different oocytes from different batches harvested from different frogs and was two or three; the representative result from one oocyte batch from each experiment is included in figures.

Statistics

Statistical analyses were performed using IBM SPSS Statistics Desktop for Japan. Significant differences were identified by a one-way analysis of variance followed by Tukey HSD ($P < 0.05$).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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