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Control of the Water Transport Activity of Barley HvTIP3;1 Specifically Expressed in Seeds

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Tonoplast intrinsic proteins (TIPs) are involved in the transport and storage of water, and control intracellular osmotic pressure by transporting material related to the water potential of cells. In the present study, we focused on HvTIP3;1 during the periods of seed development and desiccation in barley. *HvTIP3;1* was specifically expressed in seeds. An immunochemical analysis showed that HvTIP3;1 strongly accumulated in the aleurone layers and outer layers of barley seeds. The water transport activities of HvTIP3;1 and HvTIP1;2, which also accumulated in seeds, were measured in the heterologous expression system of *Xenopus* oocytes. When they were expressed individually, HvTIP1;2 transported water, whereas HvTIP3;1 did not. However, HvTIP3;1 exhibited water transport activity when co-expressed with HvTIP1;2 in oocytes, and this activity was higher than when HvTIP1;2 was expressed alone. This is the first report to demonstrate that the water permeability of a TIP aquaporin was activated when co-expressed with another TIP. The split-yellow fluorescent protein (YFP) system in onion cells revealed that HvTIP3;1 interacted with HvTIP1;2 to form a heterotetramer in plants. These results suggest that HvTIP3;1 functions as an active water channel to regulate water movement through tissues during the periods of seed development and desiccation.

Keywords: Aquaporin • Barley • Seed • TIP • Water transport activity.

Abbreviations: BiFC, bimolecular fluorescence complementation; DAP, day after pollination; DAPI, 4′,6-diamidino-2-phenylindole; MBS, modified Barth's solution; MIP, membrane intrinsic protein; NIP, Nodulin-26-like intrinsic protein; PBS, phosphate-buffered saline; PIP, plasma membrane intrinsic protein; qRT–PCR, quantitative reverse transcription–PCR; SIP, small basic intrinsic protein; TIP, tonoplast intrinsic protein; XIP, X (for unrecognized) intrinsic protein; YFP, yellow fluorescent protein.

The GeneBank accession number for each gene is: *Hordeum vulgare* HvTIP1;2 (AB540226); *H. vulgare* HvTIP2;1 (AB540222); *H. vulgare* HvTIP2;3 (AB540224); *H. vulgare* HvTIP3;1 (AB540228); *H. vulgare* HvTIP4;1 (AB540225); *Triticum aestivum* TaTIP1;2 (CK206901); and *T. aestivum* TaTIP3;1 (BQ804556).

Introduction

Aquaporins are channel proteins that have six transmembrane domains in plants, animals, fungi and bacteria. Plant growth and development are very dependent on the regulation of water movement and homeostasis, and many aquaporins facilitate water movement across membranes including the plasma membrane and tonoplast (Chrispeels and Maurel 1994, Maurel et al. 2008). A previous study suggested that low levels of aquaporins in *Graptopetalum* were responsible for its lower water transport permeability than that of the radish, the taproot system of which had a large quantity of aquaporins (Ohshima et al. 2001).

Various aquaporins have been differentiated to regulate the water balance in higher plants through their evolutionary processes. Plant aquaporins show a high multiplicity of 35, 33 and 36 homologs in Arabidopsis, rice and maize, respectively, and have been associated with many important physiological features, such as drought and salt tolerance (Suga et al. 2002, Li et al. 2008, Liu et al. 2013), cell elongation (Hukin et al. 2002), leaf and petal movement (Azad et al. 2004, Siefritz et al. 2004, Chen et al. 2013) and seed germination (Willingen et al. 2006, Liu et al. 2013). Based on amino acid sequence homology, plant aquaporins (membrane intrinsic proteins, MIPs) have been divided into five subgroups: plasma membrane intrinsic proteins (PIPs), the main roles of which are as water channels in plants; tonoplast intrinsic proteins (TIPs); Nodulin-26-like intrinsic proteins (NIPs), which are expressed in the bacteroid membranes of root nodules for N_2 (nitrogen) fixation; small basic intrinsic proteins (SIPs); and X (for unrecognized) intrinsic proteins (XIPs), which were recently identified.

Some small molecules including water in plants have recently been identified as the substrates of aquaporins. For example, PIPs have been shown to transport water (Daniels et al. 1994), glycerol (Biela et al. 1999), hydrogen peroxide (Jang et al. 2012) and carbon dioxide (Hanba et al. 2004) as their substrates. Previous studies reported that NIPs transport water, formamide, glycerol (Rivers et al. 1997), urea (Klebl et al. 2003), silicon (Ma et al. 2006) and boron (Takano et al. 2006). Some studies have reported that water (Maurel et al. 1993, Li et al. 2008), glycerol (Li et al. 2008), boron (Takano et al. 2010), urea (Liu et al. 2003) and NH_3 (Loqué et al. 2005) were substrates of TIPs.

TIP homologs belong to five subgroups, TIP1–TIP5, which have been detected in the plant genome, and their expression patterns are known to differ. Previous studies reported that the genes of the TIP1 and TIP2 subfamilies were expressed in roots and young seedlings, while those of the TIP3 and TIP5 subfamilies were expressed in the mature seeds of Arabidopsis and

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rice (Willingen et al. 2006, Li et al. 2008). The rice TIP1 genes, *OsTIP1;1* and *OsTIP1;2*, were found to be highly expressed during seed germination, whereas *OsTIP3;1* and *OsTIP3;2* were specifically expressed in mature seeds, with decreases in expression levels being reported after germination (Li et al. 2008). In addition to these tissue specificities, some of these genes are known to be regulated by stresses such as drought, salt, osmotic stress and ABA. In shoots and roots of seedlings, TIP1 genes (*OsTIP1;1* and *OsTIP1;2*) and TIP4 genes (*OsTIP4;1* and *OsTIP4;2*) were up- and down-regulated, respectively, by dehydration and high salinity, and up-regulated by ABA (Li et al. 2008, Ngnyen et al. 2013).

Most seeds form an embryo and store starch and nutrients, which are necessary for germination and development at the early stage, and are then desiccated and maintained under desiccated conditions until germination. This suggests the existence of some mechanisms to control the inner water condition of cells during the development and subsequent desiccation of seeds. For example, late embryogenesis-abundant (LEA) proteins, which accumulate during the embryo maturation process, contain hydrophilic amino acid-rich regions, indicating that they hold water molecules and then protect the structures of cells from damage by desiccation (Espelund et al. 1992, Manfre et al. 2006). Although the mechanism of a action currently remains unknown, LEA proteins may mitigate water loss and maintain cellular stability within desiccated seeds.

Some studies on LEA proteins, which play a role in the seed desiccation process, have already been published; however, the molecular mechanism involved and control of water transport in the ripening and desiccation of seeds remain unclear. Recent studies suggested that aquaporins played a role in this mechanism. For example, *OsTIP3* was shown to be expressed in rice in a seed-specific manner (Takahashi et al. 2004), and OsTIP3 was localized on the membrane of protein body type II and aleurone grain, both of which were derived from vacuoles in the aleurone cells of rice seeds (Takahashi et al. 2004). In Arabidopsis, *TIP3;1* and *TIP3;2* are known to be strongly expressed in embryos during seed maturation and the early stages of seed germination (Willigen et al. 2006, Gattolin et al. 2011). Gattolin et al. (2011) revealed that TIP3;1 and TIP3;2 co-localized to the tonoplast of the protein storage vacuole, and also appeared on the plasma membrane. Regarding their physiological functions, OsTIP3;2 was found to transport glycerol, but not water (Liu et al. 2008), whereas OsTIP3;1 did not transport water in yeast (Hayashi et al. 2015); therefore, its function as a channel protein in seeds remains unknown.

In the present study, we focused on HvTIP3;1, which accumulates in barley seeds, and its developmental and tissue-specific expression patterns were analyzed. The water transport activity of HvTIP3;1 was investigated in the heterologous expression system of *Xenopus* oocytes when it was expressed alone or in combination with HvTIP1;2, which also accumulated in barley seeds. The physiological role and regulation of water transport activity by HvTIP3;1 were also discussed.

Results

Tissue-specific expression of HvTIPs

In the present study, the expression profiles of the dormant wheat cultivar Kitakei1354 were analyzed in preliminary experiments using wheat microarray chips. The results obtained showed that some TIP genes were uniquely detected and highly up-regulated in each tissue in seed-specific, seedling-specific and root-specific manners (**Supplementary Fig. S1**). Microarray data previously revealed that the expression patterns of TIP genes were tissue specific in Arabidopsis and rice (Willingen et al. 2006, Nguyen et al. 2013).

Since the MIP family has many family members in the genome, difficulties were associated with cloning and characterizing TIP genes from hexaploid wheat. Therefore, we examined the TIPs of diploid barley, the nucleotide sequences of which were very similar to those of wheat (90% and higher). Eight *HvTIP* genes, which we previously cloned, were classified into five groups, as well as TIPs from other cereals (**Supplementary Fig. S2**).

In order to clarify the expression patterns of *HvTIP* genes, quantitative reverse transcription–PCR (qRT–PCR) was performed for five *HvTIP* genes using barley tissues. As shown in **Fig. 1**, *HvTIP2;1* mRNA constantly accumulated in all tissues such as the roots, leaves and seeds, whereas the mRNAs of *HvTIP1;2* and *HvTIP4;1* mainly accumulated in the roots and leaves. *HvTIP4;1* mRNA levels were high in young and old leaves. On the other hand, *HvTIP3;1* mRNA specifically accumulated in the seeds, and its levels were particularly high at 30 days after pollination (DAP30) during seed desiccation (**Fig. 1**). The mRNA level of *HvTIP2;3* was very low and was rarely detected in the leaves and seeds, except the roots and gynoecium (DAP0) (**Fig. 1**).

Seed-specific expression of *HvTIP3;1*

The weight and water content of barley seeds harvested every 5 days after pollination (DAP0 to DAP40, and mature seeds) and the accumulation of *HvTIP3;1* mRNA in seeds harvested every 10 days after pollination was examined to analyze the expression of HvTIP3;1 and its role during seed development.

As shown in **Fig. 2A**, seed weight increased up to DAP25 (76.9 mg) and gradually decreased after DAP25 (the fresh weight per seed of fully matured seeds was 47.8 mg). The water content was 81.2% just after pollination (DAP0) and decreased with seed development, and it was finally 6.6% in fully matured seeds.

The results of qRT–PCR using seeds harvested every 10 days after pollination showed that the accumulation of *HvTIP3;1* mRNA was low until DAP10, but increased in DAP20 and peaked at approximately DAP30. *HvTIP3;1* mRNA levels were still detected in fully matured seeds, but were lower than those in DAP30 seeds (**Fig. 2B**). Therefore, the *HvTIP3;1* gene was expressed after the midle ripening stage of seeds. It was estimated that the expression reached a peak at about DAP30–DAP40.

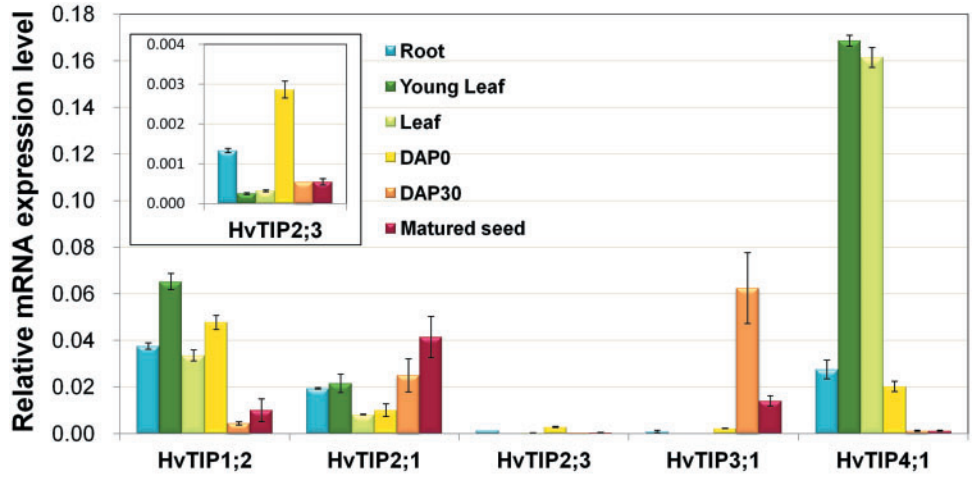

Fig. 1 Expression of *HvTIP* genes in roots, leaves and seeds during each developmental stage. qRT–PCR was performed using RNA samples prepared from the roots and young developing leaves of 4-week-old plants, leaves and ovaries collected just after pollination (DAP0), seeds at DAP30 and fully matured seeds (Matured seed) from barley. A 0.1 μg aliquot of the total RNA from each tissue was reverse-transcribed and amplified by PCR. Relative mRNA levels were normalized to the value of *Actin-1* as 1. Error bars represent the SD ($n = 3$).

Accumulation of TIP3;1 in seeds

Immunocytochemical experiments using barley seeds were performed to analyze the accumulation of the TIP3;1 protein in seeds. In developing seeds (at DAP25 and DAP30), the HvTIP3;1 signal was detected in aleurone cells and the outer layers (**Fig. 3A–F**). The outer layers had formed in the developing seeds of wheat at DAP20, and immature aleurone cells appeared (**Supplementary Fig. S4A**). The TIP3;1 signal was not detected in immature cells (**Supplementary Fig. S4B**). Aleurone cells matured after DAP30, and the TIP3;1 signal was detected in these aleurone cells (**Supplementary Fig. S4D**) as well as in embryos (the signal was weak; data not shown). Furthermore, subcellular localization to aleurone cells was analyzed by a comparison with staining with FM4-64, which is a marker for membranes. The fluorescent pattern of TIP3;1 was almost the same as that of FM4-64 staining, indicating that TIP3;1 localized to the membranes of cells and organelles (**Supplementary Fig. S4E–H**).

Water transport activity by the co-expression of HvTIP3;1 and HvTIP1;2

Previous studies reported that some TIPs in rice transported not only water, but also glycerol (Liu et al. 2008); therefore, we also examined the glycerol and water transport activities of HvTIP3;1 using a *Xenopus* oocyte heterologous expression system. However, HvTIP3;1 did not transport water (**Fig. 3**; **Supplementary Fig. S5C**) or glycerol (**Supplementary Fig. S5D**) even though HvTIP3;1 localized to the plasma membrane of the injected cells (**Supplementary Fig. S5B**).

As shown in **Fig. 1**, *HvTIP1;2* and *HvTIP2;1* were highly expressed in young and mature seeds, and the expression pattern overlapped with that of *HvTIP3;1* in seeds. HvTIP3;1 showed positive water transport activities through interacting with HvTIP1;2 as described below, whereas it did not with HvTIP2;1, Therefore, we here focus on the interaction of HvTIP3;1 with HvTIP1;2.

The effects of the co-expression of HvTIP1;2 and HvTIP3;1 was also examined using the *Xenopus* oocyte expression system, and the osmotic water permeabilities of oocytes injected with *HvTIP1;2* and/or *HvTIP3;1* cRNA were measured. As shown in **Fig. 4A**, the water permeability of *HvTIP3;1*-injected oocytes was 0.19×10^{-4} m s^{-1}, as low as that of water-injected control cells (0.16×10^{-4} m s^{-1}), indicating that HvTIP3;1 had a negligible water permeability (0.03×10^{-4} m s^{-1}). On the other hand, *HvTIP1;2*-injected oocytes showed high activity (0.75×10^{-4} m s^{-1}) and the contribution of HvTIP1;2 was estimated to be 0.59×10^{-4} m s^{-1}. When *HvTIP1;2* and *HvTIP3;1* cRNA were co-injected into oocytes, the water permeability was 0.95×10^{-4} m s^{-1}, which was significantly higher than that of *HvTIP1;2*-injected oocytes (**Fig. 4A**). The water permeability of these co-expressed TIPs (0.79×10^{-4} m s^{-1}) was obviously higher than the sum of HvTIP1;2 (0.59×10^{-4} m s^{-1}) and HvTIP3;1 (0.03×10^{-4} m s^{-1}), indicating that the water permeabilities of HvTIP1;2 and/or HvTIP3;1 were enhanced by co-expression.

An HvTIP1;2 mutant (HvTIP1;2m) that lacked the ability to transport water by substituting alanine with methionine at the 195th amino acid, the residue located inside the water channel pore (Jung et al. 1994), was constructed in order to confirm the water transport activity of HvTIP3;1 in co-expressed cells. The cRNAs of *HvTIP3;1* and/or *HvTIP1;2m* were injected into oocytes, and osmotic water permeability was then measured. As shown in **Fig. 4A**, the water transport activity of oocytes expressing HvTIP1;2m was low (0.23×10^{-4} m s^{-1}). The activity of oocytes co-expressing HvTIP1;2m and HvTIP3;1 was 0.50×10^{-4} m s^{-1}, which was lower than that of oocytes co-expressing HvTIP1;2 and HvTIP3;1, but was significantly higher than that of oocytes expressing HvTIP1;2m, suggesting that HvTIP3;1 transported water in oocytes co-expressing HvTIP1;2m and

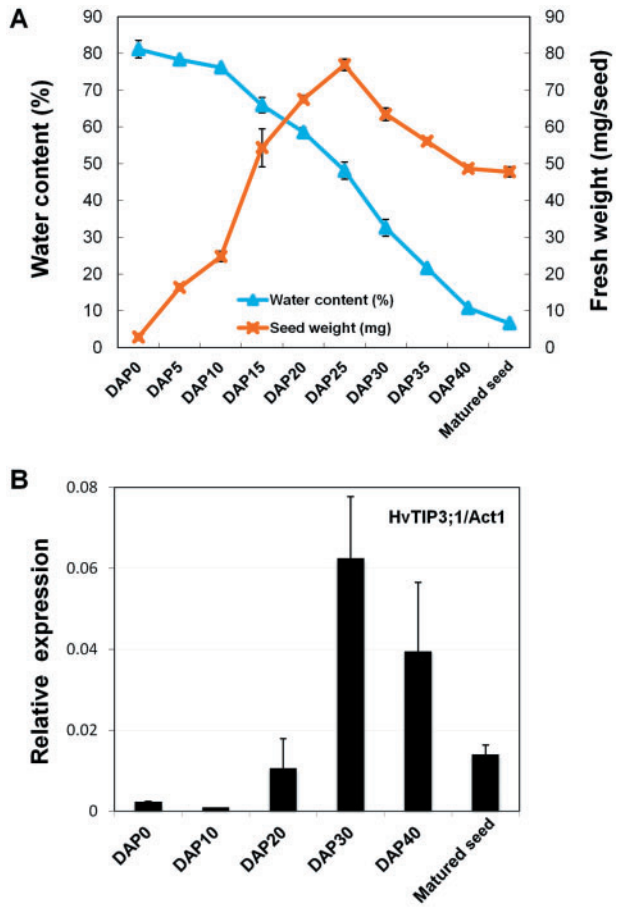

Fig. 2 Seed development and accumulation of *HvTIP3;1* mRNA in barley. (A) The water content and fresh weight of barley seeds during ripening. Seeds were harvested every 5 d during DAP0–DAP40 and matured seeds were used in these tests. Water content was measured in five seeds from each sample. Error bars represent the SD ($n = 3$). The seed weight showed the fresh weight per one grain and error bars represent the SD ($n = 15$) (B) *HvTIP3;1* expression in the ripening seeds of barley. qRT–PCR was conducted using RNA samples prepared from ovaries collected just after pollination (DAP0), seeds at DAP10, 20, 30 and 40, and matured seeds from barley. A 0.1 μg aliquot of total RNA from each tissue was reverse-transcribed and amplified by PCR. The cDNA from barley *Actin-1* was also amplified as a positive control for PCR. Relative mRNA levels were normalized to the value of *Actin* as 1. Error bars represent the SD ($n = 3$).

HvTIP3;1. The contribution of co-expressed HvTIP1;2m and HvTIP3;1 to the water permeability in oocytes was estimated as 0.33×10^{-4} m s^{-1}, and was also higher than the activity of HvTIP1;2m (0.07×10^{-4} m s^{-1}), HvTIP3;1 (0.03×10^{-4} m s^{-1}) and their sum, also indicating that HvTIP3;1 had a significant activity when co-expressed with HvTIP1;2.

In order to determine the effects of the co-expression of HvTIP1;2 and HvTIP3;1 on the water permeability of HvTIP1;2, another inactive mutant, HvTIP3;1m, was constructed in the same manner as HvTIP1;2m. The amino acid sequence of HvTIP3;1 had alanine substituted with methionine at the 202nd amino acid. As shown in **Fig. 4B**, the water permeabilities of oocytes injected with HvTIP3;1m (0.19×10^{-4} m s^{-1}) and those that co-expressed HvTIP3;1m and HvTIP1;2m (0.27×10^{-4} m s^{-1}) were as low as that of water-injected control cells (0.16×10^{-4} m s^{-1}), indicating that HvTIP3;1m actually has negligible water permeability. The water permeability of oocytes co-expressing HvTIP1;2 and HvTIP3;1m (0.70×10^{-4} m s^{-1}) was nearly equal to that of oocytes expressing HvTIP1;2 (0.69×10^{-4} m s^{-1}) (**Fig. 4B**). Therefore, the water permeability of HvTIP1;2 was not changed by the co-expression with HvTIP3;1m. Furthermore, the water permeability of oocytes co-expressing HvTIP1;2 and HvTIP3;1 was also significantly higher than that of oocytes co-expressing HvTIP1;2 and HvTIP3;1m (**Fig. 4B**). These results clearly demonstrated that HvTIP3;1 transported water when co-expressed with HvTIP1;2, and that HvTIP1;2 showed no changes in activity with the co-expression of HvTIP3;1m.

Physical interaction between HvTIP3;1 and HvTIP1;2

Bimolecular fluorescence complementation (BiFC) was used to confirm the interaction between HvTIP1;2 and HvTIP3;1. Two constructs, 'HvTIP1;2 + YFP-N' and 'HvTIP3;1 + YFP-C', which produce HvTIP2;1 and HvTIP3;1 fused with the N- and C-terminus of YFP (YFP-N and YFP-C, respectively), were transformed into onion epidermal cells by a particle bombardment method, and these cells were then observed under a confocal fluorescence microscope. If HvTIP1;2 and HvTIP3;1 interacted with each other in these cells, two non-fluorescent fragments, YFP-N and YFP-C, formed a bimolecular fluorescent complex and a complex emitted it fluorescence as an entire YFP. The images in **Fig. 5A** and **B** are two differently focused images of an onion cell. As shown in **Fig. 5A**, YFP fusion proteins were observed in the cell membranes and as a small bubble with the appearance of a vesicle near the membrane. The YFP signal in **Fig. 5B** was observed in the cytoplasm of the cell. These results showed that HvTIP1;2 and HvTIP3;1 physically interacted with each other to form a complex in the membrane and cytoplasm of these cells (**Fig. 5E**).

Discussion

A microarray analysis comparing the expression profiles of the dormant wheat cultivar Kitakei-1354 and non-dormant wheat cultivar EH47-1 (Kawakami et al. 1997) in the seeds and seedlings of wheat revealed that TIP genes were uniquely expressed in each tissue (**Supplementary Fig. S1**). When we analyze products of a large family such as aquaporins, the function or the original role might often be difficult to deipher due to the redundancy derived from the hexaploid genome. Therefore, in this study, we analyzed TIPs of barley, which has a diploid genome and is closely related to wheat. The nucleotide sequences of the TIPs from barley were very similar to those from each wheat gene; for example, *HvTIP1;2* and *HvTIP3;1* showed high homologies of 89.3% and 96.6% to their corresponding wheat genes, respectively. In the immunocytochemical experiment, we used wheat seeds because it was difficult to observe the aleurone layer of barley seeds. Images using wheat seeds are shown in **Supplementary Fig. S4**.

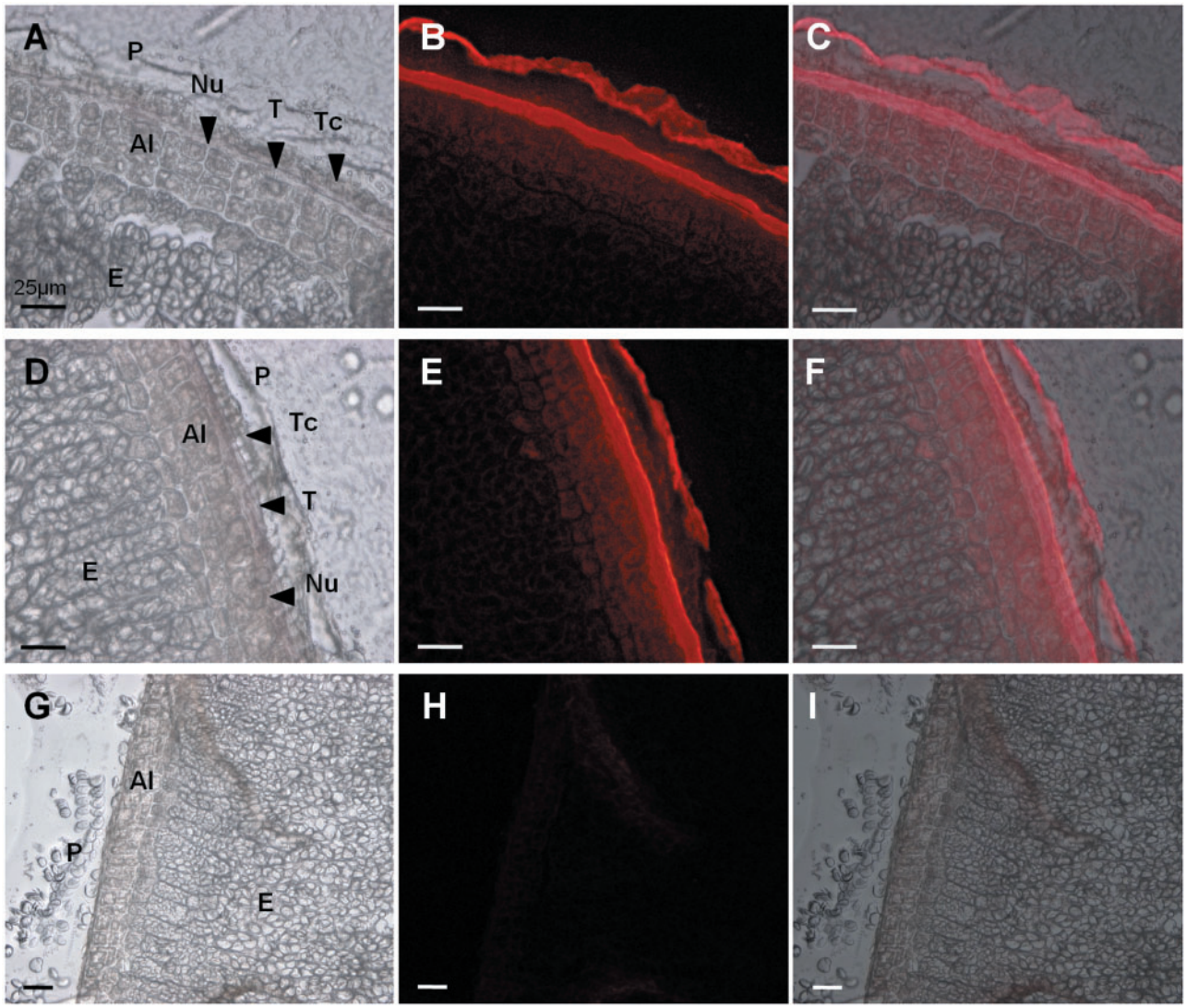

Fig. 3 Immunocytochemical localization of HvTIP3;1 in barley seeds. Immunochemical experiments were performed in barley seeds using an HvTIP3;1 antibody. (A–C) Barley seeds at DAP25. (D–F) Barley seeds at DAP30. (G–I) Barley seeds at DAP30 using pre-immune serum. (A), (D) and (G) Bright field; (B), (E) and (H) HvTIP3;1 detected by Alexa Fluor 647 (red fluorescent); (C), (F) and (I) merged image. P, pericarp; Tc, tube cells; T, testa (seed coat); Nu, nucellar; Al, aleurone cells; E, endosperm. Scale bar = 25 μm.

qRT–PCR was performed on some *HvTIP* genes found in the database by using each tissue of barley (**Fig. 1**). *HvTIP3;1* was initially expressed at DAP20, expression levels peaked at approximately DAP30 and then they decreased slightly thereafter (**Fig. 1**). These results indicated that *HvTIP3;1* was expressed in a seed-specific manner. These expression patterns were very similar to those of *OsTIP3;1*, which was expressed after the middle stage of seed development (Takahashi et al. 2004). *HvTIP2;1* was constantly expressed in all tissues, including the roots, leaves and seeds, whereas *HvTIP1;2* and *HvTIP4;1* were mainly expressed in the roots, leaves and seeds. Several aquaporins are known to be expressed in the seeds of other plants. Schuurmans et al. (2003) isolated *PsPIP1-1*, *PsPIP2-1* and *PsTIP1-1* from the cotyledon of the developing pea seed. A previous study using microarray expression profiling in Arabidopsis showed that *AtTIP3;1*, *AtTIP3;2* and *AtTIP5;1* were strongly expressed, while the expression of *AtPIP1;2* was lower in seeds (Willingen et al. 2006). In rice, *OsTIP3;1* and *OsTIP3;2* were specifically expressed in mature seeds (Li et al. 2008), and the findings of a rice microarray revealed that *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;1*, *OsTIP1;1*, *OsTIP1;2*, *OsTIP2;2*, *OsTIP3;1*, *OsTIP3;2*, *OsTIP4;1* and *OsSIP1;1* were expressed in the seeds (Nguyen et al. 2013). In the present study, our results demonstrated that *HvTIP3;1* was specifically expressed in barley seeds and *HvTIP1;2* and *HvTIP2;1* were also expressed in young and mature seeds, suggesting that some TIPs had accumulated and played some roles in these seeds, especially TIP3;1, which is involved in controlling the transport of water in the middle to late ripening stages of seeds.

Immunocytochemical experiments showed that HvTIP3;1 mainly accumulated in the aleurone layers and, to a lesser extent, in the outer layers, nucellar, testa and pericarp of barley seeds (**Fig. 3**). Wheat aleurone cells consist of a single layer (triple layers in barley) and are larger than barley cells;

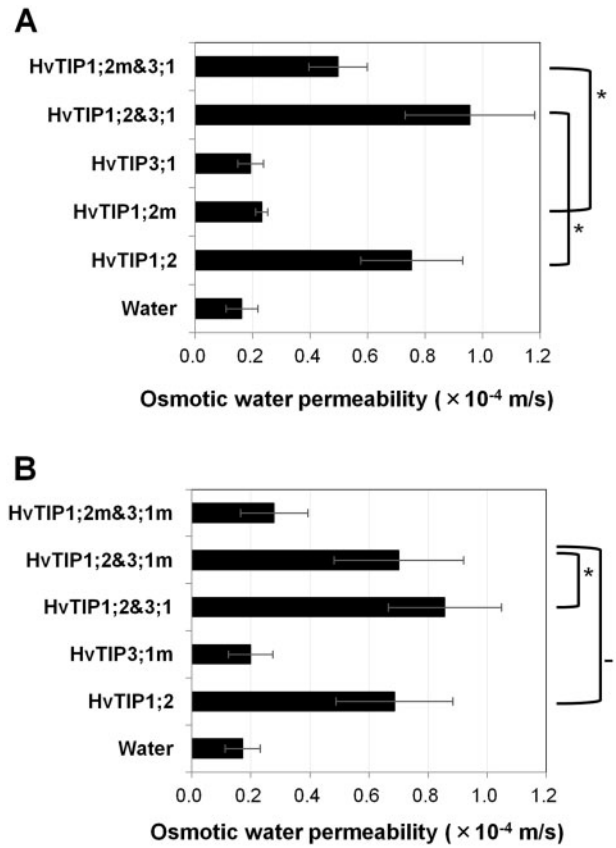

Fig. 4 Water transport activity of HvTIP3;1 in *Xenopus* oocytes. (A) The water permeabilities of HvTIP1;2, HvTIP3;1 and/or the HvTIP1;2 mutant (HvTIP1;2m), which lost its water transport activity by substituting alanine with methionine at the 195th amino acid. Error bars represent the SD ($n = 10$). (B) The water permeabilities of HvTIP1;2, HvTIP3;1 and/or the HvTIP3;1 mutant (HvTIP3;1m), which lost its water transport activity by substituting alanine with methionine at the 202nd amino acid. Error bars represent the SD ($n = 16$). A total of 50 nl of water or 20 ng of HvTIP1;2, HvTIP3;1, HvTIP1;2 mutant and HvTIP3;1 mutant cRNAs were injected into *Xenopus* oocytes and water permeability was determined from the extent of swelling when transferred into hypo-osmotic solution (1/5 strength modified Barth's solution (MBS)). Asterisks indicate *P*-values denoting significant differences ($^*P < 0.05$).

therefore, it is suitable for clearly observing subcellular localization. It is easier to follow the development of wheat seeds than barley seeds because the development of hexaploid wheat seeds (approximately 50–60 d in the field) is slower than that of barley seeds (approximately 40 d in the field). The amino acid sequence of HvTIP3;1 was similar to that of wheat TaTIP3;1 (96.6%); therefore, we could also use the same antibody in immunocytochemical experiments on wheat seeds. The TIP3;1 signal was confirmed on the membranes of the cell and aleurone grains in the mature aleurone cells of wheat, but not in the immature cells (**Supplementary Fig. S4**). Ibl et al. (2014) have also reported that HvTIP3 was accumulated in protein storage vacuoles of aleurone layers, vacuoles of the inner aleurone and endosperms in barley seeds, and the results were similar to those in this study These results suggested that the pattern of TIP3;1 was related to functions such as the control of water conditions to the storage of substances or to the maintenance of cytological structures in mature aleurone cells.

Water permeability of HvTIP3;1

HvTIP3;1 was confirmed to localize in the plasma membrane of the injected oocyte, but did not exhibit water permeability (**Fig. 4**; **Supplementary Fig. S5**). This is consistent with previous findings in rice, in which OsTIP3;1 strongly accumulated in aleurone cells, but did not exhibit any water transport activity in yeast (Hayashi et al. 2015). Li et al. (2008) reported that OsTIP3;2, which is specifically expressed in seeds, promoted glycerol permeability; however, HvTIP3;1 did not show any glycerol transport activity (**Supplementary Fig. S4**).

A previous study reported that OsPIP1;1 had low water permeability in oocytes, whereas it exhibited strong water permeability in a physical interaction with OsPIP2;1 (Liu et al. 2013). The effects of the co-expression of different TIPs on water permeability have not yet been determined. This is the first study to provide evidence for enhanced water permeability due to the co-expression of different TIPs. As shown in **Fig. 1**, HvTIP1;2 was strongly expressed in the young and mature seeds of barley, and this expression pattern partially overlapped with that of HvTIP3;1. **Fig. 4** shows that HvTIP3;1, which had no water transport activity in oocytes when expressed alone, started to exhibit significant activity in oocytes when co-expressed with HvTIP1;2. We speculated that HvTIP3;1 changed its conformation by forming a heteromer with HvTIP1;2, and then had the ability to transport water. Furthermore, **Fig. 4B** shows that the water permeability of HvTIP1;2 was not changed by heteromerization with HvTIP3;1. This phenomenon appears to be similar to that observed in oocytes co-expressing PIP1 and PIP2; however, the following points indicate that different mechanisms occur in the co-expression of TIPs: (i) HvTIP3;1 is itself able to transport to the plasma membrane (**Supplementary Fig. S4**) whereas PIP1 remains in the endoplasmic reticulum without PIP2; and (ii) water transport activity of HvTIP1;2 was not increased by its co-expression with HvTIP3;1 (**Fig. 4B**), whereas the co-expression of PIP1 and PIP2 increased the activity of the PIP2 water channel (Yaneff et al. 2014). Therefore, the activation mechanism of HvTIP3;1 may be very different from that of PIP1.

A model of water transport by HvTIP1;2 and HvTIP3;1

Fig. 6 shows a model based on the results of this study. A previous study reported that aquaporins formed tetramers (Törnroth-Horsefield et al. 2006). These findings suggest that HvTIP1;2 and HvTIP3;1 form homotetramers or heterotetramers and localize around the membranes of cells. The HvTIP1;2 homotetramer localized on membranes exhibited water transport activity, whereas the HvTIP3;1 homotetramer did not, even though it was localized on membranes. When HvTIP3;1 was co-expressed with HvTIP1;2 and they interacted with each other, they formed heterotetramers and showed higher water permeability activity than that of the HvTIP1;2 homotetramer on membranes. Therefore,

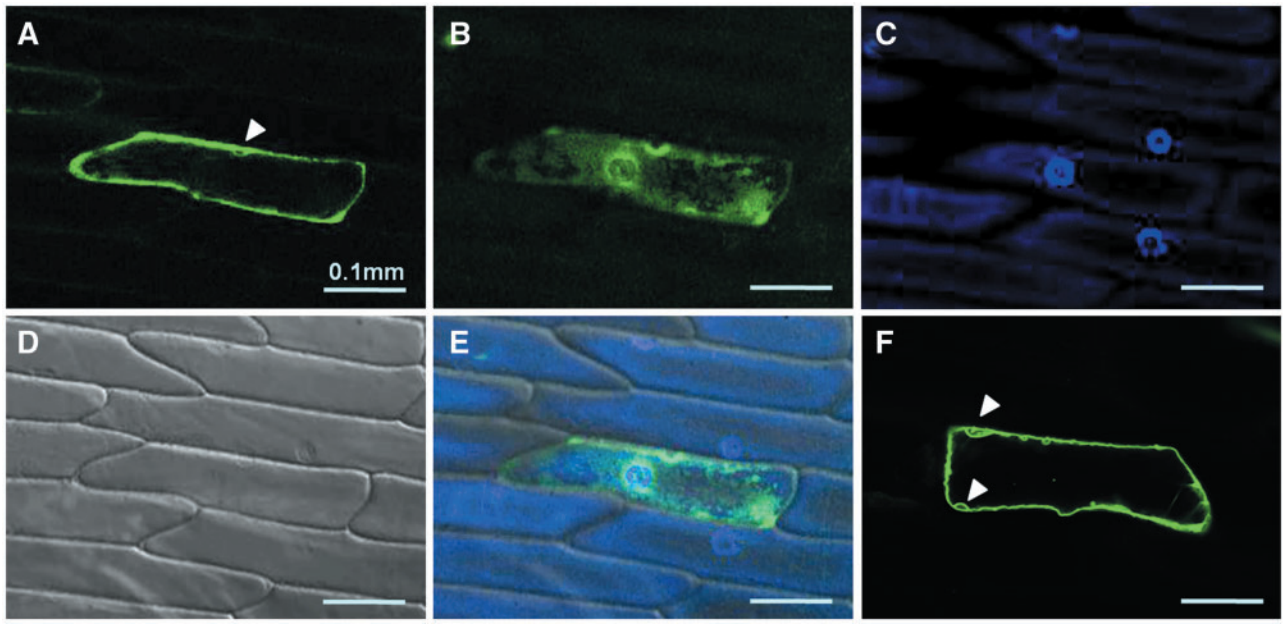

Fig. 5 Interaction between HvTIP1;2 and HvTIP3;1 in onion epidermal cells co-expressing split YFP fusion proteins. HvTIP1;2 + YFP-N and HvTIP3;1 + YFP-C were injected into onion epidermal cells using a particle bombardment method, and these cells were then observed under a confocal fluorescence microscope. (A and B) YFP, images of an onion cell taken at different focuses. (C) DAPI-stained cell, (D) bright field, (E) merged image. DAPI was used as a marker for nuclear staining. (F) GFP, a cell injected with GFP–AtVam3. Arrowheads show signals observed as small bubbles. Scale bar = 0.1 mm.

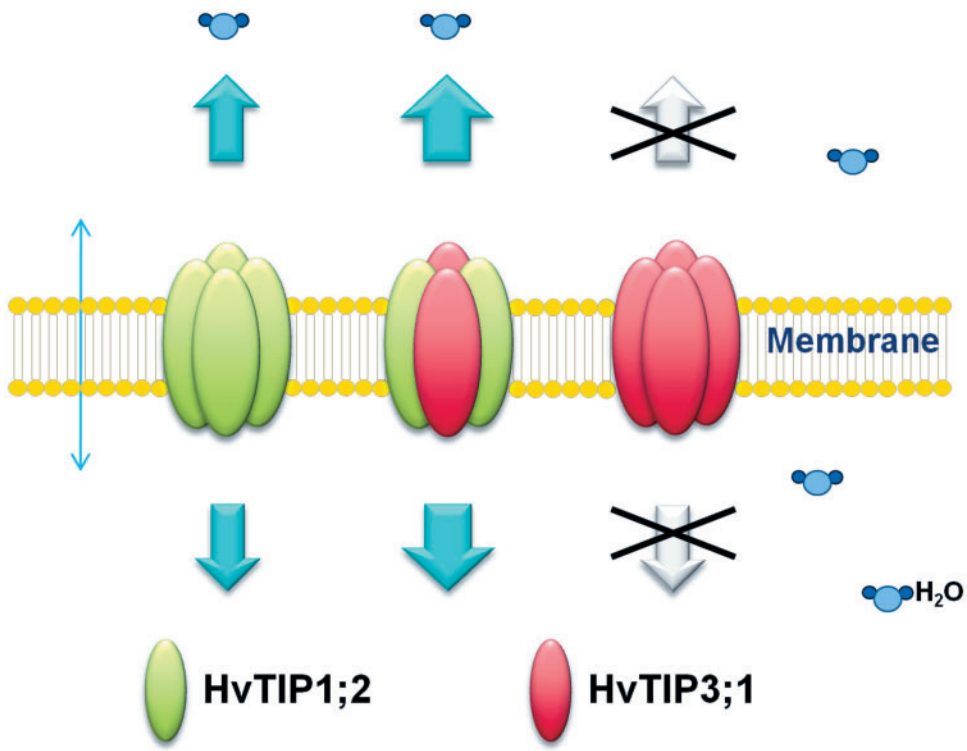

Fig. 6 A model of the interaction between HvTIP1;2 and HvTIP3;1. HvTIP1;2 and HvTIP3;1 are assumed to form a tetramer, including a homotetramer or heterotetramer, and localize around membranes. The HvTIP1;2 homotetramer, which was localized in membranes, exhibited water transport activity, whereas the HvTIP3;1 homotetramer did not, even though it was localized on membranes. HvTIP3;1 and HvTIP1;2 may form heterotetramers when co-expressed and exhibited higher water permeability than that of the HvTIP1;2 homotetramer. Some kinds of heterohexamers (HvTIP1;2 : HvTIP3;1 = 3 : 1, 1 : 1 and 1 : 3) may be contained in them. In this figure, we only showed a hexamer (HvTIP1;2 : HvTIP3;1 = 1 : 1) for clarity.

the structure or characteristics of HvTIP3;1 may be modified by the interaction with HvTIP1;2, such that the water channel (gate) opens.

Role of HvTIP3;1 and HvTIP1;2 in seeds

HvTIP1;2 was highly expressed in young and developmental tissues such as young leaves and immature seeds just after pollination, and was also expressed in late developmental tissues and mature seeds, even though its expression level was low (**Fig. 1**). The high water permeability activity of HvTIP1;2 was confirmed, suggesting that HvTIP1;2 may contribute to the cell elongation associated with young developmental stages in seeds and other tissues. In addition to *HvTIP1;2*, the level of *HvTIP3;1* markedly increased in the late ripening stage of seeds. *HvTIP3;1* expression levels increased in a seed-specific manner and peaked in the late ripening stage of barley seeds (**Fig. 1**). BiFC experiments with onion cells (**Fig. 5**) demonstrated that HvTIP2;1 and HvTIP3;1 physically interacted with each other in cells expressing both of them. Therefore, water permeability may be increased in the cells of late ripening seeds expressing both HvTIP1;2 and HvTIP3;1. A high level of water permeability may be suitable to control water conditions in the cells of seeds at this stage, and ultimately lead to seed desiccation. In addition to HvTIP1;2, as described above, some PIPs and TIPs are known to be expressed in seeds, and the interaction between HvTIP3;1 and other TIPs and its effects need to be investigated in future studies.

Materials and Methods

Materials

Barley, *Hordeum vulgare* L. cultivar Haruna-nijyo, was grown in the fields of Kurashiki, Japan. Seeds were harvested at different developmental stages ranging from DAP0 to DAP40. The harvested seeds were stored at −80°C until later use. Fully matured seeds were harvested at DAP40 and stored in a desiccator at 4°C. The dry weight of seeds was measured after incubating seeds at 150°C for 3 h. The water content of seeds was determined by (FW − DW)/FW. The dry weight of seeds was measured after incubating seeds at 150°C for 3 h. Water content was measured in five seeds from each sample in three independent biological replicates.

RNA extraction and analysis of qRT–PCR

For the expression analysis, samples including roots, leaves and seeds for RNA extraction were taken from barley plants grown in the fields of Kurashiki, Japan. The roots and young leaves of 4-week-old plants, the leaves and DAP0 seeds from plants after pollination, and DAP30 and mature seeds were used for RNA extraction. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) after seeds had been ground with a mortar and pestle. First-strand cDNA synthesis was performed using the PrimeScript™ RT regent Kit (TAKARA). qRT–PCR using SYBR premix Ex Taq (Takara) was performed by a Light cycler (Roche). Results from triplicate independent biological samples are shown, and error bars represent the SD. The primer sets of each gene are shown in **Supplementary Table S1**.

Anti-HvTIP3;1 peptide antibodies

HvTIP3 rabbit anti-peptide polyclonal antibodies used synthetic peptides produced by Operon Biotechnologies (Tokyo). These synthetic peptides were based on the common N-terminal sequence of both HvTIP3;1 and TaTIP3;1, MSTAARTTGRRGFTMGRSEDATHPDTI.

Immunocytochemical experiments

The developing seeds of wheat and barley were vacuum infiltrated for 2 h with a fixative that consisted of 4% (w/v) formaldehyde in 100 mM cacodylic acid sodium solution containing 120 mM sucrose (pH 7.5) at 4°C. After washing for 1 h with the cacodylic acid sodium solution, the seeds were dehydrated in a graded ethanol series. The samples were then embedded in Technovit 8100 (Heraeus Kulzer GmbH). Blocks were polymerized overnight at 4°C. Ultrathin sections were cut with a knife using a microtome (Yamato Kohki Industrial Co. Ltd.) and mounted on a glass slide.

In the immunocytochemical analysis, sections were treated with 0.1% pectolyase Y-23 (Kyowa Chemical Products Co., Ltd.) containing 0.1% Tween-20 in 1 × phosphate-buffered saline (PBS) (pH 7.2) for 2 h and with blocking solution containing 0.1% Tween-20 in 1 × PBS buffer (pH 7.2) for 1 h at room temperature. These sections were then incubated with a solution of the anti- HvTIP3;1 primary antibody diluted 1 : 100 in blocking solution for 1 h at room temperature. Non-immune serum was used for the negative control instead of a primary antibody. After washing with blocking solution, the sections were incubated with a solution of the Alexa Fluor 488-conjugate anti-rabbit IgG secondary antibody (Invitrogen) diluted 1 : 400 in blocking solution for 1 h at room temperature. Sections were then washed with distilled water and stained with 10 μM 4′,6-diamidino-2-phenylindole (DAPI) (Nacalai) or FM4-64 (Invitrogen). After staining, all sections were examined under a fluorescence microscope (BZ-8000, Keyence).

Water transport activity assay in *Xenopus* oocytes

The coding regions of HvTIP3;1 and HvTIP1;2 cDNA were subcloned into the pXβGev1 expression vector. Point mutations, the HvTIP3;1 mutant and HvTIP1;2 mutant, in constructs for amino acid substitution were essentially achieved using the one-step site-directed and site-saturation mutagenesis protocols described previously (Zheng et al. 2004). Constructs were linearized with *Not*I, and capped cRNA was synthesized using the mMESSAGE mMACHINE T3 in vitro transcription kit (Ambion). Oocytes were isolated from adult female *Xenopus laevis* frogs and maintained as described previously. Oocytes were injected with 50 nl of a cRNA solution containing 20 ng of HvTIP3;1 and/or 20 ng of HvTIP1;2, 20 ng of the HvTIP3;1 mutant and 20 ng of the HvTIP1;2 mutant. Water-injected oocytes were used as a negative control. The osmotic water permeability coefficient of oocytes was measured according to the procedures described previously (Katsuhara et al. 2002).

Bimolecular fluorescence complementation in onion epidermal cells

The coding regions of *HvTIP3;1* and *HvTIP1;2* were inserted separately into eYFP-N and eYFP-C expression vectors, which produced HvTIP1;2 and HvTIP3;1 fused with the N-terminal region and the C-terminal region of YFP, respectively (Kondo et al. 2013). The fusion constructs were introduced into onion epidermal cells using particle bombardment according to the procedure described by Utsugi et al. (2006). After an overnight incubation at 20°C, epidermises were peeled and observed under a confocal laser scanning microscope (FV1000, Olympus Co.). The green fluorescent protein (GFP)–AtVam3 expression vector (Uemura et al. 2002) was used as a marker localized on vacuolar membranes in cells.

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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