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Mechanisms of Water Transport Mediated by PIP Aquaporins and Their Regulation Via Phosphorylation Events Under Salinity Stress in Barley Roots

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Water homeostasis is crucial to the growth and survival of plants under water-related stress. Plasma membrane intrinsic proteins (PIPs) have been shown to be primary channels mediating water uptake in plant cells. Here we report the water transport activity and mechanisms for the regulation of barley (*Hordeum vulgare*) PIP aquaporins. HvPIP2 but not HvPIP1 channels were found to show robust water transport activity when expressed alone in *Xenopus laevis* oocytes. However, the co-expression of HvPIP1 with HvPIP2 in oocytes resulted in significant increases in activity compared with the expression of HvPIP2 alone, suggesting the participation of HvPIP1 in water transport together with HvPIP2 presumably through heteromerization. Severe salinity stress (200 mM NaCl) significantly reduced root hydraulic conductivity (Lp_r) and the accumulation of six of 10 *HvPIP* mRNAs. However, under relatively mild stress (100 mM NaCl), only a moderate reduction in Lp_r with no significant difference in *HvPIP* mRNA levels was observed. Sorbitol-mediated osmotic stress equivalent to 100 and 200 mM NaCl induced nearly identical Lp_r reductions in barley roots. Furthermore, the water transport activity in intact barley roots was suggested to require phosphorylation that is sensitive to a kinase inhibitor, staurosporine. HvPIP2s also showed water efflux activity in *Xenopus* oocytes, suggesting a potential ability to mediate water loss from cells under hypertonic conditions. Water transport via HvPIP aquaporins and the significance of reductions of Lp_r in barley plants during salinity stress are discussed.

Keywords: Aquaporin • Barley • Plasma membrane intrinsic proteins (PIPs) • Root hydraulic conductivity • Salinity stress • Water transport.

Abbreviations: DMSO, dimethylsulfoxide; Lp_r, root hydraulic conductivity; MBS, modified Barth's solution; MIP, major intrinsic protein; OA, okadaic acid; PBS, phosphate-buffered saline; P_f, osmotic water permeability coefficient; PIP, plasma membrane intrinsic protein; St, staurosporine.

Introduction

The acquisition and distribution of water are pivotal to the growth and development of plants. Water transport across cellular membranes relies largely on water channel activity mediated by aquaporins (Maurel et al. 2008). Aquaporins are pore-forming integral membrane proteins that belong to the major intrinsic protein (MIP) family (Forrest and Bhave 2007). More than 30 MIPs have been found in *Arabidopsis thaliana*, rice (*Oryza sativa*) and maize (*Zea mays*) (Chaumont et al. 2005, Sakurai et al. 2005). Plant MIPs are classified into five subfamilies, the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small intrinsic proteins (SIPs) and X intrinsic proteins (XIPs). The XIPs were identified recently in some plant species including moss (Danielson and Johanson 2008) and tomato (Sade et al. 2009). Several plant MIPs have been suggested or demonstrated to mediate the transport of small noncharged molecules in addition to water (Dordas et al. 2000, Tyerman et al. 2002, Gaspar et al. 2003, Uehlein et al. 2003, Hanba et al. 2004, Ma et al. 2006, Takano et al. 2006, Bienert et al. 2007, Dynowski et al. 2008, Ma et al. 2008). Plant PIPs can be further classified into two subgroups, PIP1 and PIP2, and have been reported to show substantial water transport activity when expressed in *Xenopus laevis* oocytes or yeast, particularly in the case of PIP2 channels (Chaumont et al. 2000). Together with the functional characterization of PIPs based on analyses of heterologous expression, results of physiological experiments showing that water uptake by roots is inhibited by mercury

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ions, shown to block the transport activity of many aquaporins, and changes in root hydraulic conductivity (Lp_r) in transgenic plants overexpressing *PIP* genes have led to the notion that aquaporins, especially PIPs, are the main pathway by which water is taken up from the soil into roots (Javot and Maurel 2002, Katsuhara et al. 2008).

Soil salinity is a major abiotic stress reducing the productivity of glycophytes including most agricultural products. Impairments of plant growth and productivity due to high concentrations of salts such as NaCl are caused by two primary factors, osmotic stress and ion toxicity (Munns and Tester 2008, Horie et al. 2009, Hauser and Horie 2010). Osmotic stress is the first stress that plants encounter in saline soil and has an immediate influence on the growth of plants under salinity stress, while ion toxicity occurs later when salt levels reach some threshold beyond which the plant cannot maintain ion homeostasis and growth (Munns and Tester 2008, Horie et al. 2009, Hauser and Horie 2010). High concentrations of salts in soil increase osmotic pressure and reduce water potential, which eventually cause a reduction in water uptake or loss of water. Furthermore, it has been reported that salinity stress induces a reduction in the Lp_r in many plant species (Azaizeh and Steudle 1991, Peyrano et al. 1997, Carvajal et al. 1999, Martinez-Ballesta et al. 2000, Martinez-Ballesta et al. 2003, Boursiac et al. 2005). Therefore, salinity stress rapidly affects water uptake in roots, and regulation of the activity of water channels in response to salinity stress is important to the viability of plants under salinity stress.

In barley (*Hordeum vulgare*), several *PIP* genes have been identified (Hollenbach and Dietz 1995, Katsuhara et al. 2002, Wei et al. 2007). The water channel activity of HvPIP1;3, HvPIP1;6 and HvPIP2;1 aquaporins has been characterized using *X. laevis* oocytes (Katsuhara et al. 2002, Katsuhara and Shibasaka 2007, Wei et al. 2007). Preliminary analyses of an expressed sequence tag (EST) database revealed that many other aquaporin genes including *PIP1* and *PIP2* exist in barley, forming a large gene family as found in other plant species (Katsuhara and Hanba 2008), raising the necessity to characterize new HvPIPs in order to understand the water transport mechanism mediated by HvPIP aquaporins in barley plants.

In this study, we present the mechanism of water transport mediated by HvPIP channels. Newly identified HvPIP1 proteins expressed in *Xenopus* oocytes showed little water transport activity, while HvPIP2 proteins showed robust activity. We show that hypertonic conditions including salinity stress trigger reductions of Lp_r in barley plants. Furthermore, we suggest that the water transport activity in intact roots requires phosphorylation by a staurosporine (St)-sensitive kinase. We also present evidence that HvPIP2 channels expressed in *Xenopus* oocytes mediate water efflux under hypertonic conditions, implying that the channels regulate water loss from cells under saline conditions, which could be a major target for down-regulation to reduce Lp_r in barley roots in response to salinity stress.

Results

Isolation of *HvPIP* cDNAs from barley roots

Several *HvPIP* cDNAs, named *HvEmip*, *HvPIP2;1*, *HvPIP1;3*, *HvPIP1;5* and *HvPIP1;6*, have been isolated previously (Hollenbach and Dietz 1995, Katsuhara et al. 2002, Wei et al. 2007). Aquaporin genes are known to form a multigene family in different plant species (Chaumont et al. 2005, Sakurai et al. 2005). We therefore searched for barley aquaporin genes in the database of HarvEST using the DNA sequence of the *HvPIP2;1* cDNA, which led to the identification of seven additional *HvPIP*-like sequence contigs (Katsuhara and Hanba 2008). Based on these sequences, putative full-length cDNAs were isolated from a cultivar of barley, Haruna-nijyo. The deduced amino acid sequences of the newly identified *HvPIP* cDNAs were found to retain six transmembrane domain regions and two NPA motifs highly conserved among aquaporins (data not shown) (Maurel et al. 2008). A phylogenetic analysis of amino acid sequences of the 10 HvPIP proteins in parallel with 11 amino acid sequences from rice (*O. sativa*) PIP proteins indicated that three of the seven newly isolated HvPIP proteins were PIP1 isoforms, while four were PIP2 isoforms (**Fig. 1**).

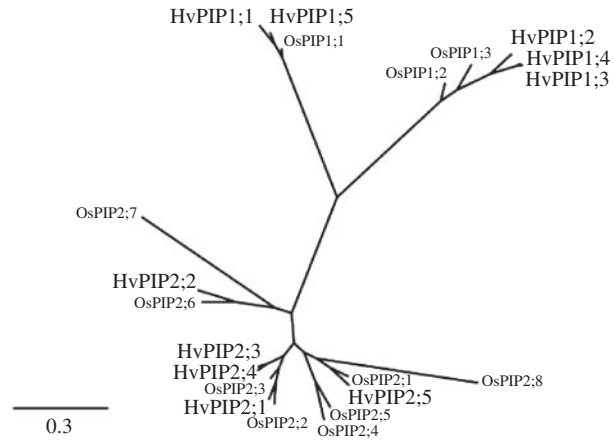


Fig. 1 A phylogenetic analysis of amino acid sequences of the plasma membrane intrinsic proteins (PIPs) from barley (*H. vulgare* cv. Haruna-nijyo) and rice (*O. sativa* cv. Nipponbare). HvPIP sequences were found to fall into two primary subfamilies as has been shown for PIP sequences from several plant species. All the HvPIP proteins identified were located on similar branches. Accession numbers for each sequence are: HvPIP1;1 (AB286964), HvPIP1;2 (AB275278), HvPIP1;3 (AB009308), HvPIP1;4 (AB275279), HvPIP1;5 (AB009309); HvPIP2;1 (AB009307), HvPIP2;2 (AB377269), HvPIP2;3 (AB275280), HvPIP2;4 (AB219525) and HvPIP2;5 (AB377270), respectively. Accession numbers for rice PIP sequences were from Sakurai et al. (2003). The tree was constructed at Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/index.cgi) (Dereeper et al. 2008). The scale bar indicates 0.3 substitutions per site.

Growth responses of barley plants and the accumulation of *HvPIP* mRNA in roots upon salinity stress

We profiled the response of the cultivar Haruna-nijyo to salinity stress imposed by 100, 200, 300 and 400 mM NaCl for 24 h. No remarkable impact on the growth of shoots or roots was observed at 100 mM, but severe growth inhibition was found at >200 mM NaCl (**Fig. 2**).

We next investigated the effects of high concentrations of NaCl on the accumulation of *HvPIP* mRNA in roots of barley, focusing particularly on the 100 and 200 mM NaCl treatments. Quantitative PCR analyses using roots of barley seedlings, with or without NaCl treatment, revealed that: (i) *HvPIP1;2*, *HvPIP1;3* and *HvPIP2;2* mRNA are the three most accumulated *HvPIP* mRNAs (**Fig. 3A**); (ii) 100 mM NaCl did not cause any significant difference in the accumulation of the 10 *HvPIP* mRNAs compared with the control (**Fig. 3A, B**); and (iii) the accumulation of *HvPIP1;2*, *HvPIP1;3*, *HvPIP1;4*, *HvPIP2;1*, *HvPIP2;2* and *HvPIP2;3* mRNAs was significantly reduced in response to 200 mM NaCl in the last 4 h of the treatment period (**Fig. 3A, C**). These results indicated that the accumulation of six out of 10 *HvPIP* mRNAs decreased under severe salinity stress where barley plants no longer cope with the stress to maintain growth.

Water transport mediated by HvPIP proteins

We performed water swelling assays using *Xenopus* oocytes to determine the water transport activity of each HvPIP channel. A 50 ng aliquot of *HvPIP* cRNA was injected into *Xenopus* oocytes and time-dependent increases in the volume of the oocytes were monitored in a half-strength modified Barth's solution (MBS). Changes in the relative volume of oocytes during the 3 min monitoring period are plotted in **Fig. 4**. All five HvPIP2 isoforms showed robust water transport activity in oocytes compared with the water-injected control (**Fig. 4**). In contrast, *HvPIP1*-expressing oocytes showed little increase in relative volume, with the exception of *HvPIP1;3*-expressing oocytes injected with 50 ng of *HvPIP1* cRNA (**Fig. 4**).

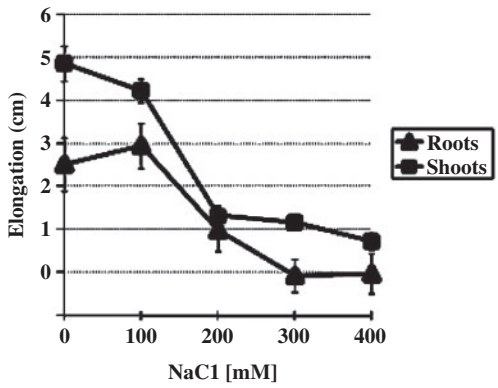


Fig. 2 Growth of the shoots and roots of barley (*H. vulgare* cv. Haruna-nijyo) exposed to high NaCl concentrations. NaCl at >200 mM but not 100 mM significantly inhibited the elongation of shoots and roots. Four-day-old seedlings were transferred to a hydroponic growth medium containing either 0, 100, 200, 300 or 400 mM NaCl and further grown for 24 h. The elongation of shoots and roots during this 24 h of stress was measured by subtracting the length of shoot and root samples at time 0 from those after 24 h. Error bars represent the standard deviations ($n = 8–10$).

A plant aquaporin from spinach has been demonstrated to form a tetramer (Törnroth-Horsefield et al. 2006). PIP1 isoforms from several plant species were reported to show little water transport activity themselves. Co-expression of *Z. mays* (Zm) PIP2 isoforms with several PIP1 isoforms has been shown to enhance net water transport activity through heteromerization of the two isoforms compared with the activity mediated by ZmPIP2 alone in *Xenopus* oocytes (Fetter et al. 2004). We therefore performed further water swelling assays to examine the role of HvPIP1 isoforms in water transport by co-expressing *HvPIP1* and *HvPIP2* in oocytes. We focused on HvPIP1;2, HvPIP1;3 and HvPIP1;4 channels as: (i) *HvPIP1;2* and *HvPIP1;3* mRNAs are the two most highly expressed of the 10 *HvPIP* mRNAs in roots of barley (**Fig. 3A**); and (ii) the accumulation of these three mRNAs was found to be decreased in response to 200 mM NaCl (**Fig. 3A, C**). When 10 ng of cRNA from each *HvPIP1* was injected into oocytes, no significant water transport activity was found compared with that in water-injected control oocytes (**Fig. 5**; see **Supplementary Fig. S1** for HvPIP1;4). When each *HvPIP2* was co-expressed with either *HvPIP1;2* or *HvPIP1;4*, there was a significant increase in the osmotic water permeability coefficient (P_f) compared with when each *HvPIP2* was expressed alone (**Fig. 5A**; see **Supplementary Fig. S1** for HvPIP1;4). Interestingly, however, co-expression of each *HvPIP2* with *HvPIP1;3* did not cause any significant enhancement of the water transport activity (**Fig. 5B**). Note that oocytes injected with <2 ng of cRNA show an increase in the P_f proportional to the amount of cRNA injected, indicating that the protein level of PIPs is properly regulated/controlled in such experiments (**Supplementary Fig. S2**). These results strongly suggested that HvPIP1;2 and HvPIP1;4, but not HvPIP1;3, mediate water transport in combination with HvPIP2 channels in barley roots.

Indirect immunofluorescence microscopy was performed using two kinds of peptide antibodies that recognize either all HvPIP1 proteins or HvPIP2;2 specifically in order to determine the tissue-specific expression of these proteins in roots of barley. Immunolabeling of HvPIP1 and HvPIP2;2 was detected through hybridization of red fluorescent Alexa 647-conjugated anti-rat IgG goat antibody and green fluorescent Alexa 488-conjugated anti-rabbit IgG goat antibody, respectively, using appropriate filter sets. Strong red fluorescence derived from HvPIP1 proteins was found in the vicinity of the xylem and a more moderate expression was also detected in the cortex layer (**Fig. 6A, B**). Strong HvPIP2;2-derived green fluorescence was found in the epidermis, especially in cells developing a root hair (**Fig. 6A, C**). The fluorescence signal was also detected in the stele (**Fig. 6A, C**). Some background green fluorescence was detected on the xylem vessels, but not on

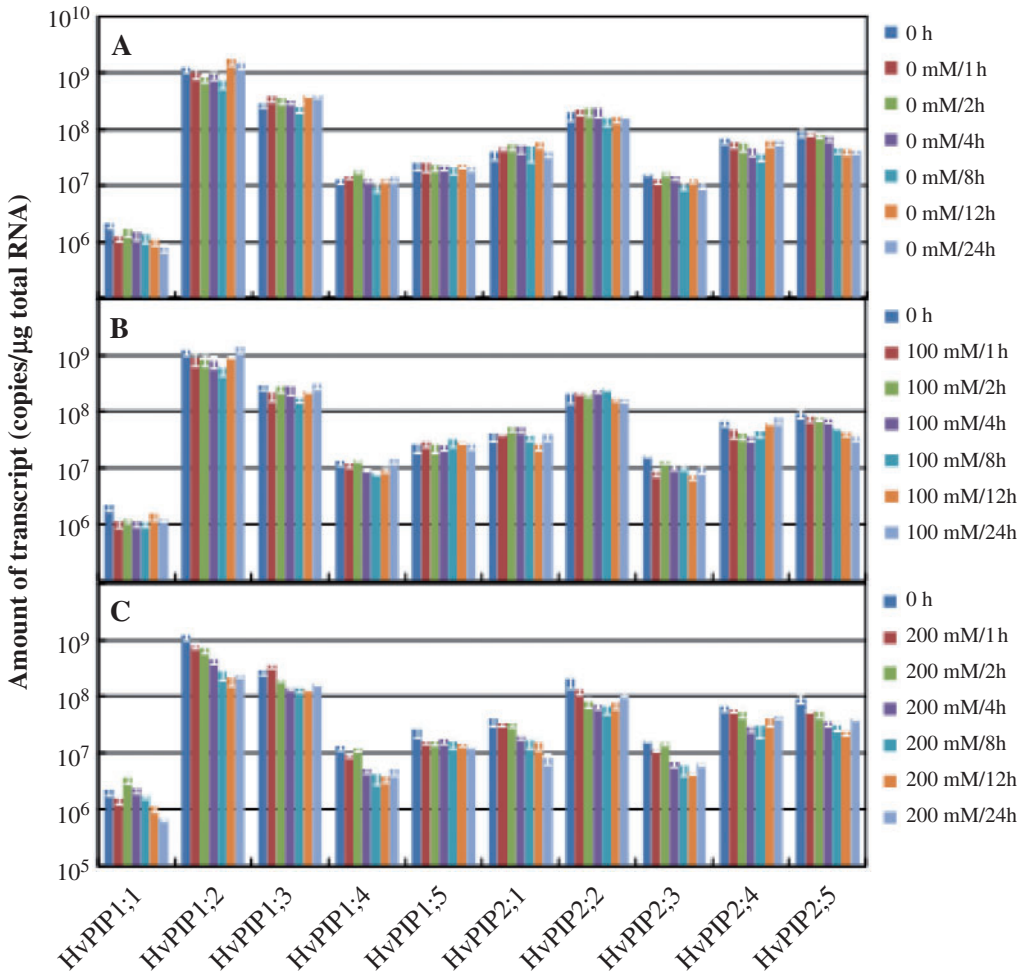


Fig. 3 Severe NaCl stress, but not relatively mild NaCl stress, decreased the accumulation of six out of 10 *HvPIP* mRNAs in barley roots. Four-day-old barley seedlings were transferred to a hydroponic growth medium containing either 0, 100 or 200 mM NaCl for 0, 1, 2, 4, 8, 12 and 24 h, and total RNA was extracted from roots. The level of each *HvPIP* mRNA was quantified by Q-PCR. Transcript levels were represented as copy numbers per µg of total RNA. Data are presented as mean values from at least three independent experiments and the error bars represent standard deviations. (A) Control (0 mM NaCl); (B) 100 mM NaCl; (C) 200 mM NaCl.

other stellar cells, as shown in **Fig. 6F**. However, the specific signals in the root stellar cells from anti-HvPIP1 and anti-HvPIP2;2 antibodies were stronger than the background levels (**Supplementary Fig. S3**). When the two images were merged, strong yellow fluorescence was revealed in the root stele (**Fig. 6A, D**), indicating that HvPIP2;2 and some or all HvPIP1 proteins were expressed in the same type of cell in the stele of barley roots.

Regulation of Lp_r in intact barley roots

To characterize the Lp_r of barley roots in response to salinity stress, we employed the pressure chamber method (Tournaire-Roux et al. 2003, Boursiac et al. 2005). Xylem sap samples were collected from barley plants exposed to either 0, 100 or 200 mM NaCl for 4 h. The average pressure–flow relationships exhibited reductions of sap flow due to salinity stress in comparison with the control (**Fig. 7A**). Comparisons of Lp_r also indicated that 100 mM NaCl caused moderate reductions ($P > 0.05$; statistically insignificant), while 200 mM NaCl induced more significant reductions ($P < 0.001$; **Fig. 7B**). The deleterious effects of salinity stress are caused by two independent factors, osmotic stress and ion toxicity (Munns and Tester 2008, Horie et al. 2009, Hauser and Horie 2010). To investigate whether the salinity-induced reductions in Lp_r are relevant to the osmotic stress phase, osmotic conditions equivalent to 100 and 200 mM NaCl were established using sorbitol. Barley plants were exposed to 177 and 354 mM sorbitol solutions for 4 h and Lp_r was measured using a pressure chamber. Both sorbitol solutions evoked nearly identical reductions in Lp_r to the corresponding NaCl treatments (**Fig. 7B**). These results indicated that the water transport in barley roots is down-regulated in response to salinity stress, particularly to the osmotic stress phase.

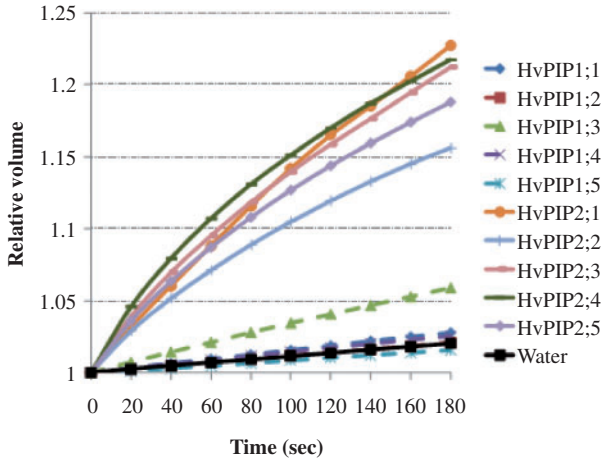


Fig. 4 The water transport activity of HvPIPs when expressed alone in *Xenopus* oocytes. HvPIP2 isoforms show robust water transport activity, while HvPIP1 isoforms show weak activity. Oocytes injected with either water or 50 ng of each *HvPIP* cRNA were incubated in an MBS for approximately 24 h at 18°C. Changes in the volume of each oocyte were monitored for 3 min after transfer of the oocyte to a hypotonic solution (half-strength MBS). Results are shown as mean values relative to the original volume at time 0 ($n = 7–9$).

We further measured Lp_r to investigate whether phosphorylation events participated in the regulation, focusing on the 100 mM NaCl treatment where no significant difference in the accumulation of *HvPIP* mRNA was found (**Fig. 3A**). Barley plants were treated with a kinase inhibitor, either K252a or St, or the phosphatase inhibitor okadaic acid (OA) for 4 h in the presence of 100 mM NaCl prior to the Lp_r measurements. Interestingly, St-treated plants, but neither K252a- nor OA-treated plants, showed significant reductions in Lp_r in the presence of 100 mM NaCl ($P < 0.01$; **Fig. 8A**). Based on these results we further tested the effect of St on the Lp_r of barley plants in the absence of NaCl. The plants were exposed to either 0, 0.1, 1.0 or 10 μM St for 4 h without NaCl prior to the Lp_r measurements. An approximately 43.6% reduction in the average Lp_r was found in barley roots in response to 0.1 μM St ($P < 0.02$; **Fig. 8B**). Ten-fold increases in the concentration of St led to dramatic reductions in the average Lp_r of barley plants, approximately 87.0 and 90.7% reductions at 1.0 and 10.0 μM St, respectively, compared with the control condition ($P < 0.01$ for both St concentrations; **Fig. 8B**). Note that a quick recovery of Lp_r was observed after removal of St (**Supplementary Fig. S4**), indicating that St did not fatally/non-specifically damage barley roots. These results further demonstrate that the Lp_r of barley roots is controlled by phosphorylation.

To see whether the activity of HvPIP2 water channels is influenced by phosphorylation in *Xenopus* oocytes, the effect of K252a and St was investigated. Among the oocytes expressing each *HvPIP2* isoform, there was no significant difference in P_f between inhibitor-treated and untreated oocytes (**Supplementary Table S1**). We further examined the effect of St on the water transport activity of HvPIP2;1 co-expressed with HvPIP1;2 in oocytes, but found no significant change in P_f (**Supplementary Fig. S5**).

HvPIP2 channels mediate water efflux under hypertonic conditions in *X. laevis* oocytes

We investigated whether HvPIP2 isoforms have the potential to mediate water efflux under hypertonic conditions such as in the presence of a large amount of NaCl. *HvPIP2*-expressing *Xenopus* oocytes were exposed to a hypertonic MBS containing an extra 100 mM NaCl, and reductions in volume (shrinking) due to water loss were monitored. Interestingly, the oocytes expressing an *HvPIP2* channel showed more robust reductions in volume than water-injected oocytes in response to the hypertonic stress caused by NaCl (**Fig. 9A**). Quantitative comparisons of the water efflux activity using P_f values further revealed that *HvPIP2*-expressing oocytes experienced more extensive water loss than control oocytes (**Fig. 9B**). These results demonstrated that HvPIP2 channels can mediate the efflux in addition to influx of water depending on the osmotic conditions.

Discussion

Water homeostasis in plants is crucial not only for growth and development but also for adaptive responses to osmotic stress caused by major abiotic factors such as salinity and drought. Water uptake by roots is an important step in the regulation of water homeostasis. Genetic evidence for the role of PIP aquaporins in the uptake of water by roots has been provided by the knockdown of *PIP* expression in tobacco and Arabidopsis plants using antisense constructs and knockout of the *PIP2;2* gene in Arabidopsis using T-DNA insertions, both of which led to significant reductions in Lp_r (Martre et al. 2002, Siefritz et al. 2002, Javot et al. 2003).

Elevated salt concentrations have been shown to inhibit water uptake in roots due to a reduction in Lp_r in several plant species including Arabidopsis and maize (Azaizeh and Steudle 1991, Peyrano et al. 1997, Carvajal et al. 1999, Martinez-Ballesta et al. 2000, Martinez-Ballesta et al. 2003, Boursiac et al. 2005). However, a previous physiological study reported that the hydraulic resistance of salinity stress-treated barley plants was relatively constant and similar to that of non-stressed control plants (Munns and Passioura 1984). These studies motivated us to investigate mechanisms of water transport via HvPIP aquaporins and regulation of the water channel activity in barley roots under salinity stress.

Plants first encounter osmotic stress in saline conditions, which leads to a reduction in water uptake or triggers water efflux depending on the extent of the stress. Our findings suggest that HvPIP2 channels predominantly function in water uptake in roots of barley plants (**Figs. 4**, **6**) via an activation process involving phosphorylation (**Fig. 8**), and that salinity

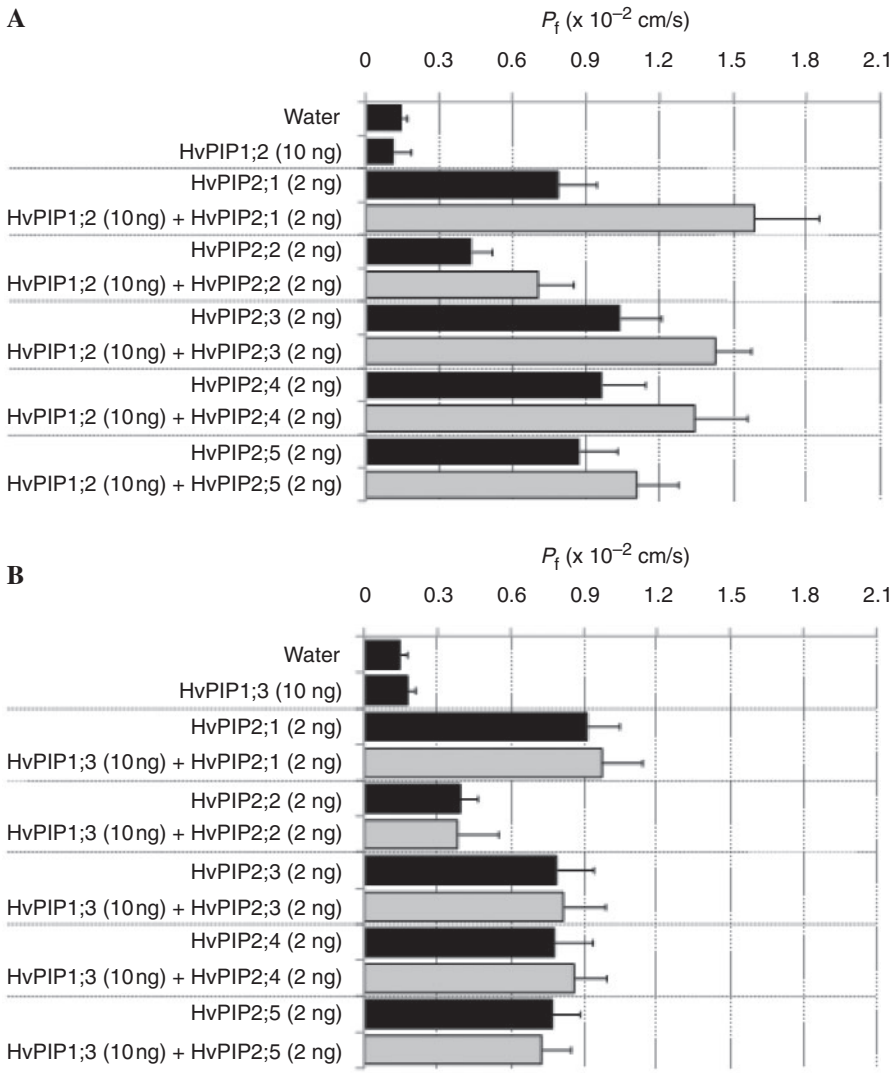


Fig. 5 Co-expression of each HvPIP2 isoform with HvPIP1;2 but not HvPIP1;3 leads to significant increases in the osmotic water permeability coefficient (P_f) of *Xenopus* oocytes. (A) Quantitative comparisons of the P_f values derived from oocytes injected with water, 10 ng of *HvPIP1;2* cRNA, 2 ng of each *HvPIP2* cRNA and 10 ng of *HvPIP1;2* cRNA together with 2 ng of each *HvPIP2* cRNA. Co-expression resulted in significant increases in the P_f of oocytes ($P < 0.001$) compared with the oocytes expressing each *HvPIP2* isoform alone ($n = 7–6$; means ± SD). (B) Quantitative comparisons of the P_f values derived from oocytes injected with water, 10 ng of *HvPIP1;3* cRNA, 2 ng of each *HvPIP2* cRNA and 10 ng of *HvPIP1;3* cRNA together with 2 ng of each *HvPIP2* cRNA. In contrast to the results shown in A, *HvPIP1;3* did not lead to a significant enhancement of the water transport activity of HvPIP2 ($n = 10–12$; means ± SD). Note that HvPIP1;3 showed weak water transport activity when 50 ng of cRNA was injected into oocytes (see **Fig. 4**).

stress causes reductions in the Lp_r, particularly severe stress due to 200 mM NaCl (**Figs. 2**, **7**), similar to what occurs in other plant species. Four-day-old plants treated with either 100 or 200 mM NaCl for 4 h were used to measure Lp_r in the present study, whereas more mature plants (20 d old) were subjected to salt stress (25–200 mM) by Munns and Passioura (1984). Therefore, it is hard to make a direct comparison between the two studies. However, the severe repression of Lp_r in response to 200 mM NaCl was reproduced in independent experiments. Note that extensive nuclear degradation due to cell death was triggered in barley roots by treatment with >300 mM NaCl for 24 h, but not by 200 mM NaCl (Katsuhara and Kawasaki 1996). These results indicate that a rigid mechanism controlling the activity of root water channels to adapt to saline conditions exists at least in the seedlings of barley plants as in other plant species. Compared with the studies of a relatively long-term (>1 d) effect of salt stress on barley plants by Munns and Passioura (1984), our studies focused on initial responses (within hours) of barley roots to salt/osmotic stress. Both short- and long-term responses should be important to fully elucidate stress-tolerant mechanisms in plants.

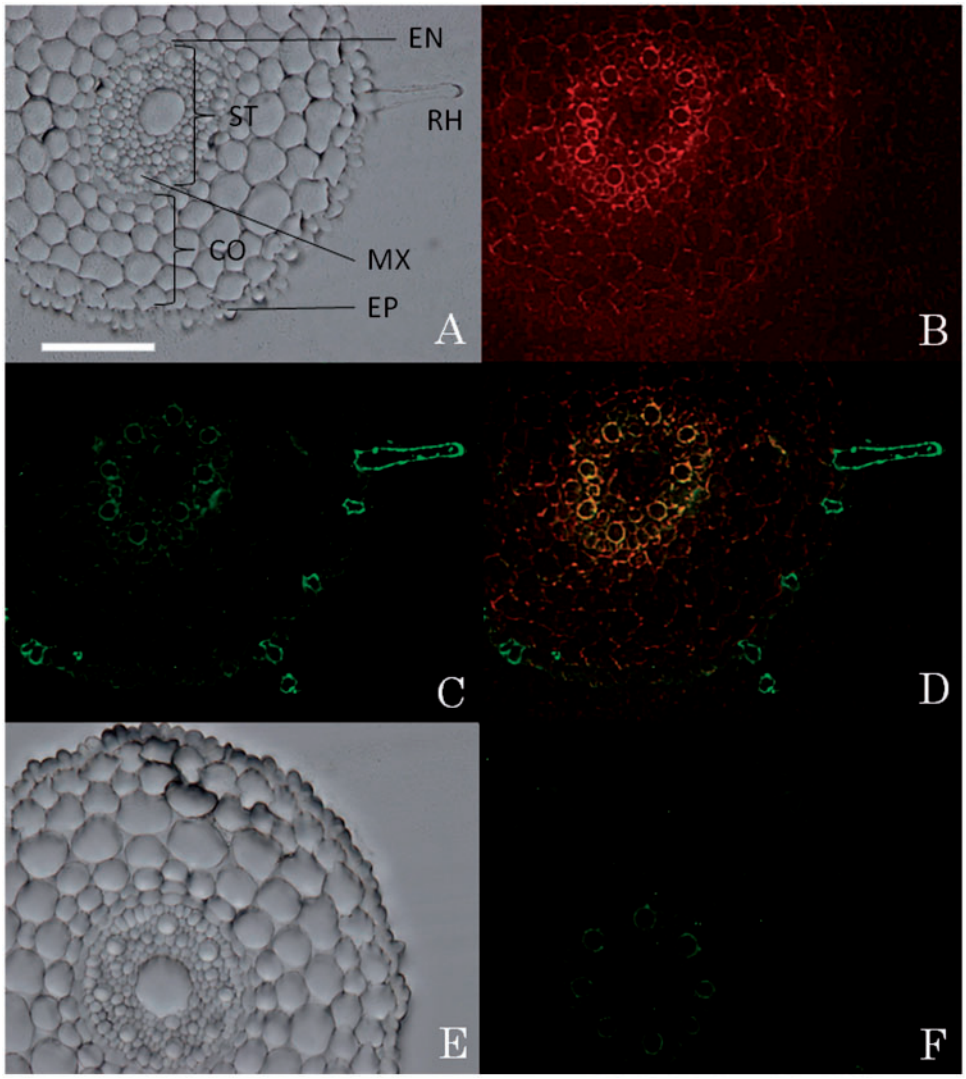


Fig. 6 In situ localization of HvPIP aquaporins in barley primary roots. A cross-section was prepared approximately 50 mm from the tip of 5-day-old seedlings. The section was hybridized with anit-HvPIP1 rat IgG (antibody) and anti-HvPIP2;2 rabbit IgG (antibody) (A–D). The negative control section was treated with non-immune rat serum plus non-immune rabbit serum (E, F). Sections were then visualized with red fluorescent Alexa 647-conjugated anti-rat IgG goat antibody and green fluorescent Alexa 488-conjugated anti-rabbit IgG goat antibody. (A) A bright field image. EP, epidermis; RH, root hair; CO, cortex; ST, stele; EN, endodermis; MX, metaxylem. The scale bar represents 100 µm. Note that the scale bar is omitted in other panels as all micrographs were recorded at the same magnification. (B) A red fluorescence image of the section in A. (C) A green fluorescence image of the section in A. (D) Overlay of B and C indicating the co-expression of HvPIP1 proteins and the HvPIP2;2 protein in the stele. (E) A bright field image of the control section. (F) Overlay of green and red background fluorescence.

The molecular mechanism of water transport mediated by HvPIP channels

PIP aquaporins are considered primary water channels functioning at the plasma membrane of plant cells (Martre et al. 2002, Siefritz et al. 2002, Javot et al. 2003). The water uptake activity of PIP aquaporins from different plant species has been investigated using *Xenopus* oocytes and yeast cells (Biela et al. 1999, Katsuhara et al. 2002, Tournaire-Roux et al. 2003, Fetter et al. 2004, Sakurai et al. 2005, Wei et al. 2007, Mahdieh et al. 2008, Matsumoto et al. 2009). PIP2 isoforms were shown to mediate robust water uptake, in contrast to PIP1 isoforms when the channels were expressed alone in heterologous cells. A similar tendency can be found in the water transport activity of HvPIP proteins. *HvPIP2*-expressing oocytes exhibited substantial water transport activity, a marked contrast to *HvPIP1*-expressing oocytes, in which little water transport activity was observed except in the case of HvPIP1;3 (**Fig. 4**). Note, however, that several plant PIP1 proteins were reported to show water transport activity (Biela et al. 1999, Tournaire-Roux et al. 2003, Wei et al. 2007, Mahdieh et al. 2008). In addition to the expression of HvPIP2;1 in the epidermis in barley

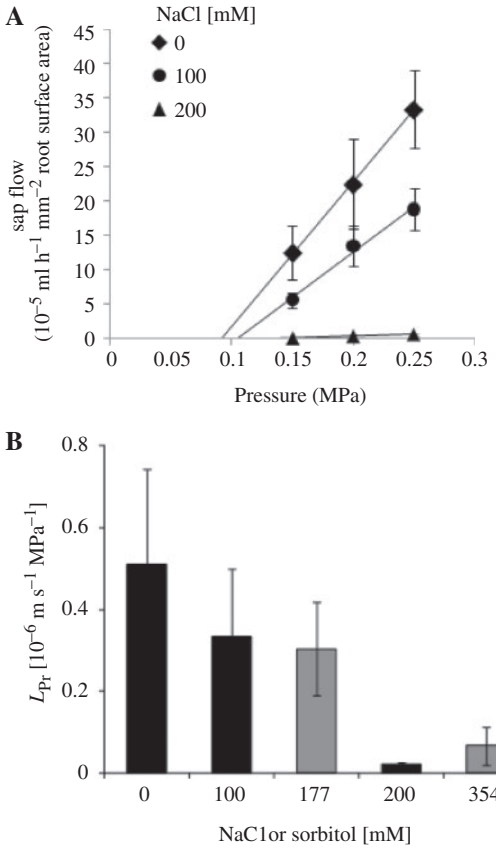
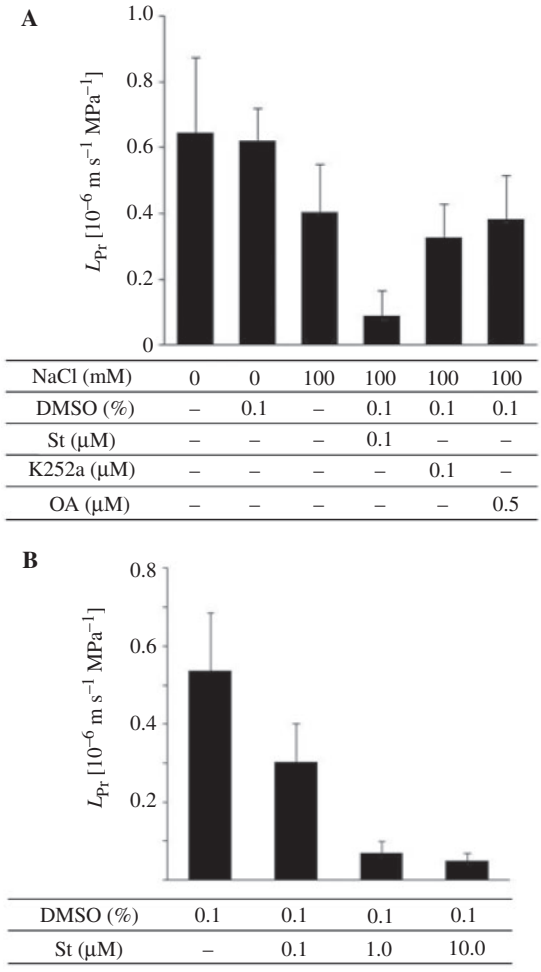


Fig. 7 The root hydraulic conductance (Lp_r) of barley plants under salinity stress. Permeability to water was measured with a pressure chamber using 4-day-old plants treated with (100 or 200 mM) or without NaCl for 4 h. (A) Average pressure–flow relationships, derived from barley plants in the absence or presence of NaCl ($n = 5–8$; means ± SE). (B) The Lp_r of plants exposed to high concentrations of NaCl or sorbitol. Lp_r values were calculated from pressure–flow relationships ($n = 5–8$; means ± SD). In contrast to the moderate reduction in response to 100 mM NaCl ($P > 0.05$), the 200 mM NaCl treatment led to substantial reductions in Lp_r ($P < 0.001$). Equivalent osmotic stress caused by sorbitol led to Lp_r reductions almost identical to those from each NaCl treatment. Black and gray bars represent results for barley plants treated with NaCl and sorbitol, respectively.



NaCl (mM)	0	0	100	100	100	100
DMSO (%)	–	0.1	–	0.1	0.1	0.1
St (μM)	–	–	–	0.1	–	–
K252a (μM)	–	–	–	–	0.1	–
OA (μM)	–	–	–	–	–	0.5

DMSO (%)	0.1	0.1	0.1	0.1
St (μM)	–	0.1	1.0	10.0

Fig. 8 Phosphorylation is an essential factor controlling water influx into barley roots. (A) Pharmacological analyses on the Lp_r of barley plants treated with 100 mM NaCl for 4 h with or without a kinase inhibitor, K252a (0.1 μM) or staurosporine (St; 0.1 μM), or a phosphatase inhibitor, okadaic acid (OA; 0.5 μM). Only the treatment with St significantly enhanced the NaCl-induced reduction in Lp_r ($P < 0.01$; $n = 5–9$; means ± SD). (B) Dose-dependent reductions in Lp_r in response to St. Four-day-old barley plants were treated with either 0, 0.1, 1.0 or 10 μM St for 4 h and Lp_r was measured by the pressure chamber method. Significant reductions in Lp_r were found in the 0.1 μM St-treated plants ($P < 0.02$; vs. DMSO-treated plants). Increasing the concentration of St further evoked substantial reductions in Lp_r ($P < 0.01$; $n = 5$; means ± SD).

roots (Katsuhara et al. 2003), HvPIP2;2, with the highest mRNA level among *HvPIP2* gene products in barley roots (**Fig. 3**), was predominantly located in epidermis, especially in root hair, and stelar cells (**Fig. 6A, C**). Together, these results indicate that HvPIP2 proteins form active water channels, and HvPIP2;2 as well as HvPIP2;1 (Katsuhara et al. 2003) mediates water uptake from the outer environment in roots of barley.

Plant aquaporins have been proposed to form a tetramer (Törnroth-Horsefield et al. 2006). Heteromerization of PIP1 and PIP2 aquaporins was further proposed in maze (Fetter et al. 2004, Zelazny et al. 2007). Co-expression of ZmPIP1;2 with either ZmPIP2;1, ZmPIP2;4, ZmPIP2;5 or even AtPIP2;3 was found to trigger significant increases in the P_f of the oocytes compared with the expression of each *PIP2* alone (Fetter et al. 2004). Co-expression of PIP1s and PIP2s was also investigated in *Mimosa* (Temmei et al. 2005), grapevine (Vandeleur et al. 2009), tobacco (Mahdieh et al. 2008) and beet (Bellati et al. 2010). Co-expression of HvPIP1;2 or HvPIP1;4 with each HvPIP2 isoform significantly increased the P_f of the oocytes (**Fig. 5A** for HvPIP1;2 and **Supplementary Fig. S1** for HvPIP1;4) despite the fact that neither HvPIP1;2 nor HvPIP1;4 shows water transport activity when expressed alone in oocytes (**Fig. 4**). HvPIP1 proteins were found to be expressed mostly in

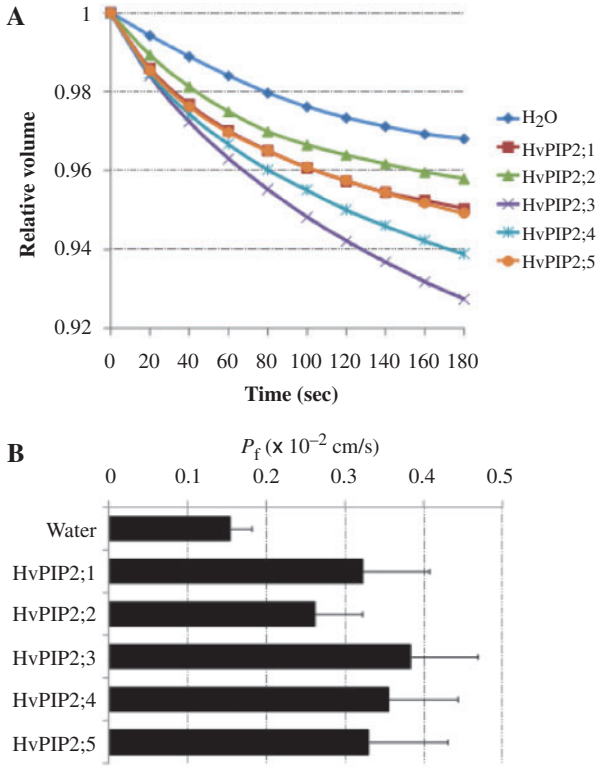


Fig. 9 HvPIP2 isoforms mediate water efflux under hypertonic conditions in *Xenopus* oocytes. (A) Relative changes in the volume of oocytes injected with either water or 50 ng of each *HvPIP2* cRNA in an MBS containing an extra 100 mM NaCl. Reductions in the volume of each oocyte (thus shrinking) were monitored for 3 min. Results are shown as mean values relative to the original volume at time 0 ($n = 8–12$). (B) Quantitative comparisons of the HvPIP2-mediated water efflux activity using a water permeability coefficient (P_f) obtained from shrinking assays shown in A. Expression of *HvPIP2* resulted in significant increases in the P_f ($P < 0.001$), representing water efflux activity, compared with water-injected oocytes ($n = 8–12$; means ± SD).

stelar cells, with moderate expression in the cortex cell layer as well (**Fig. 6A, B**). The expression of HvPIP1 in stelar cells overlapped that of HvPIP2;2 well (**Fig. 6A, D**). Taken together, these results suggested that HvPIP1 channels can also be employed in the water transport mechanism of barley roots by cooperatively functioning with HvPIP2 proteins presumably via heteromerization. Recent work also indicated a functional PIP1 in water transport (Postaire et al., 2010).

Note, however, that HvPIP1;3, which shows a high degree of similarity to HvPIP1;2 and HvPIP1;4 (**Fig. 1**), was found not to enhance water transport on co-expression with each PIP2 isoform (**Fig. 5B**). These results indicate that the co-localization of HvPIP1 and HvPIP2 in the same cell is not the only factor determining the enhancement of water transport. Elucidating the underlying mechanism including heteromerization of PIP1 and PIP2 isoforms will be important for understanding the water transport mechanism via PIP channels in planta.

Regulation of water channel activity in barley roots under salinity stress

The Lp_r of barley plants was found to be reduced upon osmotic stress established by NaCl or sorbitol (**Fig. 7**), indicating that the activity of water channels in the roots declined in response to salinity stress particularly due to the osmotic stress phase. The level of *HvPIP* mRNA was relatively stable in the culture with 100 mM NaCl compared with the control (**Fig. 3**). Interestingly, 0.1 μM St greatly reduced the Lp_r of barley plants treated with 100 mM NaCl (**Fig. 8A**). Together with the finding that the Lp_r values of non-stressed plants were significantly reduced in response to the external St (**Fig. 8B**), these results indicate that phosphorylation events control the salinity stress-induced reduction in Lp_r through the regulation of water transport activity and that phosphorylation via the St-sensitive kinase(s) is required to activate water transport in barley roots. The mechanism of salinity-induced Lp_r reductions has been well characterized in Arabidopsis (Boursiac et al. 2005, Boursiac et al. 2008a). It has recently been demonstrated that salicylic acid, like salinity stress, induces the reduction of Lp_r in Arabidopsis due to its ability to stimulate the accumulation of hydrogen peroxide (H_2O_2) (Boursiac et al. 2008a). Salicylic acid- and salinity-induced H_2O_2 accumulation was further found to invoke the internalization of AtPIP aquaporins with surrounding membranes, which in turn reduces the Lp_r of Arabidopsis plants (Boursiac et al. 2008a, Boursiac et al. 2008b). Whether these mechanisms are key factors and cooperatively function with phosphorylation events in controlling salinity-induced Lp_r reductions in barley plants will need to be addressed.

The phosphorylation-mediated reduction in Lp_r caused by salinity could be attributed to the direct regulation of HvPIP channels in barley roots. The water transport activity of several plant aquaporins has been found to be regulated by direct phosphorylation (Maurel et al. 1995, Johansson et al. 1998, Guenther et al. 2003, Wei et al. 2007, Azad et al. 2008, Van Wilder et al. 2008). We therefore tested whether the water transport activity of HvPIP2 channels is down-regulated by treatment with phosphorylation inhibitors in *Xenopus* oocytes. However, each inhibitor treatment had no significant influence on the water channel activity of HvPIP2 in *Xenopus* oocytes (**Supplementary Table S1** and **Supplementary Fig. S5**). It is possible that the water transport activity of HvPIP channels in intact roots could be blocked by an unknown regulatory mechanism (e.g. the binding of an unknown/putative regulator protein), which might be turned off upon phosphorylation in barley plants but not in *Xenopus* oocytes. Further investigation will be required to elucidate whether the water transport activity of HvPIP channels is regulated by direct phosphorylation in vivo.

Bidirectional water transport mediated by aquaporins

Bidirectional water transport has been demonstrated to be mediated by animal aquaporins, AQP0, 1, 2, 3, 4 and 5, in

X. laevis oocytes (Meinild et al. 1998). To test whether HvPIP aquaporins also regulate bidirectional water transport, we investigated the water efflux activity of HvPIP2 channels using oocytes. Although the difficulty in monitoring the shrinkage of oocytes exposed to hypertonic gradients in comparison with general swelling assays using oocytes had been pointed out (Zhang and Verkman 1991, Meinild et al. 1998), we attempted to examine the shrinkage of *HvPIP2*-expressing oocytes exposed to a hypertonic MBS including an extra 100 mM NaCl. Interestingly, all five HvPIP2 isoforms expressed in oocytes triggered a more rapid water loss than that in water-injected oocytes (**Fig. 9**), demonstrating that HvPIP2 channels mediate bidirectional water transport and thus water efflux in hypertonic conditions. Note that the water efflux activity of HvPIP2 channels is much lower than the water influx activity according to the P_f values. We assume that volume changes (and thus P_f) in oocyte-shrinking assays underestimate the actual water efflux activity of HvPIP2 channels because of potential technical limitations. Upon water loss (shrinking) due to hypertonic conditions, changes in the surface area of oocytes tend to be smaller than those in swelling assays presumably because of resistance to shrinkage by cellular components of oocytes. Furthermore, we observed that the shrinkage triggered by hypertonic conditions occurs only in limited areas, which results in small changes even if the partial shrinkage is extensive. In spite of this, however, our attempt enabled us to detect and analyze the water efflux activity of HvPIP2 channels. Note that the P_f values obtained from shrinking assays showed significant differences compared with those in water-injected control oocytes (**Fig. 9B**; $P < 0.001$), indicating that the approach used to analyze water efflux activity is semi-quantitative and can provide an index of such activity.

The data presented here indicated that water transport activity is down-regulated in barley roots under salinity stress, as has been demonstrated in many plant species (Azaizeh and Steudle 1991, Peyrano et al. 1997, Carvajal et al. 1999, Martinez-Ballesta et al. 2000, Martinez-Ballesta et al. 2003, Boursiac et al. 2005). Considering the biological significance of such a mechanism, the following possibility can be considered: prevention of excessive water loss from cells during the early stage (the osmotic stress phase) of salinity stress. A CAM plant, *Graptopetalum paraguayense*, has been found to retain far less aquaporin in the plasma membrane and tonoplast than do radish plants (Ohshima et al. 2001). *Graptopetalum* protoplasts were further shown to exhibit significantly lower water permeability than radish protoplasts, which led to a hypothesis that the suppression of aquaporin level/activity might contribute to the higher water storage feature of succulent leaves of *Graptopetalum* plants (Ohshima et al. 2001). The possibility of the down-regulation of plant aquaporins at lowered apoplastic water potentials to prevent water loss has been discussed elsewhere (Kjellbom et al. 1999, del Martínez-Ballesta et al. 2006, Hachez et al. 2006). In addition to the prevention of water loss, Lp_r reductions might represent the mode of change of barley plants from the rapid growth stage with high water absorption to the protected/tolerant stage with less water uptake. Such reversible Lp_r reductions have also been reported in some plants tolerating nutrition (especially phosphate) deprivation (Maurel et al. 2008), indicating that low Lp_r might be common in the protected/tolerant stage of plants.

More investigations are required to better understand the functions and significance of the regulation of aquaporins in physiological processes under hypertonic stress conditions brought about by major environmental stresses such as salinity and drought in planta.

Materials and Methods

Plant materials and growth conditions

The seeds of barley (*H. vulgare*) cv. Haruna-nijyo were sterilized and germinated in the dark as described previously (Katsuhara et al. 2002).

Plants were treated with salinity stress by adding appropriate amounts of NaCl to the hydroponic medium for either: (i) 24 h for the shoot/root elongation analysis; (ii) 1, 2, 4, 8, 12 and 24 h for the *HvPIP* gene expression analysis; or (iii) 4 h for the measurement of Lp_r. Note that for inhibitor treatments, 4-day-old plants were transferred from a pot to 15 ml centrifuge tubes filled with the same hydroponic nutrient solution with or without NaCl and incubated in the presence of each inhibitor for 4 h. Dimethylsulfoxide (DMSO) was used as a solvent for all inhibitors.

Extraction of RNA and quantitative PCR

Root samples were collected after NaCl treatments and immediately frozen in liquid nitrogen. Total RNA was extracted using a mortar and pestle and the RNeasy Plant Mini Kit (Qiagen).

HvPIP expression was analyzed by the quantitative real-time reverse transcription–PCR (RT–PCR) technique using gene-specific primers (**Supplementary Table S2**), and absolute quantification was performed as described previously (Mahdieh et al. 2008).

HvPIP expression in *Xenopus* oocytes

For the expression of *HvPIP2;1* and *HvPIP1;3*, published DNA constructs were used (Katsuhara et al. 2002, Katsuhara and Shibasaka 2007). All the other *HvPIP* cDNAs were excised out by appropriate restriction enzymes and both 5′ and 3′ ends were filled in using the KOD polymerase to make blunt-ended forms (TOYOBO). The resultant fragments were subcloned into the blunt-ended *Bgl*II site of pXβG-ev1. Linearized plasmid constructs were then used for cRNA synthesis with the mMESSEGE mMACHINE T3 in vitro transcription Kit (Ambion).

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 cRNA solution containing 2, 10 or 50 ng of each *HvPIP* cRNA for the expression of HvPIP channels. For the co-expression analysis, 50 nl of a

cRNA solution containing 2 ng of each *HvPIP2* and 10 ng of each *HvPIP1* was injected. As a negative control, water-injected oocytes were used in all experiments. Injected oocytes were incubated in an MBS for approximately 24 h at 18°C. The oocytes were transferred from the MBS (200 mOsm) to either a 2-fold diluted MBS (100 mOsm) for water influx (swelling) assays or an MBS with 100 mM NaCl added (400 mOsm) for water efflux (shrinking) assays. Changes in cell volume were recorded and P_f values were calculated as described previously (Mahdieh et al. 2008).

In water influx (swelling) assays using kinase inhibitors, injected oocytes were incubated in an MBS supplemented with 1 μM K252a (Calbiochem) or 1 or 10 μM St (Calbiochem) for 30 min prior to the swelling assays. DMSO was used as a solvent for both inhibitors and the final concentration of DMSO in inhibitor-containing and control DMSO solutions was 0.1%.

Indirect immunofluorescence microscopy

Root samples (3–5 cm from the root tip) were fixed in a 4% (w/v) formaldehyde solution (pH 7.4) supplemented with 20 mM cacodylic acid sodium salt (Nacalin) and 60 mM sucrose for 4 h at 4°C. Fixed samples were washed three times with the washing solution (20 mM cacodylic acid sodium salt and 60 mM sucrose) at 4°C. Samples were embedded in Technovit 8100 (Kulzer) supplemented with 5% (v/v) butoxyethanol. Micro-sliced samples 20 μm thick were surrounded with a thin hydrophobic barrier using PAP PEN (Dido Sangyo) and treated with 0.1% (w/v) pectolyase Y-23 (Kyowa Chemical) in PBS-T [phosphate-buffered saline, 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, supplemented with 0.1% Tween-20 (Nacali)] for 1 h at 25°C in a moisture chamber. After washing with PBS and blocking with 5% skim milk (Becton Dickinson) in PBS-T for 1 h, rat antibody against HvPIP1s (generated against the synthetic peptide MEGKEEDVRLGANRY, Medial & Biological Laboratories Co.) and anti-HvPIP2;2 rabbit antibody (generated against the synthetic peptide VSEEPEHAAPAR, Medial & Biological Laboratories Co.) were applied to samples. For a negative control, non-immune rat serum plus non-immune rabbit serum was applied. Primary antibodies or non-immune sera were reacted for 1 h at 25°C, and then secondary antibodies [anti-rat IgG goat antibody conjugated with Alexa 647 and anti-rabbit IgG goat antibody conjugated with Alexa 488 (Invitrogen)] for another 1 h. After washing twice with the blocking solution and once with PBS, samples were analyzed with a fluorescence microscope (BZ-8000, Keyence). For the quantitative fluorescence analysis, images were converted into monochrome images. Fluorescence intensity was measured with the lines indicated in images using WinRoof software (ver. 3.51, MITANI Corporation, 2000).

Measurements of Lp_r

The stem was fixed on a metal plant holder and the gap between the holder and sample was filled with dental paste (PROVIL® novo Light; Heraeus Kulzer). The fixed sample was set in a pressure chamber filled with either the nutrient solution or the solution supplemented with NaCl. Pressure (P) was applied to the chamber using an air compressor (Super Oil Free Bebicom; Hitachi, Ltd.) and exuded sap was collected into a glass tube at each pressure point. The sap flow rate of individual samples (J) was determined by measuring sap weight per unit time. The surface area of the root of individual samples (A) was measured using the WinRHIZO system (Regent Instruments Inc.). J/A was plotted against P, and the regression line was determined between $P = 0.15$ MPa and $P = 0.25$ MPa. Lp_r was calculated as the slope of the regression line.

Supplementary data

Supplementary data are available at PCP online.

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