

CO₂ Transport by PIP2 Aquaporins of Barley

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CO₂ permeability of plasma membrane intrinsic protein 2 (PIP2) aquaporins of *Hordeum vulgare* L. was investigated. Five PIP2 members were heterologously expressed in *Xenopus laevis* oocytes. CO₂ permeability was determined by decrease of cytosolic pH in CO₂-enriched buffer using a hydrogen ion-selective microelectrode. HvPIP2;1, HvPIP2;2, HvPIP2;3 and HvPIP2;5 facilitated CO₂ transport across the oocyte cell membrane. However, HvPIP2;4 that is highly homologous to HvPIP2;3 did not. The isoleucine residue at position 254 of HvPIP2;3 was conserved in PIP2 aquaporins of barley, except HvPIP2;4, which possesses methionine instead. CO₂ permeability was lost by the substitution of the Ile254 of HvPIP2;3 by methionine, while water permeability was not affected. These results suggest that PIP2 aquaporins are permeable to CO₂, and the conserved isoleucine at the end of the E-loop is crucial for CO₂ selectivity.

Keywords: Aquaporin • Barley • Carbon dioxide • Plasma membrane intrinsic protein 2.

Abbreviations: P_{CO₂}, CO₂ permeability; P_f, osmotic water permeability; PIP, plasma membrane intrinsic protein.

Sequence data from the article can be found in the DNA Data Bank Japan (DDBJ) data library under the following accession numbers: *HvPIP2;1*, AB219366; *HvPIP2;2*, AB377269; *HvPIP2;3*, AB275280; *HvPIP2;4*, AB219525; and *HvPIP2;5*, AB377270, respectively.

Introduction

It is widely accepted that carbon dioxide is transported across biomembranes through aquaporins in a cell (for a review, see Kaldenhoff, 2012). Plant aquaporins are classified into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic

proteins (NIPs), small basic intrinsic proteins (SIPs) and X intrinsic proteins (XIPs) (Danielson and Johanson 2008, Maurel et al. 2008). PIP aquaporins consist of two major subgroups, PIP1 and PIP2. In general, PIP2s have a higher capacity to facilitate water transport in heterologous expression systems, while PIP1s show low or no water transport activity (Chaumont et al. 2000, Johanson et al. 2001, Moshelion et al. 2002, Horie et al. 2011, Shibasaki et al. 2012). The tobacco PIP1 aquaporin, NtAQP1, displayed CO₂ transport activity in *Xenopus laevis* oocytes (Uehlein et al. 2003). NtAQP1 facilitated CO₂ transport when expressed in yeast cells, but the tobacco PIP2, NtPIP2;1 did not (Otto et al. 2010). The Arabidopsis PIP1, AtPIP1;2, was also shown to be permeable to CO₂, while the PIP2, AtPIP2;3, was not, in a yeast expression system (Heckwolf et al. 2011). The involvement of NtAQP1 in mesophyll CO₂ conductance was demonstrated by overexpression and RNA interference (RNAi) suppression experiments (Flexas et al. 2006, Uehlein et al. 2008). The knockout mutant of the *AtPIP1;2* gene exhibited a decreased mesophyll conductance, indicating that AtPIP1;2 facilitates the diffusion of CO₂ in leaves (Uehlein et al. 2012a). Ectopic expression of the ice plant PIP1, *McMIPB*, in tobacco plants increased the CO₂ assimilation rate and mesophyll CO₂ conductance (Kawase et al. 2013). These reports imply a physiological significance of the CO₂ permeation of PIP1 aquaporins. On the other hand, Hanba et al. (2004) showed that overexpression of the barley PIP2, *HvPIP2;1*, in rice plants increased their photosynthetic rate and mesophyll CO₂ conductance. This suggested that some PIP2 aquaporins are possibly permeable to CO₂. Recently, the CO₂ permeability of NtPIP2;1 was shown by means of polymer-embedding experiments (Uehlein et al. 2012b), although the CO₂ permeability of NtPIP2;1 was not detected in the heterologous expression system (Otto et al. 2010). CO₂ permeability of PIP2s has not been assessed in detail. In this study, we examined the CO₂ permeability of five barley PIP2 aquaporins by assessing the pH decrease of the cytosol of *X. laevis* oocytes heterologously expressing HvPIP2s in CO₂-enriched buffer. In addition, we

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apparently more rapid than with water-injected oocytes (Fig. 1B). The cytosolic pH reached a plateau within 5 min in *HvPIP2;1* cRNA-injected oocyte in the typical experiments. As the acidification of the cytosol by CO₂-enriched buffers apparently followed an exponential curve, the time constant (τ) of the acidification was determined by exponential curve fitting. The reciprocal of τ ($1/\tau$) was significantly higher in *HvPIP2;1* cRNA-injected oocytes compared with the water-injected controls, regardless of the CO₂/H₂CO₃ concentration (2.2 and 6.5 mM) (Fig. 1C). This indicates that CO₂ or H₂CO₃ in the bath solution migrated across the cell membrane into the frog oocytes via *HvPIP2;1* and immediately dissociated to H⁺ and HCO₃⁻ in the cell to acidify the cytosol.

In addition to *HvPIP2;1*, we examined the CO₂ permeability of the other four PIP2 members identified from barley (Horie et al. 2011) to gain insight into the CO₂ transport of PIP2 aquaporins. The cytosolic pH of *X. laevis* oocytes was 7.5–7.6 in the bath solution containing 0.01 mM CO₂/H₂CO₃ (Fig. 2A). Upon replacement of the bath solution with CO₂-enriched buffer (6.5 mM CO₂/H₂CO₃), the cytosolic pH decreased promptly toward 7.0–7.1 in *HvPIP2;3* cRNA-injected as well as *HvPIP2;1* cRNA-injected oocytes (Fig. 2A). In *HvPIP2;4* cRNA-injected oocytes, the pH decrease was not as fast as in *HvPIP2;1* and *HvPIP2;3* cRNA-injected oocytes (Fig. 2A). The acidified pH of the oocytes returned by replacement of the buffer with the low CO₂ buffer (0.01 mM CO₂/H₂CO₃). The CO₂ permeability (P_{CO_2}) of the cell membrane of oocytes was calculated from the time constant, final cytosolic pH after the acidification and surface to volume ratio of oocytes (Yang et al. 2000) (Fig. 2B). The P_{CO_2} values of the water-injected oocytes and *HvPIP2;4* cRNA-injected oocytes were low and no significant difference was observed between the two. On the other hand, the oocytes injected with cRNAs of *HvPIP2;1*, *HvPIP2;2*, *HvPIP2;3* and *HvPIP2;5* demonstrated increased P_{CO_2} (3- to 5-fold). This strongly suggests that a subset of barley PIP2s, *HvPIP2;1*, *HvPIP2;2*, *HvPIP2;3* and *HvPIP2;5*, are CO₂ permeable in the heterologous expression system in *X. laevis* oocytes. Our previous report demonstrated that all five *HvPIP2*s were permeable to water when expressed in oocytes (figs. 4 and 5 in Horie et al. 2011).

Isoleucine 254 (I-254) is an important factor for CO₂ permeability of *HvPIP2;3*

The amino acid identity between *HvPIP2;3* and *HvPIP2;4* is very high, and only six amino acids are different out of 296 (Fig. 3A). However, the results described above suggested that *HvPIP2;3* was permeable to CO₂/H₂CO₃, but *HvPIP2;4* apparently was not. We examined the CO₂ permeability of the amino acid-substituted aquaporins of *HvPIP2;3* and *HvPIP2;4* (Fig. 3B) to determine the structural basis of the difference in CO₂ permeability between the two aquaporins.

Simultaneous substitution of four amino acids of *HvPIP2;3*, A-268, L-281, R-289 and S-290, to L-268, F-281, S-289 and R-290 [*HvPIP2;3*^(LFSR)] did not affect the acidification of the cytosol of

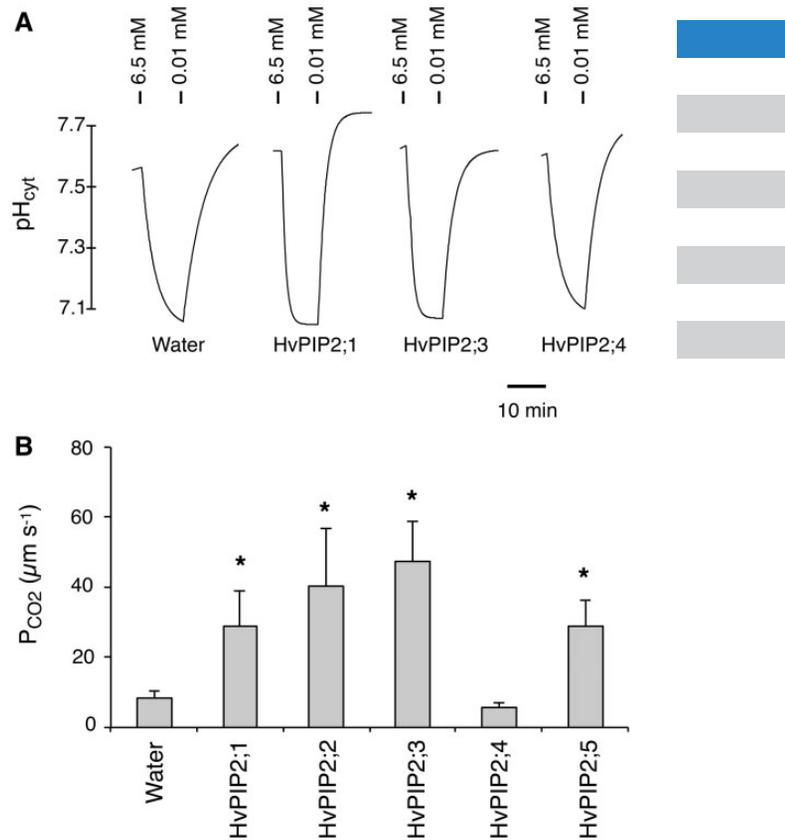


Fig. 2 CO₂ permeability of PIP2 aquaporins of barley. (A) Representative traces of cytosolic pH change of water-, *HvPIP2;1* cRNA-, *HvPIP2;3* cRNA- and *HvPIP2;4* cRNA-injected *X. laevis* oocytes. cRNAs and carbonic anhydrase were injected 24–48 h before the measurements. Ticks above the traces indicate where the bath solutions were replaced with the modified Barth's solution containing the designated concentrations of CO₂/H₂CO₃. (B) P_{CO_2} of the cell membrane of *X. laevis* oocytes injected with water ($n = 14$), *HvPIP2;1* cRNA ($n = 10$), *HvPIP2;2* cRNA ($n = 5$), *HvPIP2;3* cRNA ($n = 6$), *HvPIP2;4* cRNA ($n = 7$) or *HvPIP2;5* cRNA ($n = 3$). Error bars indicate the SEM. Asterisks indicate significant difference of the mean from the water-injected control (Water) by Student's *t*-test at $\alpha = 0.05$.

HvPIP2;3^(LFSR) cRNA-injected oocytes compared with *HvPIP2;3*-injected oocytes (Supplementary Fig. S1B). The osmotic water permeability (P_f) of *HvPIP2;3* and *HvPIP2;3*^(LFSR) was not significantly different ($P > 0.05$, Supplementary Fig. S1C). This indicates that the four amino acids in the C-terminal stretch are not involved in the CO₂ permeability of *HvPIP2;3*. Substitution of I-254 of *HvPIP2;3*, which is localized at the edge of the E-loop, by methionine (Fig. 3C) resulted in substantial repression of the P_{CO_2} (Fig. 3D). Substitution of methionine 254 (M-254) of *HvPIP2;4* by isoleucine, *HvPIP2;4*^(M254I) (Fig. 3B) caused activation of P_{CO_2} (Fig. 3D). The P_f of *HvPIP2;3*^(I254M) and *HvPIP2;4*^(M254I) was examined to test whether the mutated aquaporins were functional (Fig. 3E). The substitution of I-254 of *HvPIP2;3* and M-254 of *HvPIP2;4* did not show any apparent effect on P_f . This indicates that I-254 of *HvPIP2;3* is crucial to the CO₂ permeability.

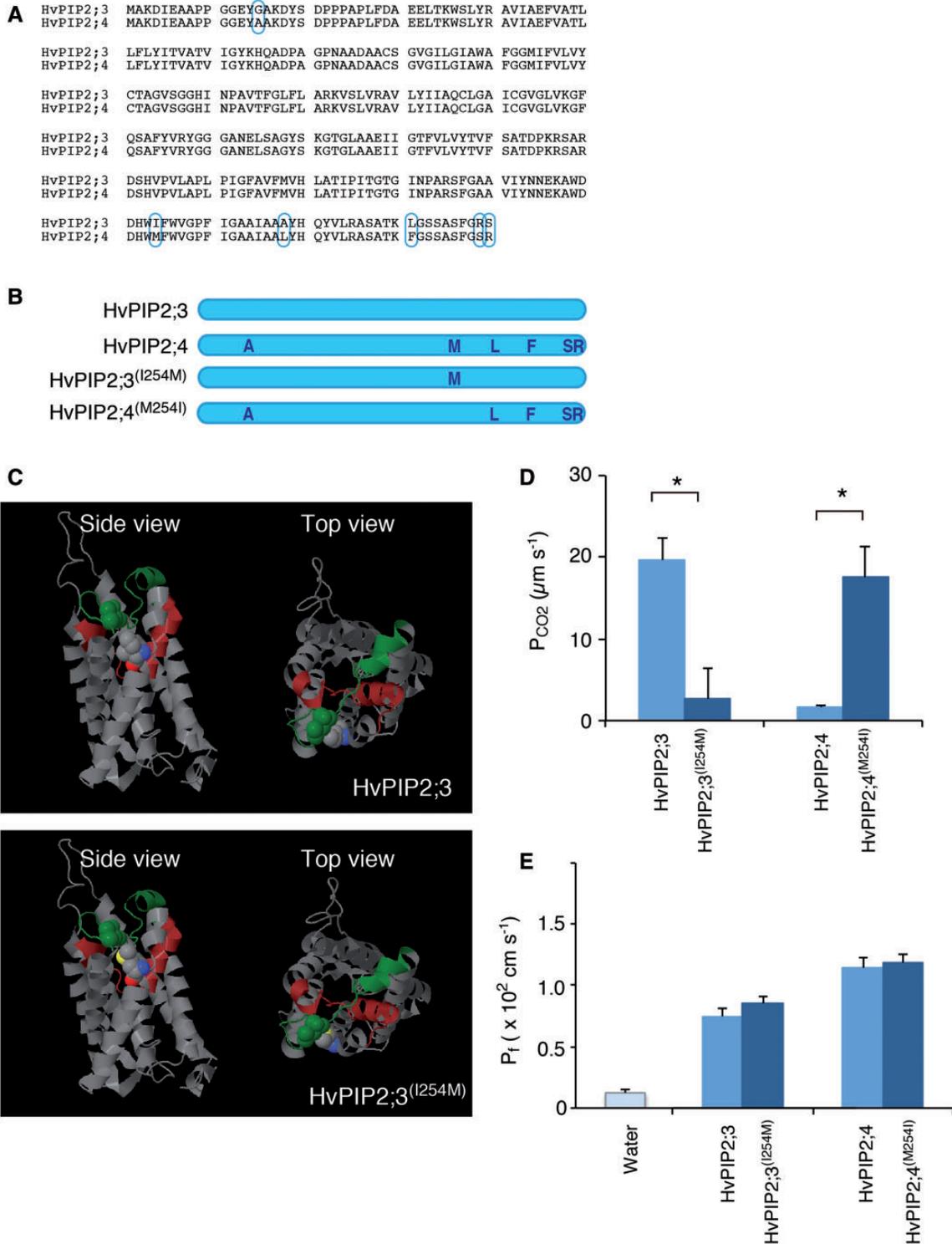


Fig. 3 Isoleucine 254 of HvPIP2;3 is one of the key factors determining CO₂ permeability. (A) Alignment of amino acid sequences of HvPIP2;3 and HvPIP2;4. Six different amino acids are designated by cyan ellipsoids. (B) Illustrated representation of amino acid substitution constructs. Indigo letters indicate the amino acids of HvPIP2;4 origin; the remainder are those of HvPIP2;3. (C) Three-dimensional homology modeling of HvPIP2;3 and HvPIP2;3^(I254M) molecules. The model was constructed based on an X-ray diffraction structural model of spinach SoPIP2;1. The yellow ball shape indicates the sulfur atom of M-254. Red and blue ball shapes indicate oxygen and nitrogen atoms, respectively, of the 254th amino acid. The green ball shape indicates L-165 close to M-254. The green stretch indicates the C-loop. The brown strand and helix indicate the E-loop. (D) CO₂ permeability (P_{CO_2}) of the cell membrane of *X. laevis* oocytes injected with HvPIP2;3 ($n = 6$), HvPIP2;4 ($n = 3$) and the amino acid-swapped constructs, HvPIP2;3^(I254M) ($n = 7$) and HvPIP2;4^(M254I) ($n = 3$). cRNAs and carbonic anhydrase were injected 24–48 h before the

(continued)

Discussion

We provide evidence that barley PIP2 aquaporins, HvPIP2;1, HvPIP2;2, HvPIP2;3 and HvPIP2;5, but not HvPIP2;4, facilitate CO₂ transport across biomembranes (Figs. 1, 2). The CO₂ permeability of PIP1 aquaporins has been demonstrated using heterologous expression systems. The CO₂ permeability of NtAQP1 was examined in a *Saccharomyces cerevisiae* expression system and a *X. laevis* oocyte expression system (Uehlein et al. 2003, Heckwolf et al. 2011). The CO₂ permeability of AtPIP1;2 was shown in a yeast expression system. These expression systems demonstrated that PIP2 aquaporins neither NtPIP2;1 or AtPIP2;3 did not facilitate transport of CO₂ (Otto et al. 2010, Heckwolf et al. 2011). Meanwhile, Uehlein et al. (2012b) demonstrated the CO₂ permeability of NtPIP2;1 by embedding the isolated protein in a polymer membrane. The discrepancy in CO₂ permeability of NtPIP2;1 described by Otto et al. (2011) and Uehlein et al. (2012b) was discussed in terms of the biological membrane having a certain background CO₂ permeability (Uehlein et al. 2012b). Hanba et al. (2004) suggested that HvPIP2;1 is permeable to CO₂ by using overexpression in plants. However, direct evidence for the CO₂ permeability of PIP2s had not been provided previously. In this study, we successfully demonstrated the CO₂ permeability of barley PIP2s in a *X. laevis* oocyte expression system. Surprisingly, our data suggested that four out of five known PIP2s from barley were permeable to CO₂. More PIP2s might be CO₂ permeable than we expected.

In this study, we examined the CO₂ permeability of only PIP2s of barley. The present work does not deny the possibility that PIP1s might be permeable to CO₂, as HvPIP1s were not examined. It was previously shown that tobacco NtAQP1 localized on the cell membrane of the oocytes and was permeable to CO₂ (Uehlein et al. 2003). However, for barley PIP1s, we did not observe efficient localization to the cell membrane (Supplementary Results; Supplementary Fig. S2). This corresponds to the lack of water transport activity of HvPIP1;2 (Horie et al. 2011). One of the reasons for the undetected water permeability of HvPIP1s in the previous study (Horie et al. 2011) may be the low efficiency of targeting of PIP1s to the oocytes' cell membrane. For this reason, we did not examine the CO₂ permeability of PIP1s of barley in this study. The permeability of HvPIP1s to CO₂ remains an open question.

It was suggested in this study that HvPIP2;3 is permeable to CO₂ but HvPIP2;4 is not, although only six amino acids are different (Fig. 2). Taking advantage of this difference, we determined the amino acids essential for CO₂ permeability. We identified I-254 of HvPIP2;3 as a critical amino acid residue

(Fig. 3; Supplementary Fig. S1). The substitution of I-254 by methionine substantially impaired CO₂ permeability. However, the P_f was not lost (Fig. 3E).

Zhang et al. (2010) reported that valine/isoleucine (V/I) in the membrane-spanning helix 2 of the rice PIP2s was crucial for water permeation activity. The amino acid corresponding to this position in OsPIP1s is alanine. The substitution of V/I by alanine decreased the P_f (Zhang et al. 2010). In HvPIP2;3 and HvPIP2;4, the corresponding position is V. This residue coincides with significant P_f of HvPIP2;3 and HvPIP2;4. Suga and Maeshima (2004) reported that the specific V residue in the E-loop of radish PIP2s was important for water transport. The substitution of V with I, as seen in radish PIP1s, substantially decreased the water permeability. The corresponding amino acid in HvPIP2;3 and HvPIP2;4 was V. There has been no previous report of finding a structural basis for the CO₂ permeability of aquaporins.

I-254 is located at the C-terminal end of the E-loop. When substituted with methionine, this residue was predicted to be sited proximal to the oxygen molecule of the carboxyl group of the main chain close to leucine 165 in the C-loop, with the sulfur atom facing the C-loop (Fig. 3C). The sulfur atom of methionine interacts with the nucleophilic oxygen atom (Fig. 3C) (Chakrabarti and Bhattacharyya 2007). This interaction may result in a distortion of the C-loop and hamper the CO₂ permeability of HvPIP2;3. Importantly, this isoleucine is highly conserved in PIP1 and PIP2 of barley, except HvPIP2;4 (Supplementary Fig. S3). The importance of isoleucine at the C-terminal end of the E-loop has not been examined previously. Recently an aquaporin from cyanobacterium was reported to be permeable to CO₂ (Ding et al. 2013). The corresponding position was leucine instead of isoleucine in the algal aquaporin. If the mechanism of CO₂ impermeability of the M-254-substituted aquaporin is attributed to the interaction with the C-loop via the sulfur atom, it can be easily understood why leucine did not disrupt the CO₂ permeability. Supplementary Table S1 shows a list of PIP members whose CO₂ permeability has been examined and the amino acid sequence at the C-terminal end of the E-loop. Supplementary Fig. S4 shows the alignment of amino acid sequences of PIP1s and PIP2s at the corresponding region of Arabidopsis, rice, maize and barley. It is noticeable that the V residue of AtPIP1;2 is capable of substituting for the I residue. NtPIP2;1 and AtPIP2;3 possess I at this position, but they were not permeable to CO₂ in the heterologous expression systems (Otto et al. 2010, Heckwolf et al. 2011). These indicate that there are other factors for CO₂ selectivity besides the specific amino acid residue at the C-terminal end of the E-loop. Comparative analyses of aquaporin polymorphism in

Fig. 3 Continued

measurements. Error bars indicate the SEM. Asterisks indicate a significant difference of the mean (Student's *t*-test, $\alpha = 0.05$). (E) Osmotic water permeability (P_f) of the cell membrane of *X. laevis* oocytes injected with water ($n = 8$), HvPIP2;3 cRNA ($n = 9$), HvPIP2;3^(I254M) cRNA ($n = 9$), HvPIP2;4 cRNA ($n = 9$) and HvPIP2;4^(M254I) cRNA ($n = 10$). Error bars indicate the SEM. No significant difference was observed between HvPIP2;3 and HvPIP2;3^(I254M), or between HvPIP2;4 and HvPIP2;4^(M254I) (Student's *t*-test, $\alpha = 0.05$).

the future will provide new insights with regard to CO₂ permeability. It may lead to new technologies to regulate CO₂ conductance in plants via aquaporins facilitating CO₂ permeation.

Materials and Methods

Water transport activity assay in *Xenopus laevis* oocytes

The cDNAs of HvPIP2;3, HvPIP2;4, HvPIP2;3^(I254M) and HvPIP2;4^(M254I) were subcloned into the pXβGev1 expression plasmid vector, as reported previously (Horie et al. 2011). The plasmid was linearized with *NotI*, and capped cRNA was synthesized using the mMESSAGE mMACHINE T3 in vitro transcription kit (Ambion). Oocytes were isolated from adult female *X. laevis* and maintained as described previously (Katsuhara et al. 2002). Oocytes were injected with 50 nl of a cRNA solution containing 2 ng of RNAs 24–48 h before measurement. As a negative control, water-injected oocytes were used. The osmotic water permeability coefficient of oocytes was measured according to the procedures described previously (Katsuhara et al. 2002, Mahdiah et al. 2008).

Construction of the hydrogen ion-selective microelectrode

A glass capillary with a filament (1.5 mm outer diameter/1.12 mm inner diameter, World Precision Instruments) was pulled with a micropipet puller (model P-1000, Sutter Instruments) under the conditions described in **Supplementary Table S2**. The glass pipet was then filled with a cocktail of hydrogen ionophore [1:5:4 mixture of hydrogen ionophore I cocktail A (Selectophore grade, Fluka), 0.5% polyvinyl chloride dissolved in tetrahydrofuran and tetrahydrofuran]. The glass pipet was left to stand for a day for the ionophore mixture at the tip of the pipet to solidify.

CO₂ permeability assay in *Xenopus laevis* oocytes

Xenopus laevis oocytes were prepared as described for the water transport activity assay (Horie et al. 2011). Each oocyte was injected with 25 ng of HvPIP2;1, HvPIP2;2, HvPIP2;3, HvPIP2;4 or HvPIP2;5 cRNA. A 25 ng aliquot of carbonic anhydrase (Catalog No. C-3934, Sigma-Aldrich) was simultaneously injected into the oocytes (**Supplementary Results; Supplementary Fig. S5**). The oocytes were impaled with a membrane potential microelectrode and a hydrogen ion-selective microelectrode. The membrane potential microelectrode was made in the same way as the hydrogen ion-selective microelectrode, except for filling with the ionophore cocktail; in this case, it was filled with 0.5 M KCl. The hydrogen ion-selective microelectrode was backfilled with the electrode solution containing 0.5 M KCl, 0.2 M MES and Tris (pH 6.0). The membrane potential microelectrode and hydrogen ion-selective microelectrode were attached to head stages (HS-9A and HS-2, respectively, Axon Instruments).

The reference electrode was immersed in the bath solution via an agar bridge containing 3 M KCl. The dimensions of the chamber made of acrylic resin were 3 mm (depth) × 3 mm (width) × 25 mm (path length). The estimated time for solution exchange in the proximity of the oocyte with a flow rate of 400 μl min⁻¹ was approximately 10 s. An amplifier, Axoclamp 900A (Axon Instruments), a digitizer, Digidata 1440A (Axon Instruments), and software, pCLAMP 10 (Axon Instruments), were used to acquire the voltage output of the microelectrodes. The recording rate was 1,000 Hz. The pH of the cytosol of the oocytes was determined from the difference between the two microelectrodes. The electrodes were calibrated with calibration solutions whose pH was buffered to 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 with either 0.1 M MES/Tris or PIPES/Tris, and contained 0.1 M KCl.

The P_{CO₂} was determined according to a previous report (Yang et al. 2000). The final cytosolic pH was determined from the actual measurement at the end of the recordings. The time constant (τ) was obtained from the trace of the difference of the two microelectrodes by exponential curve fitting. The surface to volume ratio of oocytes was determined from the average of the diameter of 18 representative oocytes.

Construction of HvPIP2;3^(I254M), HvPIP2;4^(M254I) and HvPIP2;3^(LFSR)

Point mutation of the constructs for the amino acid substitution, HvPIP2;3^(I254M) and HvPIP2;4^(M254I), was done essentially according to a previous report (Zheng et al. 2004). The primers utilized are listed in **Supplementary Table S3**. cDNA of HvPIP2;3^(LFSR) happened to be isolated naturally along with the cloning of the cDNA of HvPIP2;3. The corresponding sequence might exist in a natural population of *Hordeum vulgare* cv. Haruna nijyo to a minor extent.

Homology modeling

Homology modeling was performed by the Workspace at the Swiss-Model website, URL: <http://swissmodel.expasy.org/> (Arnold et al. 2006).

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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