Functional Characterization of a Novel Plasma Membrane Intrinsic Protein2 in Barley *

Mineo Shibasaka*, Sizuka Sasano, Sigeko Utsugi and Maki Katsuhara

Institute of Plant Science and Resources, Okayama University, Kurashiki 710-0046, JAPAN

*Correspondence to: Mineo Shibasaka, Institute of Plant Science and Resources, Okayama University, Chuo-2-20-1, Kurashiki 710-0046, Japan, Tel.: +81-86-434-1223; Fax: +81-86-434-1249; E-mail: smine@rib.okayama-u.ac.jp

*Running title: *A Novel PIP2 from barley*

KEY WORDS

aquaporin, plasma membrane intrinsic protein, PIP, HvPIP2;8, water transport, barley, *Hordeum vulgare*,*Xenopus laevis* oocytes

ABBREVIATIONS

EGFP, enhanced green fluorescence protein; P_f, osmotic water permeability coefficient; PIPs, plasma membrane intrinsic proteins;

ABSTRACT

Water homeostasis is crucial to the growth and survival of plants. Plasma membrane intrinsic proteins (PIPs) have been shown to be primary channels mediating water uptake in plant cells. We characterized a novel PIP2 gene, *HvPIP2;8* in barley (*Hordeum vulgare*). HvPIP2;8 shared 72-76% identity with other HvPIP2s and 74% identity with rice OsPIP2;8. The gene was expressed in all organs including the shoots, roots and pistil at a similar level. When *HvPIP2;8* was transiently expressed in onion epidermal cells, it was localized to the plasma membrane. HvPIP2;8 showed transport activity for water in *Xenopus* oocytes, however its interaction with HvPIP1;2 was not observed. These results suggest that HvPIP2;8 plays a role in water homeostasis although further functional analysis is required in future.

INTRODUCTION

Plasma membrane intrinsic proteins (PIPs), members of the plant aquaporin family, have been shown to be primary channels mediating uptake in plant cells.[1] Plant PIPs can be classified into two distinct clades, PIP1s and PIP2s, according to their sequence similarity.[2-4] PIP1 and PIP2 display different transport activity when expressed alone in *Xenopus* oocytes. PIP2 increases oocyte membrane water permeability, while PIP1 displays no or low water permeability.[5] This differential behavior seems to be related to PIP trafficking; PIP2 is able to reach the plasma membrane whereas

PIP1 seems to be retained in the endoplasmic reticulum.[6]

In the genome of *Arabidopsis* and rice, there are 13 and 11 PIPs, respectively.[7,8] In barley five PIP1s and six PIP2s have been isolated so far.[9-11] They showed different expression patterns.[11] For example, total expression of PIP1s was comparable between leaf regions, whereas PIP2 expression was up to six times higher in the elongation zone. In the present study, we characterized a novel PIP2 gene, HvPIP2;8 in terms of transport activity, expression pattern and subcellular localization.

RESULT AND DISCUSSION

Search of novel PIPs in barley

A number of aquaporin has been identified in many plant species. In rice genome, there are 33 aquaporin genes.[8] Among them 8 OsPIP2s were annotated. In *Arabidopsis*, there are 8 AtPIP2s among 35 aquaporin genes.[7] Since the genome sequence of barley has not been completed, the number of aquaporin in barley is still unknown. So far 6 members belonging to PIP2 (HvPIP2;1 to 2;5, HvPIP2;7) have been isolated in barley.[9-11] Recent release of barley full-length cDNAs let us to find more PIPs.[12] TBLASTN search by using amino acid sequence of all HvPIPs as a query resulted in 570 hits. On the other hand, BLASTn search by using nucleotide sequnence of whole coding regions of all HvPIPs known as a query yielded 1387 hits. Exclusion of repeated hits from the two merged searches resulted in 17 clones. Among them, 4 clones including AK355531, AK356299,

AK373720, and AK361542, have not been reported before. In the present study, we functionally characterized one of them AK356299, which was annotated HvPIP2;8.

Phylogenic analysis of HvPIP2;8

A phylogenic tree analysis was conducted with other PIP members from barley, rice and *Arabidopsis.* HvPIP2;8 forms a different clade with other PIPs (Fig. 1A). The identity of HvPIP2;8 to other HvPIPs ranged from 72 to 76%. In the same clade, there are four members including HvPIP2;8, OsPIP2;8, AK361542 and AF366565. There are no members from dicots in this clade, suggesting that this clade is specific for monocots.

Characteristic of the primary structure of HvPIP2;8

HvPIP2;8 exhibits typical MIP motif, six transmembrane domains and two NPA motifs (Fig. 1B). Furthermore, following highly conserved amino acid residues, which are considered to be involved in the regulation of activation, Ser^{280} and Ser^{285} via phosphorylation[13], His^{206}, Lys^{199}, Arg^{200}, and Arg^{203} in Loop D, and Asp^{13}, Asp^{16}, Glu^{18}, Asp^{27}, Asp^{30}, Asp^{38}, and Glu^{41} in acidic N-terminus, via cytosolic pH,[14] are also present in HvPIP2;8 (Fig. 1B). Interestingly, HvPIP2;8 exhibits three histidine residues in Loop C, which are not conserved in other members.

Transport activity in *Xenopus* oocyte

To determine the transport activity for water, *HvPIP2;8* was expressed in *Xenopus* oocytes. Compared to the control injected with water, oocytes expressing *HvPIP2;8* showed significantly higher transport activity for water (Fig. 2). It was reported that PIP1 and PIP2 interact each other[15-17]. For example, co-expression of ZmPIP1;2 with ether ZmPIP2;1, ZmPIP2;4, ZmPIP2;5 significantly increased the osmotic water permeability coefficient (P_f) of the oocytes compared with the expression of each PIP2 alone.[18] Co-expression of HvPIP1;2 with HvPIP2;1, 2;2, 2;3, 2;4, and 2;5 also resulted in increased water permeability coefficient of *Xenopus* oocytes.[10] To investigate whether HvPIP2;8 is able to activate other PIP1 protein, we co-expressed HvPIP2;8 with HvPIP1;2. As a result, the transport activity was not enhanced (Fig. 2). As a positive control, we also co-expressed HvPIP1;2 and HvPIP2;4 to test whether HvPIP1;2 functions in oocytes. The result showed that different from HvPIP2;8, HvPIP2;4 interacted with HvPIP1;2 by further increasing the water permeability coefficient (Fig. 2). This result suggests that the water transport by HvPIP2;8 is relatively constant and there is no need to change dynamically. HvPIP2;8 might be expressed in the cells located inner tissues where water environment is relatively stable.

Expression patterns of *HvPIP2;8*

The organ-dependent expression of *HvPIP2;8* was investigated with semi-quantitative RT PCR.

HvPIP2;8 was expressed in all organs tested including the shoots, roots and pistil (Fig. 3). In the shoots, both mature and young leaf showed similar expression level (Fig. 3). This expression pattern is different form that of *HvPIP2;7* (Fig. 3), which was specifically expressed in the leaves.

Subcellular localization

The subcellular localization of HvPIP2;8 was investigated by transiently introducing its fusion with EGFP into the onion epidermal cells. Observation with a fluorescence microscope showed the EGFP signal was localized out of the nuclei, stained with DAPI (Fig. 4A-C), indicating that HvPIP2;8 was localized to the plasma membrane. By contrast, the signal was observed in the cytosol and nuclei in the cells expressing EGFP alone.

As a conclusion, HvPIP2;8 is a plasma membrane-localized water channel. It is universally expressed in all tissues. Although the exact role of HvPIP2;8 remains to be further examined, our results suggest that it may be involved in water homeostasis.

MATERIALS AND METHODS

Phylogenetic analysis

A Blast search using the query of new HvPIPs hits AF366565 from wheat and FP100796 from

bamboo. The amino acid sequences used for analysis are as follows: all barley and *Arabidopsis* PIPs, OsPIP2;6, OsPIP2;7, OsPIP2;8, AF366565, and FP100796. After the gaps were eliminated the phylogenetic tree was constructed at Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/index.cgi).[19]

RNA extraction and analysis of gene expression by RT-PCR

Hordeum vulgare cv. Haruna-nijo was used. Samples including mature leaves and pistils for RNA extraction were taken from barley plants grown in fields of Kurashiki, Japan. Samples of the roots and shoots were excised from 4-day-old seedlings and young leaves were excised from one-week-old seedlings. Growth conditions were the same as described previously.[9] Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) after grounded with a mortar and pestle. First-strand cDNA synthesis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems). PCR was performed using primer sets in Table 1 according to Utsugi et al.[20]

Transient expression in onion epidermis cells

The coding sequence of *HvPIP2;8* was inserted into pBI221-EGFP[21] to be fused in-frame to the C terminus of EGFP coding sequence. The fusion construct was introduced into onion epidermal cells using the particle bombardment according to the procedure described by Utsugi et al.[20] After overnight incubation at 23˚C, epidermises were peeled and observed with a fluorescence microscope (BZ-8000, Keyence, Osaka, Japan) using a filter set for GFP (OP-66836, Keyence, Osaka, Japan).

DAPI (final concentration 2μM, NACALAI TESQUE INC, Kyoto, Japan) was used as necessary to stain nuclei. DAPI staining images were observed using a filter set for DAPI (OP-66834, Keyence, Osaka, Japan) merged with the corresponding EGFP image.

Water transport activity assay in *Xenopus laevis* oocytes

The coding regions of *HvPIP2;8* cDNA was subcloned into the pXβGev1 expression vector. The construct was linearlized with NotI, 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.[28] Oocytes were injected with 50 nl of a cRNA solution containing 2 ng of *HvPIP2;8* and/or 10 ng of *HvPIP1;2*; 2 ng of *HvPIP2;4* and 10 ng of *HvPIP1;2* as a positive control. As a negative control, water-injected oocytes were used. The osmotic water permeability coefficient of oocytes was measured according to the procedures described previously.[22]

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FIGURE LEGENDS.

FIGURE 1. Phylogenetic analysis and predicted protein structure. A. Phylogenetic tree of the plasma membrane intrinsic protein (PIP) from barley (*H. vulgare* cv. Haruna-nijo), *Arabidopsis thaliana*, and rice (*O. sativa* cv. Nipponbare). The scale bar indicates 0.2 substitutions per site. B. Predicted structure of HvPIP2;8.

FIGURE 2. Water transport activity of HvPIP2;8. The amounts and kind of cRNAs injected were shown on the left of the graph. P_f values are shown. Error bars represent the standard deviation (n=10). Different letter denotes significant difference ($p<0.05$).

FIGURE 3. Organ-specific expression of *HvPIP2;8* and *HvPIP2;7* in barley plants. Semi quantitative RT-PCR analysis in the shoot of 5-day-old seedling, root and young leaves of 10-week-old plants, mature leaves, and pistil were used. *ACT1* was used as an internal standard.

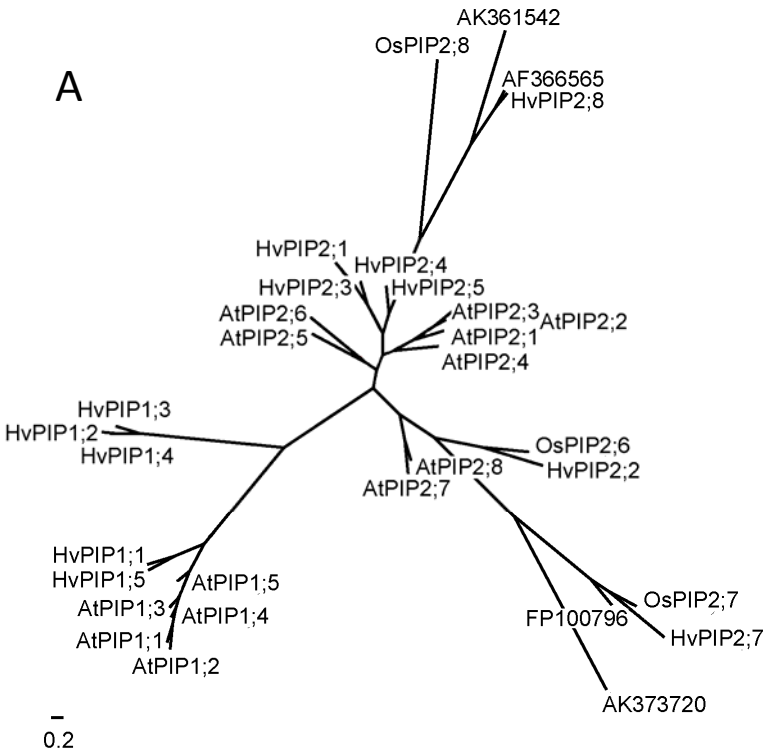
FIGURE 4. Subcellular localization of EGFP:HvPIP2;8 in onion epidermal cells.

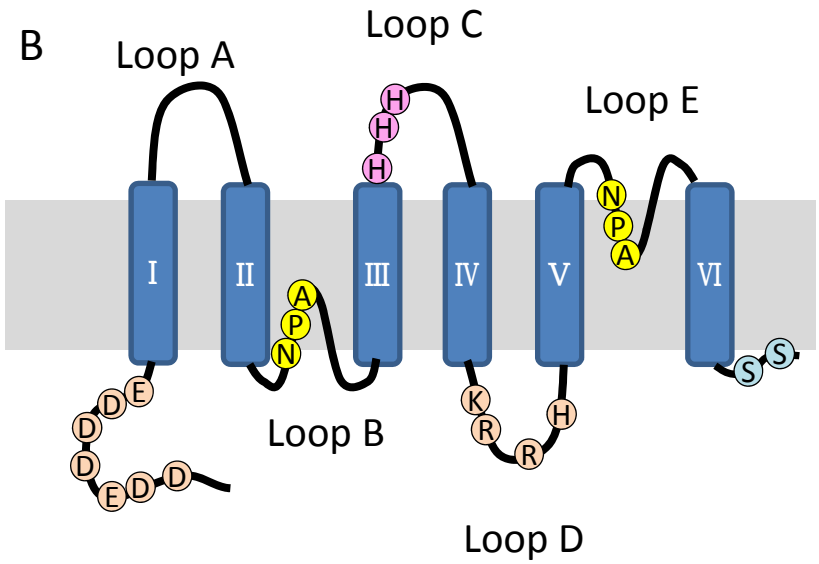
EGFP:HvPIP2;8 (a), DAPI stained (b), the merged image of a and b (c), and EGFP alone (d) as a control. The white scale bars represent 0.1 mm.

Table 1 Gene-specific primer pairs used in the RT–PCR experiments

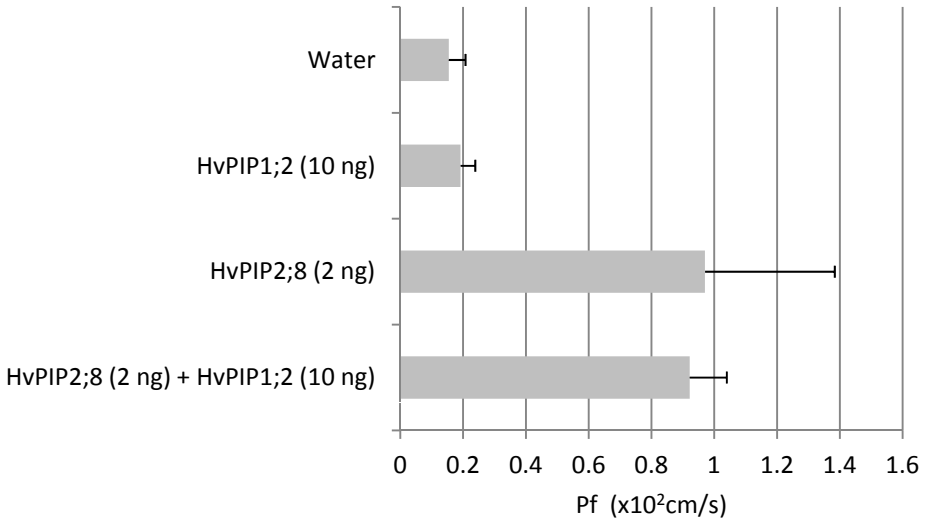
Gene	Primer[a]
HvPIP2;8	Forward: 5' ^{213}ACACAAGCGCCAGACCGACG^{232} 3'
	Reverse: 5' ^{870}GGCGGCATCGTAGTTGGACCG^{850} 3'
HvPIP2;7	Forward: 5' ^{200}AGAGCCAGTCCTCCGCCCAG^{219} 3'
	Reverse: 5' ^{907}GGAGCTTCGACCGATCACACG^{887} 3'
ACT1	Forward: 5' ^{1}ATGGCTGACGGTGAGGACATCC^{22} 3'
	Reverse: 5' ^{422}ACGGCCTGAATAGCGACGTAC^{402} 3'

[a] Numbers for each primer correspond to nucleotide positions from the ATG initiation codon.

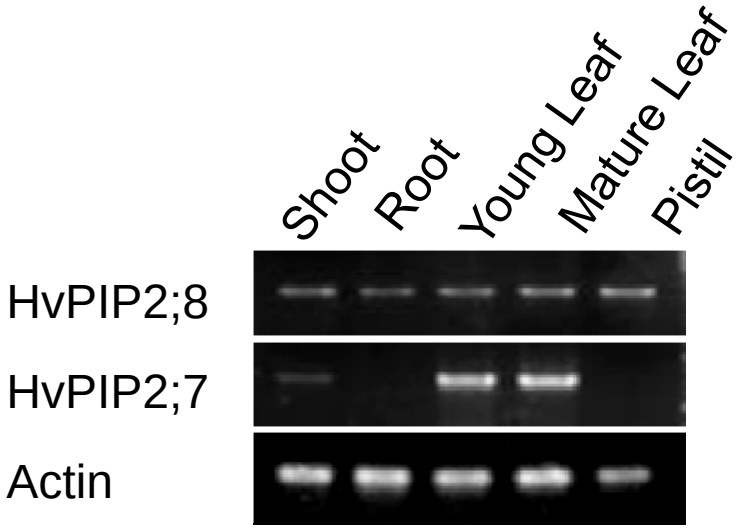




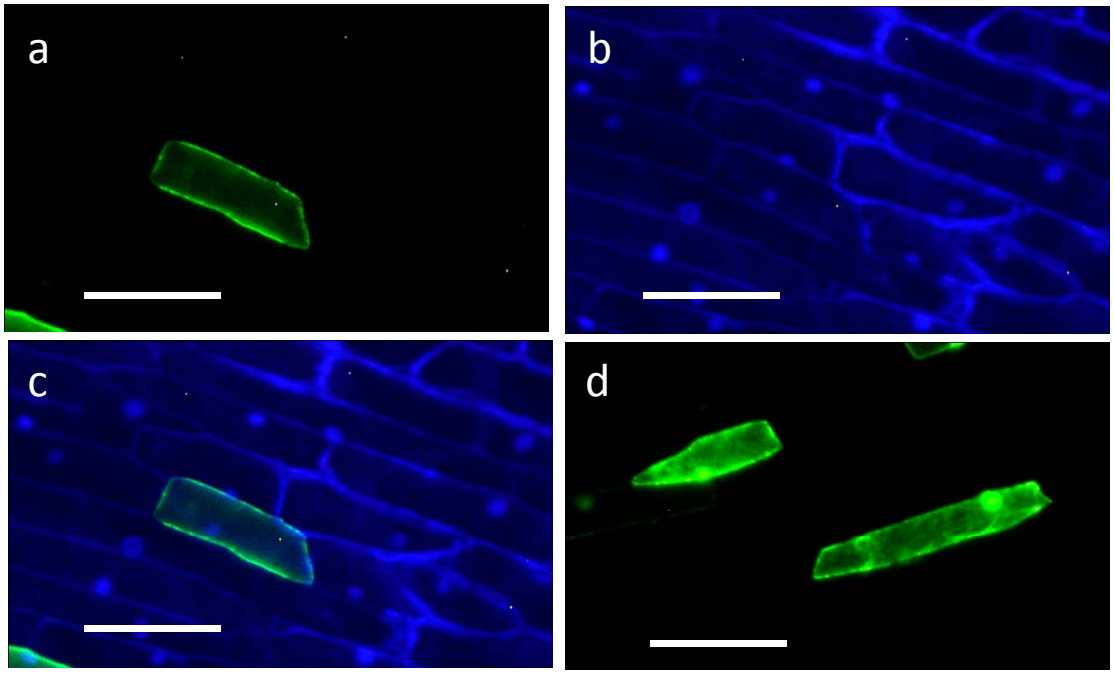
Shibasaka et al. Figure 1



Shibasaka et al. Figure 2



Shibasaka et al. Figure 3



Shibasaka et al. Figure 4