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HvPIP1;6, a Barley (*Hordeum vulgare* L.) Plasma Membrane Water Channel Particularly Expressed in Growing Compared with Non-Growing Leaf Tissues

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The aim of the present study was to identify water channel(s) which are expressed specifically in the growth zone of grass leaves and may facilitate growth-associated water uptake into cells. Previously, a gene had been described (*HvEmip*) which encodes a membrane intrinsic protein (MIP) and which is particularly expressed in the base 1 cm of barley primary leaves. The functionality of the encoding protein was not known. In the present study on leaf 3 of barley (*Hordeum vulgare* L.), a clone was isolated, termed *HvPIP1;6*, which has 99% amino acid sequence identity to *HvEmip* and belongs to the family of plasma membrane intrinsic proteins (PIPs). Expression of *HvPIP1;6* was highest in the elongation zone, where it accounted for >85% of expression of known barley PIP1s. Within the elongation zone, faster grower regions showed higher expression than slower growing regions. Expression of *HvPIP1;6* was confined to the epidermis, with some expression in neighboring mesophyll cells. Expression of *HvPIP1;6* in *Xenopus laevis* oocytes increased osmotic water permeability 4- to 6-fold. Water channel activity was inhibited by pre-incubation of oocytes with 50 μ M HgCl₂ and increased following incubation with the phosphatase inhibitor okadaic acid or the plant hormone ABA. Plasma membrane preparations were analyzed by Western blots using an antibody that recognized PIP1s. Levels of PIP1s were highest in the elongation and adjacent non-elongation zone. The developmental expression profile of *HvPIP2;1*, the only known barley water channel belonging to the PIP2 subgroup, was opposite to that of *HvPIP1;6*.

Keywords: Barley (*Hordeum vulgare* L.) — Cell elongation zone — Epidermis — Leaf growth — Membrane intrinsic proteins — Plasma membrane water channel.

Abbreviations: LHCb, light harvesting chlorophyll *a/b*-binding protein; MBS, modified Barth's saline; MIP, membrane intrinsic protein; NIP, NOD26-like intrinsic protein; OA, okadaic acid; PBS, phosphate-buffered saline; Pf, osmotic water

permeability; PIP, plasma membrane intrinsic protein; SIP, small basic intrinsic protein; TIP, tonoplast intrinsic protein.

The nucleotide sequence reported in this paper has been submitted to the EMBL database under accession number CO720031.

Introduction

Aquaporins belong to the group of membrane intrinsic proteins (MIPs). MIPs have been found in a wide range of organisms and account for a considerable portion of membrane proteins (Schäffner 1998, Kjellbom et al. 1999, Johansson et al. 2000, Maurel et al. 2002, Chaumont et al. 2005, Hachez et al. 2006, Katsuhara 2007). Classification of MIPs is according to their subcellular location (TIPs, tonoplast intrinsic proteins; PIPs, plasma membrane intrinsic proteins), their organ of discovery (NIPs, nodule or NOD26-like intrinsic proteins) or their molecular size (SIPs, small basic intrinsic proteins). Some aquaporins transport primarily water and increase osmotic water permeability (P_f) several-fold when expressed in oocytes; these aquaporins may be classified as 'water channels' (Tyerman et al. 2002). Other aquaporins are less specific and also transport solutes such as glycerol (Biela et al. 1999), urea (Gaspar et al. 2003), boron (Dordas et al. 2005), hydrogen peroxide (Henzler and Steudle 2000, Bienert et al. 2007), silicon (Ma et al. 2006) or, as recently suggested, ammonia (Loqué et al. 2005), carbon dioxide (Uehlein et al. 2003, Hanba et al. 2004) or selenium (Zhang et al. 2006). It has also been suggested that aquaporins fulfill less of a transport role but function either as osmo- and turgor sensors (Hill et al. 2004) or as markers for targeting vesicles to the central vacuole (Ma et al. 2004).

Plant development requires coordination between uptake, partitioning and loss of water. The degree to which aquaporins play a controlling role in these

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processes depends on the contribution of water flow through membranes to overall tissue water flow (Steudle and Henzler 1995, Steudle and Peterson 1998, Tyerman et al. 1999). Expression of aquaporins has been observed particularly in those tissues which are expected to have a high transcellular flow of water (Dixit et al. 2001, Hakman and Oliviusson 2002, Siefritz et al. 2004, Eisenbarth and Weig 2005) and has been linked to diurnal changes in hydraulic conductivity (Henzler et al. 1999) and leaf movements (Moshelion et al. 2002). Some studies point to a role for aquaporins in regulating water transport in growing root, stem or inflorescence tissues (Kaldenhoff et al. 1995, Chaumont et al. 1998, Barriau et al. 1999, Hukin et al. 2002). However, little information exists for growing tissues of leaves (Hollenbach and Dietz 1995, Schünmann and Ougham 1996).

Grasses are of major economic importance. They also provide a convenient experimental system to study growth-related processes in leaves (see also Fig. 1). The meristem and elongation zone are located near the base of leaves (point of leaf insertion) and are enclosed by sheaths of older leaves. Further along this enclosed leaf region is another leaf zone, the non-elongation zone, in which cells have ceased elongation and show some residual lateral expansion (Fricke and Flowers 1998). Cells in that portion of the developing leaf which has emerged from sheaths of older leaves (emerged blade) experience a very different micro-environment with respect to light and humidity and driving forces for radial water movement (transpiration). If aquaporins play a role in these processes, this should show as a developmental pattern in their expression, protein abundance or regulation.

More than a decade ago, Hollenbach and Dietz (1995) identified a gene of the MIP family (*HvEmip*) which was expressed particularly in the base 1 cm of barley primary leaves, that region which includes the growth zone of leaf 1. The functionality of *HvEmip* was not tested, nor were detailed expression studies carried out for different leaf developmental zones or growing regions at higher spatial resolution. In the present study, a barley cDNA clone with high (99%) amino acid sequence identity to *HvEmip* was cloned. The clone, which encoded a member of the PIP1 subfamily, was termed *HvPIP1;6* (EMBL accession No. CO720031). The tissue distribution of *HvPIP1;6* was studied by in situ PCR. The expression of *HvPIP1;6* was analyzed at high spatial resolution along the developing leaf 3 of barley and compared with the developmental profile of expression of *HvPIP2;1*, the only previously known barley water channel (Katsuhara et al. 2002), and of other known barley PIPs. The functionality of *HvPIP1;6* was studied through expression of *HvPIP1;6* in *Xenopus laevis* oocytes. The effect on water transport of phosphatase and kinase inhibitors and of HgCl_2 was tested; ABA, which increases

hydraulic conductance at the cell and root level (Hose et al. 2000), was also tested in terms of its effect on water channel activity. Plasma membranes were isolated from different leaf zones and Western analyses were used to probe for levels of PIPs.

Results

Isolation of cDNA clones

Based on the sequence of *HvEmip*, primers were designed to isolate and functionally characterize cDNA clones of closely related PIPs in the barley cultivar Golf. Two cDNA clones were isolated from RNA extracted from leaf tissues, a full-length clone and a shorter clone.

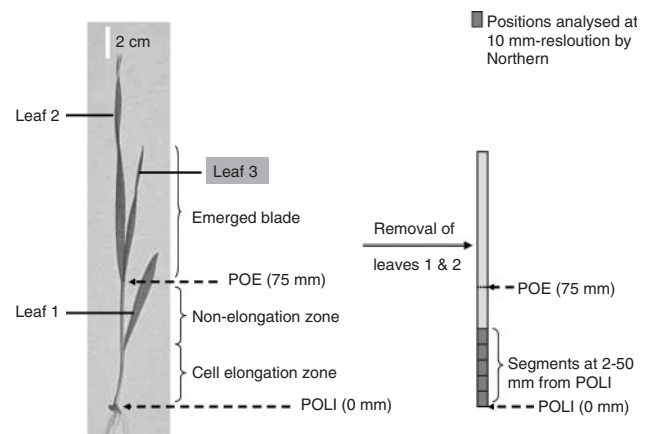


Fig. 1 Scheme of a barley plant detailing leaf regions analyzed for expression of *HvPIP1;6* (and other genes; Northern analyses) and for abundance of PIPs (Western analyses). The growing leaf 3 was analyzed at a developmental stage when it was elongating at (near-)maximum velocities and blade elongation contributed most to elongation of the leaf. The only exception was an experiment shown in Fig. 3B (left-hand column), in which plants were analyzed 1–2 d later in development to study the expression of *HvPIP1;6* in growing sheath tissue. Prior to extraction of RNA or isolation of plasma membrane proteins, the older leaves 1 and 2 were peeled off and the younger leaf 4 was removed from within leaf 3. The elongation zone (EZ) of leaf 3 stretches from about 2 mm to about 40 mm from the point of leaf insertion (POLI). Above the EZ is the ‘non-elongation zone’ (NEZ) in which cells have ceased elongation yet show residual lateral expansion. This zone extends to the point of emergence (POE) of leaf 3 from the sheath of leaf 2. The POE is typically between 70 and 80 mm from POLI. Above the POE is the emerged blade portion (EmBL) of the growing leaf 3. For Northern analyses, the central portion of the EZ (10–30 mm from POLI) was analyzed, which shows the highest relative elemental growth rates, or the base 50 mm were analyzed at 10 mm resolution (see Fig. 3). In addition, 20 mm long mid-portions of the non-elongation zone and of the emerged blade portion were analyzed. Western analyses required more plant material and the EZ, NEZ and EmBL samples consisted of the entire elongation zone (2–40 mm from POLI), the entire non-elongation zone (40 mm from POLI to POE) and the entire emerged blade portion (POE to tip of leaf 3), respectively.

The shorter clone lacked the entire N-terminal half of the sequence, including one of the two NPA motifs which are characteristic and essential for functioning of water channels. Therefore, all further analyses were carried out with the longer, full-length transcript.

Sequence alignment of the full-length clone with known (barley) PIP1 aquaporins classified the clone as a PIP that belongs to the subgroup of PIP1s (Fig. 2). The present PIP1 was termed *HvPIP1;6* (EMBL accession No. CO720031). Identity at the nucleotide level of the entire sequence (coding and 3' untranslated region) of *HvPIP1;6* (1,088 bp) to other, known barley PIP1s was as follows: *HvEmip* (1,133 bp, 99% identity), *HvPIP1;5* (1,065 bp, 72%), *HvPIP1;3* (879 bp, 83%), *HvPIP1;2* (1,039 bp, 70%) and *HvPIP1;4* (1,120 bp, 66%). Sequence identity of the encoded protein between *HvPIP1;6* (288 amino acids) and other barley PIP1s was: *HvEmip* (288 amino acids, 99% identity), *HvPIP1;5* (290 amino acids, 93%), *HvPIP1;3* (292 amino acids, 87%), *HvPIP1;2* (292 amino acids, 87%) and *HvPIP1;4* (292 amino acids, 88%). [During revision of this manuscript, a barley PIP1 was annotated as *HvPIP1;1*; this PIP1 encodes a protein which is identical to the protein encoded by *HvPIP1;6* (see also Fig. 2).]

HvPIP1;6 expression

Different expression reference systems were used to test to what degree differences in expression of *HvPIP1;6* between leaf zones were specific for this particular gene. Both actin and 18S rRNA proved not to be suitable references for comparison between growing and non-growing leaf tissues since expression was considerably higher in growing compared with non-growing tissues. However, within the leaf elongation zone, expression of 18S rRNA varied little between fast- and slow-growing regions and could be used as reference (see Fig. 3). Another possible reference, tubulin, has also been shown to vary in expression between growing and non-growing tissues (Schröder et al. 2002). Therefore, the most suitable reference for comparison of growing and non-growing leaf regions proved to be ethidium bromide staining of gels (together with spectrophotometric checking of RNA concentration in samples), confirming equal loading and uniform running of (total) RNA on gels. [After completing the bulk of the analyses, we observed in a related study on cuticle development in barley (Richardson et al. 2007) that expression of ubiquitin changes little between leaf zones and is a suitable expression reference. This reference gene was used for real-time PCR analyses, where expression of *HvPIP1;6* was compared with the expression of other barley PIP1s (see Fig. 5).]

Using Northern analyses, expression of *HvPIP1;6* along the growing leaf 3 of barley was highest in the elongation zone and hardly detectable in the non-elongation

zone and emerged blade (Fig. 3A). Within the elongation zone, tissue segments elongated at the highest relative elemental rates (change in length per segment length and time) in the center region (see also Fricke 2002). Expression of *HvPIP1;6* was highest in these regions and lowest in the most proximal and distal regions, showing the lowest relative elemental growth rates (Fig. 3B).

Expression of *HvPIP1;6* was also followed along roots. As for leaves, expression was highest in tissue encompassing the growth zone (Fig. 3B). Whether *HvPIP1;6* is also expressed in seeds or reproductive tissue was not tested.

To test whether high expression of *HvPIP1;6* was specific to growing blade leaf tissue, leaves were harvested at a later developmental stage, when sheath elongation contributed significantly to the elongation of the leaf. The length of the elongation zone at this developmental stage was reduced from about 40 mm to 30 mm, and sheath tissue accounted for most of the elongation zone (Fig. 3B). Again, expression of *HvPIP1;6* was highest in the region showing the highest relative elemental growth rate.

The expression pattern along the developing barley leaf of *HvPIP2;1* was opposite to that of *HvPIP1;6* (Fig. 3C). While *HvPIP1;6* was expressed most highly in the elongation zone, *HvPIP2;1* was expressed most highly in the emerged portion of the blade. In the non-elongation zone, expression of *HvPIP2;1* varied between being hardly detectable and being intermediate between expression levels in the other leaf zones.

The Northern blot of *HvPIP2;1* showed two bands, one at 2.20 kb and one at 1.69 kb. The probe used for Northern analysis was directed at the coding region of *HvPIP2;1* and, therefore, might have hybridized with other as yet unidentified barley PIP2(s). If so, these PIP2s must have shown an expression pattern either similar to or not interfering with the expression pattern of *HvPIP2;1* (e.g. no high expression in the elongation zone).

Tissue localization of *HvPIP1;6* expression

In situ PCR was used to study the tissue distribution of *HvPIP1;6* expression in mature and growing leaf tissue. Expression in the elongation zone was confined to the epidermis, with some expression in mesophyll cells bordering the epidermal cells (Fig. 4). Under the experimental conditions (32 cycles of PCR), none of the other leaf tissues (bundle sheaths, xylem and phloem) showed an expression signal (Fig. 4). In the mature blade, an expression signal was non-detectable in all tissues, including the epidermis (not shown).

Expression of *HvPIP1;6* in relation to expression of other barley PIP1s

Towards the end of this study, two additional barley PIP1s were annotated, *HvPIP1;2* and *HvPIP1;4*. *HvPIP1;1*,

HvPIP1_6	MEGKEEDVRLGANKYSERQPIGTAAQGGSE----DKDYKEPPPAPLFEFGELKSNWSFYRAG	56
HvEmip	MEGKEEDVRLGANKYSERHAIQTAAQGGSE----DKDYKEPPPAPLFEFGELKSNWSFYRAG	56
ZmPIP1_3	MEGKEEDVRLGANKFSERQPIGTAAQAGAGDDDDKDYKEPPPAPLFEFGELKSNWSFYRAG	60
OsPIP1_1	MEGKEEDVRLGANRYSERQPIGTAAQAGAG--DDKDYKEPPPAPLFEFGELKSNWSFYRAG	57
HvPIP1_5	MEGKEEDVRLGANRYSERQPIGTAAQGGG---DDKDYKEPPPAPLFEFGELKSNWSFYRAG	57
AtPIP1_3	MEGKEEDVRLGANKFPERQPIGTSAQT-----DKDYKEPPPAPLFEFGELSSWSFYRAG	54
SoPIP1_2	MEGKDEDVRLGANKYGERKQGLGTVAQ-----DRDYKEPPPAPLFEFGELTWSFYRAG	53
HvPIP1_2	MEGKEEDVRLGANRYSERQPIGTAAQGGG--ADEKDYKEPPPAPLFEAEELTWSFYRAG	58
HvPIP1_4	MEGKEEDVRLGANRYSERQPIGTAAQGGG--ADEKDYKEPPPAPLFEAEELSSWSFYRAG	58
HvPIP1_3	MEGKEEDVRLGANRYSERHPIGTAAQGGG--ADEKDYKEPPPAPLFEAEELTWSFYRAG	58
TaPIP1	MEGKEEDVRLGANRYSERHPIGTAAQGGG--ADKKDYKEPPPAPLFEAEELTWSFYRAG	58
HvPIP2_1	-MAKDIEAAPQGGGFS-----SKDYSDFPPAPIVDFFELTKWSLYRAV	42
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HvPIP1_6	IAEFMATFLFLYVTLTVMGYSG-----AAS--KCATVGIQGIAWSFGGMI FALVYCTA	108
HvEmip	IAEFMATFLFLYVTLTVMGYSG-----AAS--KCATVGIQGIAWSFGGMI FALVYCTA	108
ZmPIP1_3	IAEFVATFLFLYITVLTVMGVSK-----STS--KCATVGIQGIAWSFGGMI FALVYCTA	112
OsPIP1_1	IAEFVATFLFLYITVLTVMGVSK-----SSS--KCATVGIQGIAWSFGGMI FALVYCTA	109
HvPIP1_5	IAEFVATFLFLYITVLTVMGVSK-----APS--KCATVGVQGIAWSFGGMI FALVYCTA	109
AtPIP1_3	IAEFVATFLFLYITVLTVMGVSK-----APN--MCASVGIQGIAWAFGGMI FALVYCTA	106
SoPIP1_2	IAEFVATFLFLYISILTVMGYSR-----ADN--KCKTVGIQGIAWAFGGMI FALVYCTA	105
HvPIP1_2	IAEFVATFLFLYISVLTVMGVVVG-----NPSGSKCGTVGIQGIAWSFGGMI FVLYVYCTA	112
HvPIP1_4	IAEFVATFLFLYISVLTVMGVVVG-----NPSGSKCGTVGIQGIAWSFGGMI FVLYVYCTA	112
HvPIP1_3	IAEFVATFLFLYISVLTVMGVVVG-----NPSGSKCGTVGIQGIAWSFGGMI FVLYVYCTA	112
TaPIP1	IAEFVATFLFLYISVLTVMGVVVG-----NPSGSKCGTVGIQGIAWSFGGMI FVLYVYCTA	112
HvPIP2_1	IAEFVATFLFLYITVATVIGYKHQSDPTVNTDAACSGVGLGIAWAFGGMI FVLYVYCTA	102
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HvPIP1_6	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQGLYMGNGGGAN	168
HvEmip	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQGLYMGNGGGAN	168
ZmPIP1_3	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQGLYMGNGGGAN	172
OsPIP1_1	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQGLYMGNGGGAN	169
HvPIP1_5	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQGLYMGNGGGAN	169
AtPIP1_3	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQPNPYQLGGGAN	166
SoPIP1_2	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQPPYQVGGGGSN	165
HvPIP1_2	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQTTLYMGNGGGAN	172
HvPIP1_4	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQTTLYMGNGGGAN	172
HvPIP1_3	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQTTLYMGNGGGAN	172
TaPIP1	GISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQTTLYMGNGGGAN	172
HvPIP2_1	GVSGGHINPAVTFGLFLARKVSLIRALLYIIAQCLGAICGAGVVKGFQSSYYVRYGGGAN	162
	*:*****:*****: **.*: * * * * * : * * * * * : * * * * * : * * * * *	
HvPIP1_6	VVASGYTKGSGGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	228
HvEmip	VVASGYTKGSGGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	228
ZmPIP1_3	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	232
OsPIP1_1	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	229
HvPIP1_5	VVASGYTKGSGGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	229
AtPIP1_3	VVASGYTKGSGGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	226
SoPIP1_2	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	225
HvPIP1_2	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	232
HvPIP1_4	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	232
HvPIP1_3	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	232
TaPIP1	VVASGYTKGSGGLGAEIVGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	232
HvPIP2_1	ELVAGYTKGSGGLGAEIIGTFVLVYTVFSATDAKRNARDSHVPI LAPLPIGFVAVFLVHLAT	222
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HvPIP1_6	IPITGTGINPARSLGAAIYINREH-AWSDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	284
HvEmip	IPITGTGINPARSLGAAIYINREH-AWSDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	284
ZmPIP1_3	IPITGTGINPARSLGAAIYINRDH-AWSDHWIFWVGPFIGAALAAIYHQVIR--AIPF-	288
OsPIP1_1	IPITGTGINPARSLGAAIYINKDH-AWSDHWIFWVGPFIGAALAAIYHQVIR--AIPF-	285
HvPIP1_5	IPITGTGINPARSLGAAIYINRDH-AWSDHWIFWVGPFIGAALAAIYHQVIR--AIPF-	286
AtPIP1_3	IPITGTGINPARSLGAAIYINKDH-AWSDHWIFWVGPFIGAALAAIYHQVIR--AIPF-	282
SoPIP1_2	IPITGTGINPARSLGAAIYINKQ--SWDDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	282
HvPIP1_2	IPITGTGINPARSLGAAIYINKQ--SWDDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	288
HvPIP1_4	IPITGTGINPARSLGAAIYINKQ--AWDDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	288
HvPIP1_3	IPITGTGINPARSLGAAIYINKQ--AWDDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	288
TaPIP1	IPITGTGINPARSLGAAIYINKQ--AWDDHWIFWVGPFIGAALAAIYHQVVIR--AIPF-	288
HvPIP2_1	IPITGTGINPARSLGAAIYINTDK-AWDDHWIFWVGPFIGAALAAIYHQVLRASAALG	281
	*****:*****: **.*: * * * * * : * * * * * : * * * * * : * * * * *	
HvPIP1_6	KTKS- 288	
HvEmip	KTKS- 288	
ZmPIP1_3	KSRs- 292	
OsPIP1_1	KSRs- 289	
HvPIP1_5	KSRs- 290	
AtPIP1_3	KSRs- 286	
SoPIP1_2	KSK-- 285	
HvPIP1_2	KSRD- 292	
HvPIP1_4	KSRD- 292	
HvPIP1_3	KSRG- 292	
TaPIP1	KSRD- 292	
HvPIP2_1	SYRSN 286	
	: :	

Fig. 2 Sequence alignment of PIP1 and PIP2 isoforms. Amino acid sequences of the PIP isoforms HvPIP1;6 (*Hordeum vulgare*, accession No. CO720031; deduced protein sequence shown here), HvEmip (X76911), HvPIP1;2 (BAF33067), HvPIP1;3 (AB009308), HvPIP1;4 (BAF33068), HvPIP1;5 (AB009309), ZmPIP1;3 (*Zea mays*, AF326487), OsPIP1;3 (*Oryza sativa*, BAD22920), TaPIP1 (*Triticum aestivum*, AF366564), AtPIP1;3 (*Arabidopsis thaliana*, BT006313), SoPIP1;2 (*Spinach oleracea*, AY372191) and of the PIP2 isoform HvPIP2;1 (AB009307) were aligned using ClustalW. The NPA boxes are highlighted with a light gray background. The motif recognized by the PIP1 antibody is boxed. Note the absence of this motif in the only PIP2 shown. The two amino acids which differ between HvEmip and other PIP1s, including HvPIP1;6, are also highlighted with a light gray background (positions 19 and 20). HvPIP1;1 (BAF41978), which is identical in protein sequence to HvPIP1;6, is not shown.

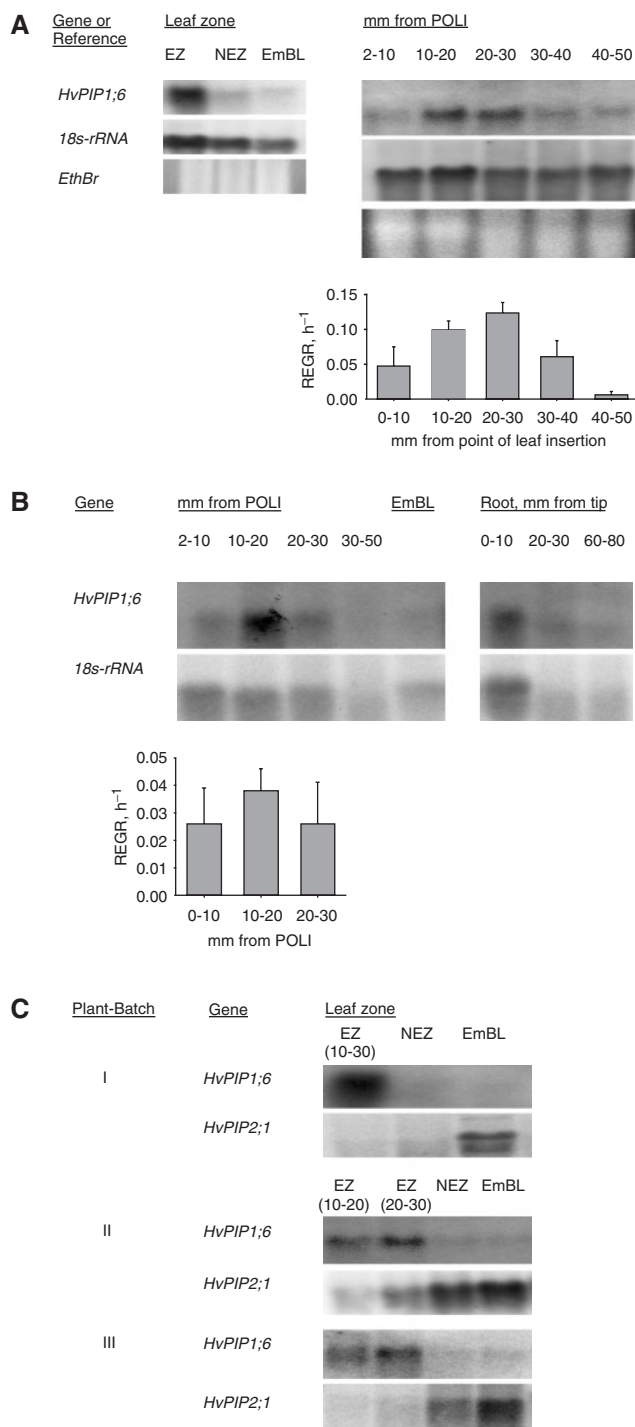


Fig. 3 (A) Expression of *HvPIP1;6* along the growing leaf 3 of barley. Left-hand column: expression was studied in the central portion of the leaf elongation zone, EZ [10–30 mm from the point of leaf insertion (POLI)], in the non-elongation zone, NEZ (50–70 mm) and in the emerged blade (2 cm long mid-portion), EmBL. Different expression reference systems were used for Northern analyses: 18S rRNA, actin (not shown) or ethidium bromide staining of gels. Right-hand column: expression of *HvPIP1;6* along the leaf elongation zone at higher spatial resolution.

which was also annotated, is identical in protein sequence to *HvPIP1;6*. This made it possible to compare the expression of *HvPIP1;6* with that of several other barley PIPs, which might well comprise the entire set of barley PIPs (see Discussion). Expression of genes was quantified by real-time PCR using primers designed for the gene-specific untranslated region. Expression of PIPs was related to expression of the housekeeping gene ubiquitin. The value obtained for roots was set to 1.0 for comparison between tissues. Fig. 5A shows that *HvPIP1;6* was not the only barley PIP1 that was expressed most highly in the leaf elongation zone, but that *HvPIP1;5* showed a similar expression profile. In contrast, *HvPIP1;3* was expressed primarily in root and mature leaf tissue. Expression of *HvPIP1;2* was even more root specific (Fig. 5). Expression of *HvPIP1;4* was near the limit of detection and there was no consistent pattern in expression between root and leaf tissues (not shown).

Real-time PCR is more sensitive than Northern analyses, and this could explain why expression of *HvPIP1;6* was also detectable in non-growing leaf tissue. On average, expression of *HvPIP1;6* was 3.6 times higher in growing compared with non-growing leaf tissues.

Using expression values of PIPs related to ubiquitin, the quantitative contribution of each PIP1 isoform to expression of 'total' PIPs for a particular leaf tissue could

The spatial distribution of average relative elemental growth rates (REGRs) of 10 mm long segments along the leaf elongation zone is also shown (error bars represent the SD; between 18 and 22 individual values were averaged for each segment; residual elongation within the segment 40–50 mm from POLI is due to elongation of tissue just distal to 40 mm). REGR profiles were determined according to Fricke and Peters (2002). (B) Expression of *HvPIP1;6* in growing sheath tissue (left-hand column) and along seminal roots (right-hand column). Expression of *HvPIP1;6* was analyzed when sheath elongation contributed most to elongation of leaf 3. Sh1 and Sh2 refer to sheath tissue at 2–10 and 10–20 mm from POLI; B1 and B2 refer to adjacent blade tissue segments at 20–30 and 30–50 mm from POLI. Expression is also shown for the emerged blade portion (EmBL). Average REGRs for segments Sh1, Sh2 and B1 are shown, together with the SD (error bars; $n=25-31$ individual values for each leaf segment). At this advanced leaf developmental stage, REGR was negligible at locations distal to 30 mm from POLI. Expression of *HvPIP1;6* along seminal roots was analyzed at 0–10 mm (this region includes the growth zone), at 20–30 mm (zone where lateral roots start) and at 60–80 mm (zone where root hair formation commences) from the tip. (C) Comparison of expression of *HvPIP1;6* and *HvPIP2;1* along the growing leaf 3. Results of three batches of plants are shown; for each batch, the same gel and (blotted) membranes were used for Northern analysis of *HvPIP1;6* and *HvPIP2;1*, rendering the use of an expression reference such as 18S rRNA unnecessary. Within the elongation zone, expression was followed within the central portion showing the highest relative elemental growth rates, at 10–30 mm (batch I) or at 10–20 and 20–30 mm POLI (batches II and III).

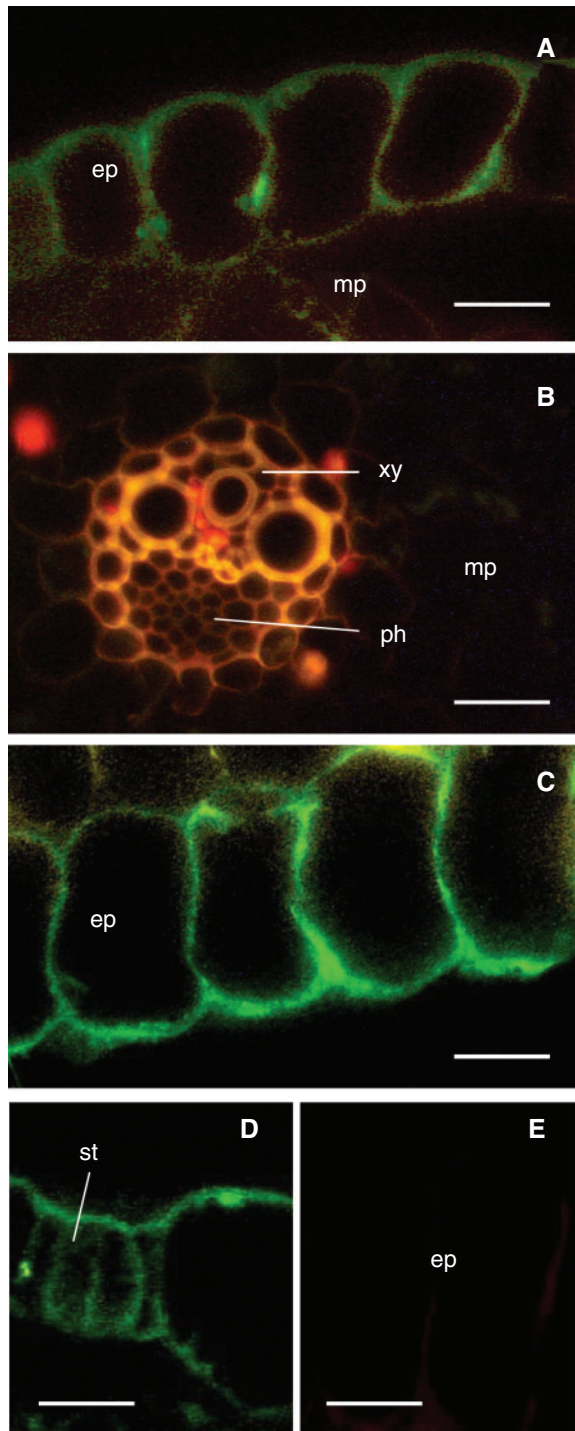


Fig. 4 In situ PCR localization of *HvPIP1;6* expression in the elongation zone of barley leaves. *HvPIP1;6* expression is shown by green fluorescence; orange-red color (as in B) is due to autofluorescence of tissue (cell walls and chloroplasts). (A) Cross-section of upper epidermal cells. (B) Cross-section of a vascular bundle and mesophyll. (C) Cross-section showing lower epidermal cells. (D) Stomatal guard cells. (E) Background control. The bar equals 20 μm (A, B) and 15 μm (C–E). ep, epidermis; st, stomatal guard cells; mp, mesophyll; xy, xylem; ph, phloem.

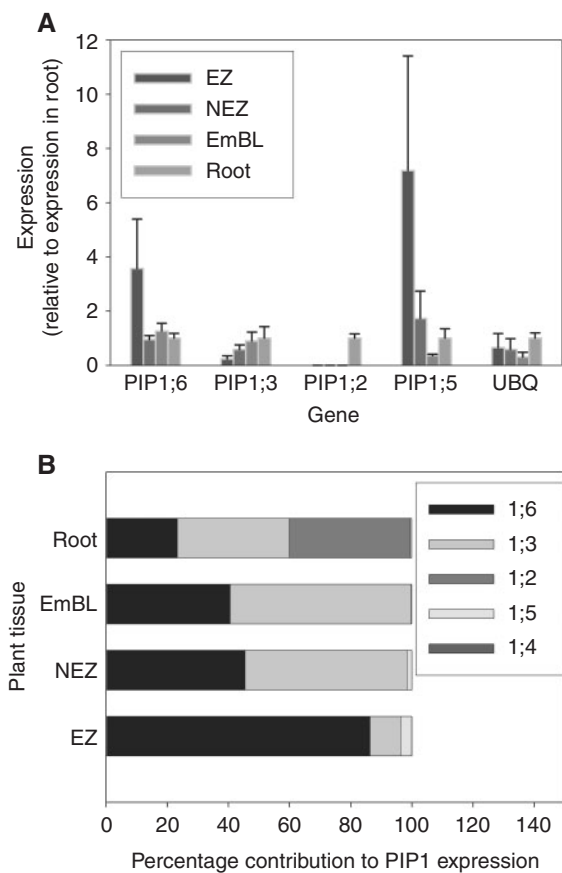


Fig. 5 Real-time RT-PCR expression analyses of barley PIP1 isoforms. Three batches of plants were harvested, RNA isolated, reverse-transcribed into cDNA and expression quantified by real-time RT-PCR. Expression of PIP1s was standardized by relating values to expression of the housekeeping gene ubiquitin. (A) For comparison between plant tissue regions, the value for the root was set to 1.0 (the root sample comprised both growing and mature tissue at a fresh weight ratio of $\sim 1:1$). Data are means of three experiments, error bars represent the SD; the error bar for roots represents the SD of PIP:ubiquitin expression ratios for three experiments. Expression data for ubiquitin are also shown. Expression data for *HvPIP1;4* are not shown since values were near the limit of detection; this would have resulted in artificially high/low values when calculating ratios. (B) Contribution to total expression of PIP1s in leaf and root tissue of each PIP1 isoform (*HvPIP1;6*, 1;3, 1;2, 1;5 and 1;4). The relative contents of PIP1 mRNAs are expressed as the percentage on the basis of relative (PIP gene : ubiquitin) copy numbers of mRNAs. Values are averages of three experiments. For clarity of the figure, SDs are not shown. For some isoforms, the percentage contribution was smaller than the thickness of lines. EZ, elongation zone; NEZ, non-elongation zone; EmBL, emerged blade.

be calculated (Fig. 5; 'total', if we assume that *HvPIP1;2–1;6* represent the entire set of barley PIP1s). The only tissue in which expression of one PIP1 isoform dominated expression of PIP1s was the leaf elongation zone. Here, *HvPIP1;6* accounted for 86.4% of expression of

PIP1s; the only other PIP1s showing significant expression were *HvPIP1;3* (10%) and *HvPIP1;5* (3.6%). The same three PIP1s accounted for the bulk of PIP1 expression in the non-elongation zone and emerged blade, except that expression of *HvPIP1;3* accounted for most of the PIP1 expression and that the contribution of *HvPIP1;6* halved, to 41–46%. In root tissue, the contribution of *HvPIP1;6* further decreased, while *HvPIP1;3* and *HvPIP1;2* each accounted for >35% of expression; in leaf tissues, expression of *HvPIP1;2* accounted for <0.01% of expression of PIP1s. Although the contribution of the expression of *HvPIP1;5* to PIP1 expression was consistently low (0.3–3.6%), it was present in all leaf tissues. This contrasted with the situation for *HvPIP1;4*, where expression was inconsistent between tissues and even lower (<0.0001%)—so low that it did not permit any reliable estimate of expression levels [although expression was at the limit of detection of real-time PCR, PCR product could be detected by analyzing samples by agarose gel electrophoresis and staining with ethidium bromide (not shown)].

The sum of PIP1 expression, related to ubiquitin, for leaf regions and root was 9.51 ± 6.25 (elongation zone), 4.35 ± 0.78 (non-elongation zone), 6.31 ± 2.29 (emerged blade) and 10.25 ± 2.05 (root), respectively (means \pm SD of three harvests).

Water channel activity (oocyte assay)

Expression of *HvPIP1;6* in oocytes increased the P_f of oocytes between 4- and 6-fold, from about $0.9 \times 10^{-3} \text{ cm s}^{-1}$ in H_2O -injected oocytes (control) to $5.2 \times 10^{-3} \text{ cm s}^{-1}$ in mRNA-injected oocytes (Fig. 6A, B; $P < 0.001$). The increase in P_f was prevented by pre-incubation of oocytes with $50 \mu\text{M}$ HgCl_2 (Fig. 6C).

Pre-incubation of oocytes with the phosphatase inhibitor okadaic acid (OA) almost doubled the mRNA-dependent P_f (Fig. 6D; $P < 0.001$ when comparing mRNA-injected oocytes with or without pre-treatment with OA); OA had no significant effect on the P_f of water-injected oocytes. Pre-treatment of oocytes with the kinase inhibitor k252a had no significant effect on mRNA-dependent P_f , but the response varied considerably between oocytes (Fig. 6D, large error bars).

Pre-treatment of oocytes with $50 \mu\text{M}$ ABA increased P_f in both mRNA- and water-injected oocytes (Fig. 6E), and by a similar proportion (66 and 61%, respectively). The ABA-dependent increase in P_f in mRNA-injected oocytes was highly significant ($P < 0.001$, comparing P_f in mRNA-injected oocytes in the presence or absence of ABA).

Plasma membrane isolation and Western blotting

Plasma membranes were isolated from different developmental zones of the growing leaf 3 of barley and analyzed

by SDS-PAGE and Western blotting. Coomassie-stained gels showed bands at molecular mass positions typical for monomeric (28–29 kDa) and dimeric isoforms of PIPs (55–58 kDa, Fig. 7). The emerged portion of the leaf showed a dimeric band at slightly higher molecular mass. Dimeric and, particularly, monomeric bands were strongest in the emerged zone.

Anti-PIP1 antibody reacted most in the elongation zone and adjacent non-elongation zone. Reaction in the emerged blade was less than in the other leaf zones and varied between plant batches (Fig. 7, batches I–III).

Anti-*HvPIP2;1* antibody reacted more weakly with plasma membrane preparations than did anti-PIP1 antibody (not shown). In addition, the anti-*HvPIP2;1* antibody reaction varied between plasma membrane preparations and produced variable results for leaf regions. In the lower molecular weight region, the antibody reacted at around 28–29 kDa, the expected region for PIP2 monomers, but also reacted at a slightly higher molecular weight (~34–35 kDa, not shown, see also band pattern in Katsuhara et al. 2002).

Some of the plasma membrane preparations of the emerged leaf portion appeared greenish, which suggested the presence of chloroplast-derived proteins, whereas preparations of the non-elongation zone were less green and those of the elongation zone clear in color. One of the most abundant proteins in chloroplasts are light-harvesting chlorophyll *a/b*-binding proteins (LHCbs). Their monomeric molecular weight is similar to that of aquaporin monomers, and LHCb contamination of samples of the emerged leaf region could have explained strong Coomassie-stained bands at 28–29 kDa. This was confirmed by Western analyses using a mix of antibodies directed at various isoforms of LHCb (Fig. 7).

Discussion

HvPIP1;6

The barley PIP1 characterized here, *HvPIP1;6*, has high sequence identity to the previously studied *HvEmip* (Hollenbach and Dietz 1995) and is identical to a recently annotated barley PIP1, *HvPIP1;1*, the biochemical functions of which are not known. The coding region of *HvPIP1;6* and *HvEmip* differs in only two amino acids (and nucleotides); their untranslated region is identical too, except for an additional 6 bp repeat in *HvEmip* (*HvPIP1;6* with TGCTCT at bp positions 1,018–1,023; and *HvEmip* with an additional TGCTCT at bp positions 1,024–1,029). Most probably, *HvPIP1;6* and *HvEmip* represent cultivar-specific alleles of the same gene (but see also next paragraph). If the two genes co-existed in the barley cultivar studied (Golf), this should have resulted in the isolation of both clones when using the primers for

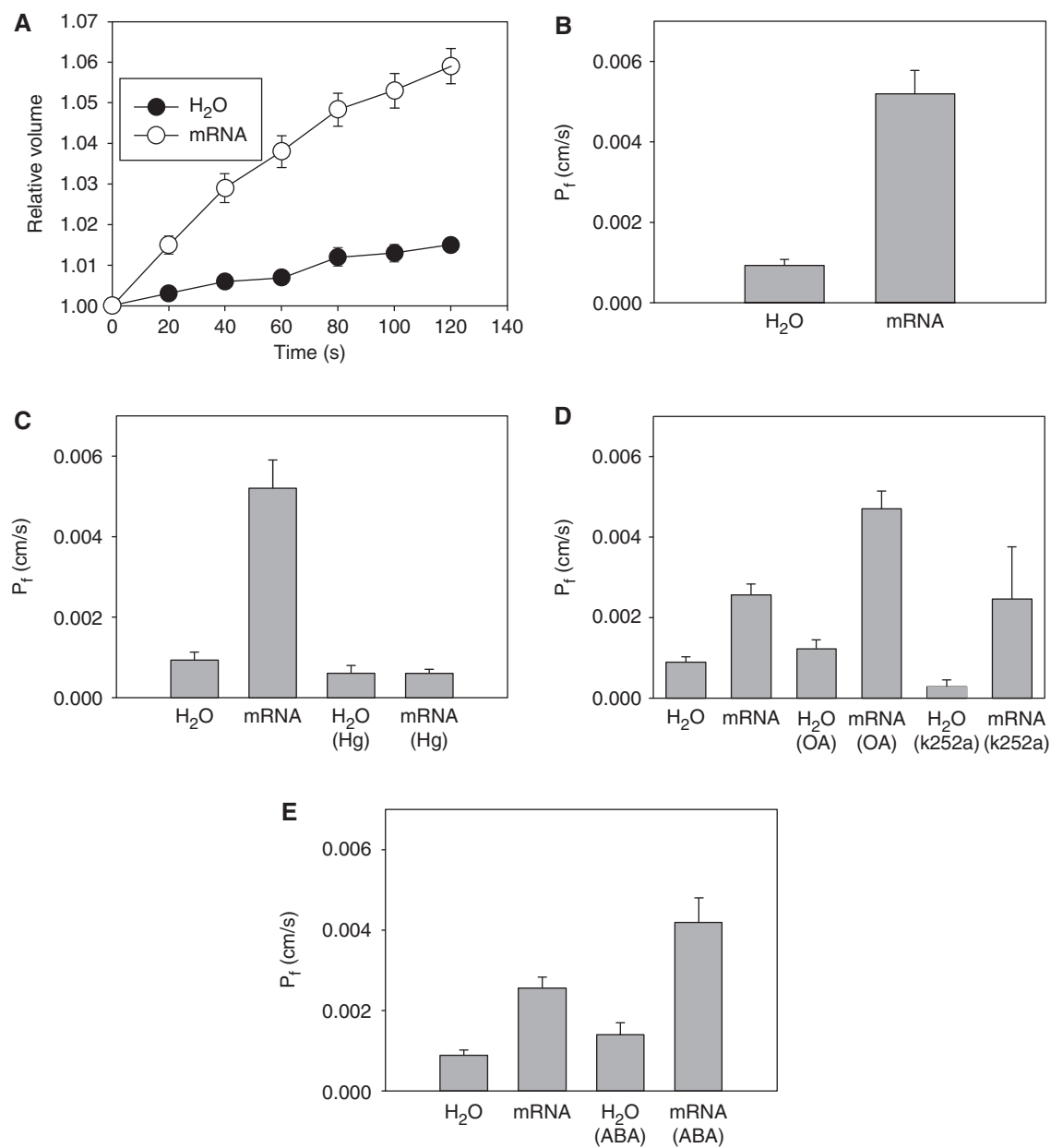


Fig. 6 (A, B) Osmotic water permeability, P_f , in oocytes expressing mRNA of HvPIP1;6, and the effects of (C) HgCl₂, (D) OA and k252a, and (E) ABA on P_f . Control oocytes were injected with H₂O instead of mRNA. In A, a time course of increase in relative oocyte volume following transfer to hypo-osmotic medium is shown; P_f values are shown in B. Results are means \pm SE; the number (n) of oocytes analyzed was: (A–C) H₂O (24), mRNA (29), H₂O–Hg (7), mRNA–Hg (7); (D) H₂O (18), mRNA (14), H₂O–OA (18), mRNA–OA (20), H₂O–k252a (5) and mRNA–k252a (4); (E) H₂O (18), mRNA (14), H₂O–ABA (5) and mRNA–ABA (6).

isolation of *HvPIP1;6* (which were designed based on the sequence of *HvEmip*). More than 30 transformed colonies were sequenced and none showed the sequence of *HvEmip*.

HvPIP1;6 has a glutamine and proline at positions 19 and 20, respectively, similar to PIP1s in, for example, maize, rice and *Arabidopsis*, and other known barley PIPs (Fig. 2), including PIP1-related contig sequences at

www.barleybase.org. In contrast, HvEmip has a histidine and alanine at positions 19 and 20, respectively. We could identify only one further PIP1 among *Arabidopsis*, rice, maize and barley, which also has a histidine and alanine at positions 19 and 20 (maize ZmPIP1;1). Possibly, both HvEmip and ZmPIP1;1 fulfil functions that are different from those of close homologs or have sequencing errors in common.

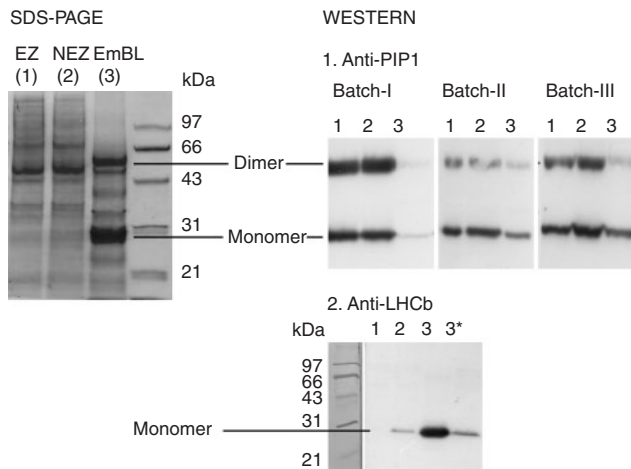


Fig. 7 SDS-PAGE and Western blot analyses of plasma membrane proteins isolated from different developmental zones of the growing leaf 3 of barley. Plasma membranes were isolated from the elongation zone (1, EZ), the adjacent non-elongation zone (2, NEZ) and from the emerged portion (3, EmBL) of the growing blade. Between 7.5 and 15 μ g of plasma membrane proteins were loaded per lane; for each gel, equal amounts of protein were loaded of the three leaf zones. Gels were either stained for protein (Coomassie, SDS-PAGE) or Western blotted with an antibody recognizing barley PIP1s or LHCb. The PIP1 antibody stained bands at positions where monomeric (~28–29 kDa) and dimeric (56–58 kDa) forms of aquaporins would be expected. Anti-PIP1 blots for three different batches of plants (batches I–III) are shown. One Western blot with anti-LHCb antibody is shown for leaf zones 1–3; two samples (3 and 3*) of the emerged leaf zone were analyzed in the anti-LHCb blot. One sample (3, from batch II) gave a strong Coomassie-stained band [see SDS-PAGE '(3)'] and a strong LHCb band; the other sample (3*, from batch III) gave a weak Coomassie-stained band (not shown) and a weaker LHCb band.

Because of the above, we could not rule out with certainty that *HvPIP1;6* and *HvEmip* encode proteins of different function, and therefore decided to term the clone isolated here differently, as *HvPIP1;6*.

HvPIP1;6 is expressed particularly in the leaf epidermis, in the elongation zone. In comparison, expression of *HvEmip* was studied in leaf 1 and at an advanced developmental stage, possibly with little residual blade elongation; expression was highest in the base 1 cm ('basiplast' of leaf 1; Hollenbach and Dietz 1995) comprising both the cell division and elongation zone, and, on a total RNA basis, expression was highest in epidermal peels, which were most probably taken from mature tissue (Hollenbach and Dietz 1995). Leaf 1 differs from all subsequent leaves of barley in several respects. The morphology of leaf 1 is particular; the blade elongation zone is enclosed in a 1–2 cm short coleoptile and not in sheaths that are 6–10 cm long, and leaf 1 relies entirely on seed storage reserves for mineral nutrient supply.

Water channel activity of *HvPIP1;6*

Expression of *HvPIP1;6* in oocytes increased P_f up to 6-fold. Pre-incubation with 50 μ M $HgCl_2$ prevented the increase in P_f . It therefore appears justified to classify *HvPIP1;6* as a water channel.

Water channel activity is generally associated with PIP2s rather than PIP1s, a noticeable exception being PIP1s of *Arabidopsis* (Kammerloher et al. 1994). Water channel activity has been tested for PIP1s of other species (Biela et al. 1999, Dixit et al. 2001), including three PIP1s of maize, *ZmPIP1;1*, *ZmPIP1;2* and *ZmPIP1;5* (Chaumont et al. 1998, Chaumont et al. 2000, Gaspar et al. 2003), one PIP1 of rice (Li et al. 2000) and three PIP1s of radish (Suga et al. 2004). However, these PIP1s showed either no or only a slight increase (a factor of 1.8–2.2) in P_f when expressed individually in oocytes or yeast, with the exception of radish PIP1;3, which increased P_f in yeast to 3.2 times the control value. Fetter et al. (2004) observed that when maize *ZmPIP1;1* and *ZmPIP1;2* were co-expressed in oocytes, they displayed water channel activity, raising the possibility that some of the other PIP1s tested previously for water channel activity through individual expression in oocytes may well act as water channels in planta. So far, the PIP1 characterized here, *HvPIP1;6*, is the first PIP1 described for grasses which increases P_f by a factor generally associated with PIP2s when expressed individually in oocytes.

Unlike PIP2s, PIP1s may transport boron or glycerol (Biela et al. 1999, Dordas et al. 2000). The ability to transport these neutral solutes functionally defines the PIP1 family but does not prove that they move through the proteins in planta (Zhou et al. 1998). Movement of glycerol and boron through *HvPIP1;6* was tested qualitatively, using oocytes expressing *HvPIP1;6* mRNA (results not shown). Oocytes were pre-loaded with either glycerol or boron and subsequently transferred to hypo-osmotic media devoid of these solutes. Pre-loading reduced the swelling rate by >80% compared with oocytes which had not been pre-loaded and was most probably due to competition for passage through *HvPIP1;6* between boron and glycerol (moving out of the oocyte) and water (moving into the oocyte). This suggests that *HvPIP1;6* allows some passage of boron and glycerol. The latter could assist in wax ester production in the barley leaf epidermis (Richardson et al. 2005).

HvPIP1;6 and its role in elongation growth

The observation that *HvPIP1;6* is highly expressed in growing blade and sheath leaf tissues and in the growth zone of seminal roots suggests that the over-riding factor influencing expression of *HvPIP1;6* is growth.

Biophysical analyses of the barley leaf elongation zone suggest that growth is limited by the hydraulic properties of tissues (Fricke and Flowers 1998, Fricke 2002). Seven large

lateral veins supply water to the elongating leaf epidermis (Fricke 2002). If water moved along the transcellular path, it would have to pass up to 30 epidermal cells to reach those cells that are located halfway between two adjacent veins, and epidermal water transport could become growth limiting (Fricke 2002). Cell pressure probe studies suggest that symplastic water transport between epidermal cells is negligible in barley, at least in mature tissue (Fricke 2000). This leaves the plasma membrane as the most likely site where water transport is regulated. In a recent study on barley, we observed that plasma membrane water permeability of leaf epidermal cells is 34% higher in the elongation zone compared with the emerged blade (Volkov et al. 2007). Some of this difference may be due to the action of HvPIP1;6, which is expressed 3–4 times more in the elongation zone (based on real-time PCR data; Fig. 5A). Western analyses suggest that protein levels of HvPIP1;6 are also considerably higher in the elongation zone compared with the emerged blade: the signal of the antibody that recognized (most likely) all barley PIP1 isoforms was higher in the elongation zone, where expression of HvPIP1;6 accounted for almost 90% of expression of PIP1s.

In addition to the above expression analyses, we tested two treatments for their effects on changes in expression of HvPIP1;6 (not shown). Both salinity (100 mM NaCl) and source reduction have been shown previously to reduce the leaf elongation velocity of the growing barley leaf 3 (reviewed in Fricke 2002, Fricke et al. 2006). It was hypothesized that an increase in water permeability (and water channel activity) would facilitate maintenance or recovery of some growth during stress. We followed expression of HvPIP1;6 in two replicate experiments where plants were subjected for 1–2 d to source reduction or for 10 min, 30 min, 1, 2 and 4 h to 100 mM NaCl. Although there were increases in HvPIP1;6 expression (when related to that of the reference 18S rRNA using densitometric analyses), these increases were far smaller (20–40%) than predicted based on biophysical analyses. If an increase in HvPIP1;6 water channel activity assists growth under these stresses, it must occur primarily through post-translational modification. A candidate could be ABA (Fricke et al. 2006; see also below).

Phosphorylation of HvPIP1;6

It is not possible to say whether application of phosphatase and kinase inhibitors in the oocyte swelling assay caused direct effects on the HvPIP1;6 protein or affected water permeability indirectly, for example through modifying intracellular trafficking (for a review, see Miller and Zhou 2000). Dependence of the activity of aquaporins on phosphorylation has been shown for PIP2 isoforms (e.g. Johansson et al. 1996, Johansson et al. 1998), as well as

for TIP and NIP isoforms (Maurel et al. 1995, Guenther et al. 2003), but not for PIP1s. The only barley PIP tested previously in the oocyte expression system (HvPIP2;1) showed no increase in water channel activity in response to OA (Katsuhara et al. 2002).

Phosphorylation of PIP2s at Ser274 is well documented (Johansson et al. 1996, Johansson et al. 1998), but this phosphorylation site is missing from HvPIP1;6 (and other PIP1s). There are, however, proteomic data suggesting an N-terminal phosphorylation of PIP1s (Santoni et al. 2003). In PIP2s, Ser114 is also believed to be phosphorylated (Johansson et al. 1998), and a potential, corresponding phosphorylation site in HvPIP1;6 is Ser130 (Netphos v2.0 at <http://www.cbs.dtu.dk>). Using Netphos v2.0, other potential phosphorylation sites (outside transmembrane domains and within cytosolic loops) of HvPIP1;6 are predicted at Ser28, Ser49, Ser196 and Ser207.

Regulation of HvPIP1;6 through ABA

ABA increased osmotic water permeability of oocytes expressing HvPIP1;6 when applied at physiological concentrations (50 μ M; Fricke et al. 2004). The effect was highly significant, and oocytes originating from the same batch were compared (with and without ABA) to avoid difficulties arising from batch to batch variation in P_f . ABA increased P_f by a similar proportion in mRNA- and water-injected oocytes. This suggests that ABA affects HvPIP1;6 and oocyte-endogenous water channels to a similar extent. ABA has been shown to increase cell and tissue hydraulic conductance (Hose et al. 2000), and has been proposed to gate water channels (Wan et al. 2004) and to be involved in the regulation of some maize MIP genes in response to salt (Zhu et al. 2005). In addition, there exist reports which demonstrate effects of ABA on membrane transport properties in non-plant systems (mammalian tissue, cyanobacteria), and ABA has been detected in animal tissue (reviewed in Huddart et al. 1986). Therefore, the idea that ABA affects P_f of a plant water channel in a non-plant experimental system (oocytes) is not so far-fetched.

Developmental changes in expression and abundance of PIP1s and PIP2s

On the one hand, HvPIP1;6 and HvPIP2;1 showed opposite expression patterns along the developing barley leaf. This could suggest that PIP1s and PIP2s fulfil different roles in plants (Chaumont et al. 2000, Johansson et al. 2000, Fotiadis et al. 2001). On the other hand, differences in expression pattern along the blade were also observed within the PIP1 subgroup (Fig. 5A). In addition, in a recently completed microarray study using barley Affymetrix chips (www.barleybase.org), we observed that about 50% of all MIPs are expressed differentially between leaf zones, without a strict developmental separation

between PIP1s (elongation zone) and PIP2s (emerged blade) (not shown).

Real-time PCR analyses identified one further PIP1, *HvPIP1;5*, that is particularly expressed in the leaf elongation zone. The difference in expression between elongation and other leaf zones was even larger than for *HvPIP1;6*, but the level of expression of *HvPIP1;5* was only about 5% that of *HvPIP1;6*. Real-time PCR data confirm the observation of Katsuhara et al. (2002) that *HvPIP1;3* is expressed rather evenly between root and (emerged) leaf tissue. The present data also suggest that *HvPIP1;2* is expressed in an even more root-specific manner than *HvPIP2;1* (Katsuhara et al. 2002). It remains to be shown what the functionality of *HvPIP1;2* and *HvPIP1;5* is and whether expression of these genes is confined to particular tissues within roots and leaves, respectively.

The antibody used here for Western analyses reacted with the N-terminus of *HvPIP1;6*. While this N-terminus is very different between PIP1 and PIP2 proteins and therefore ideal for design of antibodies that specifically recognize PIP1s, it is also highly conserved among plant PIP1s (see also Fig. 1). The antibody most probably reacted with all barley PIP1s, not just *HvPIP1;6*. Therefore, the Western

data suggest that the total level of PIP1 protein is higher in enclosed compared with emerged leaf tissue in barley. Real-time PCR expression analyses of the (possibly) entire set of barley PIP1s showed that the sum of expression, when related to ubiquitin, is twice as high in the elongation compared with the non-elongation zone and intermediate in the emerged blade. The discrepancy in results between real-time PCR and Western analyses when comparing the elongation and non-elongation zone could have various causes, most probably that expression levels do not necessarily reflect protein levels. Also, although ubiquitin was the most suitable reference gene for expression between leaf zones, expression was 1.5- to 2-fold higher in enclosed compared with emerged leaf tissue (Fig. 5A). This reduced values of expression ratios (PIP1:ubiquitin) in enclosed leaf tissue.

Sequence identity of barley PIPs to PIP1 in www.barleybase.org, maize and rice

There exist three plant species for which the entire set of full-length sequences of MIPs is known: *Arabidopsis* (35; Johanson et al. 2001), maize (33; Chaumont et al. 2001) and rice (33; Sakurai et al. 2005). Both rice and maize are grasses and more closely related to barley than

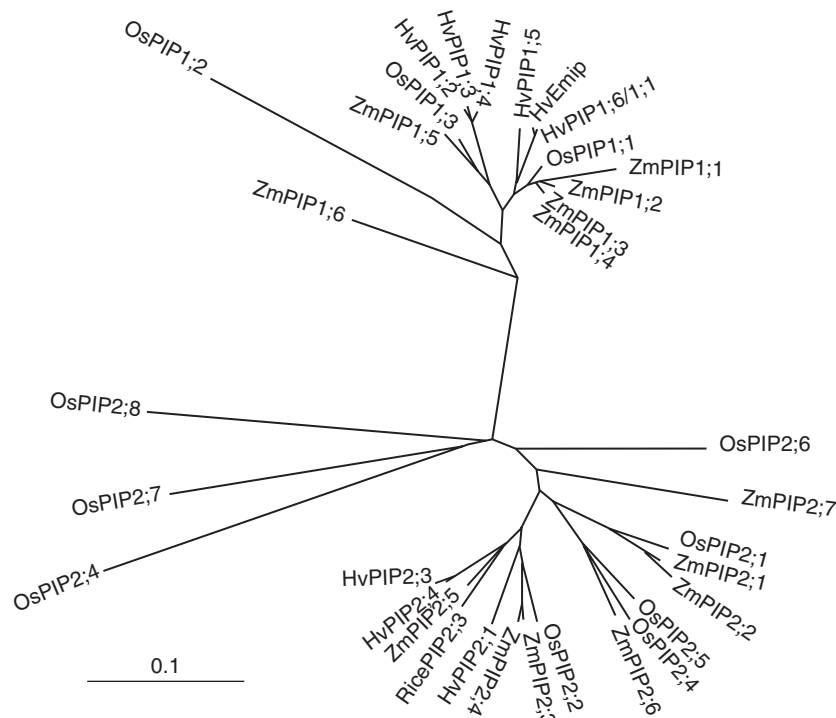


Fig. 8 Phylogenetic tree of plasma membrane intrinsic proteins of barley, maize and rice. Protein sequences were aligned using ClustalW. The distances shown (see scale bar) represent the evolutionary distance expressed in number of substitutions per amino acid. Annotations of genes are given in Fig. 1 (barley), and in Sakurai et al. (2005; rice) and Chaumont et al. (2001; maize). Note that for rice and maize, the entire set of PIPs is shown, whereas for barley, only those that are known in full sequence are shown and there are likely to be more barley PIPs, in particular PIP2s (see text for more details).

to *Arabidopsis*. In maize, there exist six PIP1s and seven PIP2s; in rice, there are only three PIP1s and eight PIP2s. In barley, five different PIP1s are known (HvPIP1;2–1;6, HvPIP1;1 being identical to HvPIP1;6 and assuming that HvEmip is identical to HvPIP1;6); in addition, three PIP2s are known (HvPIP2;1, 2;3 and 2;4). At www.barleybase.org, there are nine (contig or gene) sequences which are related to PIP1s and seven sequences related to PIP2 (summarized in Supplementary Table S1). The only PIP1-related sequence that is not identical in either full-length or partial sequence to a known barley PIP1 is contig1235, and this contig is very short (51 bp). It is therefore possible that there are no additional PIP1s contained in barleybase and that HvPIP1;2–1;6/1;1 represent the entire set of barley PIP1s. In contrast, there exist several barleybase PIP2-related contigs with sequence identity <85% to known barley PIP2s, including two full-length coding sequences contigs (contig1222 and contig1216). This suggests the existence of at least two more barley PIP2s (see also next paragraph).

A phylogenetic tree of barley, rice and maize PIPs clearly separates PIP1s from PIP2s (Fig. 8). Due to the small number of known full-length barley PIP2s, it is difficult to say whether barley PIP2s are more closely related to rice or maize, or if rice is more closely related to maize than to barley. The tree suggests the existence of at least two more barley PIP2s, one that groups with ZmPIP2;6, OsPIP2;4 and OsPIP2;5; and one that groups with ZmPIP2;1, ZmPIP2;1 and OsPIP2;1. Concerning PIP1s, the phylogenetic tree shows a closer relationship between maize and rice than between either of these two species and barley. HvPIP1;6 (and HvPIP1;5) cluster together with OsPIP1;1 and ZmPIP1;1–1;4. OsPIP1;1 does not show water channel activity (Sakurai et al. 2005) when expressed in yeast, whereas ZmPIP1;1 and ZmPIP1;2 display water channel activity when co-expressed in oocytes (Fetter et al. 2004). Possibly, all PIP1s, or all PIP1s on this branch are able to act as water channels in planta, depending on the presence or co-expression in cells of other PIP1 isoforms.

Materials and Methods

Plant material and growth conditions

Barley [*Hordeum vulgare* L. cv Golf (Svalöf Weibull AB, Svalöv, Sweden)] was grown hydroponically on modified Hoagland solution as detailed previously (Fricke and Peters 2002). All plants, except those used for isolation of plasma membranes (Lund, Sweden), were grown at Paisley, in a growth chamber (Microclima MC1000HE, CEC Technology) at 21°C day and 15°C night temperatures. Photosynthetically active radiation at the third leaf level was 350–400 μmol photons m⁻² s⁻¹, relative humidity was 70%, and the light/dark period lasted 16 h/8 h. Growth conditions at Lund were similar, except that relative humidity varied between 40 and 70% and temperature between 21 and 25°C.

Plants were analyzed at the time leaf 3 was elongating at a (near-)maximum velocity (typically 2.5–3 mm h⁻¹). This developmental stage covered the period when leaf 3 had emerged by 4–10 cm from the sheath of leaf 2. At this stage, leaf 3 grew primarily through elongation of blade tissue, and sheath elongation contributed little to leaf growth. The total length of leaf 3 ranged from about 12 to 18 cm. In one experiment, in which the expression of *HvPIP1;6* was compared between growing sheath and blade tissue, plants were harvested 1–2 d later in development.

Plant harvest and preparation of RNA extracts

Plants were harvested at the same time of day (around 10 a.m., 4 h into the photoperiod) to avoid interference from diurnal changes in aquaporin expression (Yamada et al. 1997, Henzler et al. 1999). Samples were taken from three developmental zones along the elongating leaf 3 of barley, the elongation zone, non-elongation zone and emerged blade (a scheme detailing sampling positions is shown in Fig. 1); samples from 10–20 plants were pooled for each location. The elongation zone of leaf 3 of barley stretches to about 40 mm from the point of leaf insertion and shows a bell-shaped spatial distribution of relative elemental growth rates. RNA extracts from the elongation zone were prepared either from the center region (10–30 mm section) showing the highest relative elemental growth rates (Fricke and Peters 2002) or from 10 mm long segments throughout the elongation zone (0–10, 10–20, 20–30 and 30–40 mm from the point of leaf insertion). RNA extracts were also prepared from the non-elongation zone, with tissue harvested between 50 and 70 mm from the point of leaf insertion, and from a 2 cm section halfway along the emerged blade portion.

During two experiments, root tissue was sampled. Samples were taken at the tip 10 mm, which included the elongation zone, at 20–30 mm from the tip (zone where lateral roots start) and at the root hair region.

RNA extraction and isolation of cDNA clones

Total RNA was prepared using an Ambion Totally RNA kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Contaminating DNA was removed by adding 10 U of RNase-free DNase I (Promega, Madison, WI, USA). RNA extracts were stored at –80°C.

An equal amount of total RNA (0.4 μg), which had been extracted from the growing leaf 3, was reverse transcribed into the first strand of cDNA with MMLV reverse transcriptase (Promega) and two-base anchored oligo(dT) primers '-T₍₁₂₎AC-3'' following the manufacturer's instructions. The resulting cDNAs were amplified by PCR with primers (5'GAGGACAAG GACTACAAGGA/5'-ACTACACAACAACTGAGCAC). These primers were designed based on the sequence of HvEmip (accession No. X76911) to cover a 1,004 bp long segment that included a large portion of the coding region and most of the 3' non-coding region. One unit of DyNAzyme^{EXT} DNA polymerase (Flowgen, Lichfield, UK) was used in each PCR. The resulting PCR products were purified using a QIAgen Quick PCR purification kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions, cloned into the pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* DH5α competent cells. The cloned cDNAs were then sequenced by MWG-Biotech (Ebersberg, Germany).

Oocyte expression assay

The full coding region of HvPIP1;6 cDNA was produced by PCR with two primers, 5'-CAGGATCCATGGAGGGCAAGGAGGAGG and 5'-CTGGATCCTTAGGACTTGGTCTTGAAT, both primers with an added *Bam*HI restriction site at the 5' ends. The resulting PCR product was cloned into a *Xenopus* oocyte expression vector pGEMHE (supplied by Dr. Ingela Johansson, Glasgow University, UK). Plasmid DNA was digested with *Nhe*I (Promega), and mRNA was produced with an Ambion In vitro Transcription kit (mMessage mMachine Ultra) following the manufacturer's instructions. A 50 ng aliquot of mRNA or 50 nl of water was injected into stage V and VI oocytes obtained from *X. laevis*. The oocytes were incubated in modified Barth's saline [MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES], 10 µg ml⁻¹ sodium penicillin and 10 µg ml⁻¹ streptomycin sulfate, pH 7.4 at 18°C for 2 d. Swelling tests were carried out by transferring oocytes to 20% MBS, and the images were recorded on a video tape using a low magnification (×20) dissection microscope and video camera. To study the effect of mercury on oocyte swelling, some of the oocytes were incubated in MBS containing 50 µM HgCl₂ for 10 min and then assayed as usual in 20% MBS devoid of HgCl₂.

The effect of a kinase inhibitor (k252a), phosphatase inhibitor (OA) and ABA on oocyte swelling was tested as follows. Oocytes injected with mRNA or water were incubated in MBS at 18°C for 2 d and then incubated for 30 min in MBS containing either 0.1% dimethylsulfoxide (DMSO; control), 1 µM k252a and 0.1% DMSO, 5 µM OA and 0.1% DMSO, or 50 µM ABA and 0.1% DMSO.

The P_r of oocytes was calculated from the initial slope of the time course of relative change in oocyte volume following transfer to 20% MBS (Johansson et al. 1998).

Northern analysis

Between 10 and 20 plants were harvested for extraction of RNA, as detailed above, during each experiment. A 20 µg aliquot of total RNA was loaded on agarose/formaldehyde gels, run in 1× formaldehyde gel-running buffer (Sambrook and Russell 2001) and transferred to a nylon membrane. The cDNA probes (see below) were labeled with [³²P]dATP (ICN) using the Prime-a Gene labeling system (Promega). Northern hybridization was carried out following Sambrook and Russell (2001) with 50% (v/v) formamide, 5× SSPE, 0.1% (w/v) SDS, 100 g ml⁻¹ denatured, fragmented salmon sperm DNA and 2× Denhardt's reagents at 42°C for 18 h. Stringency conditions during washes were as follows: first wash with 1% SSC and 0.1% SDS for 20 min at room temperature, and three subsequent washes with 0.2% SSC and 0.1% SDS for 20 min at 68°C.

The cDNAs used as probes for expression analysis of HvPIP1;6 were prepared as follows: the PCR product with the specific aquaporin gene primers (see above) was cloned into pGEMT Easy vector (Promega). The plasmid insert of successful (aquaporin) transformants was amplified with the same primers and purified using a Qiagen PCR Purification kit. The same Northern expression profiles were obtained with probes that were directed either exclusively at the (i) entire coding region; (ii) or the 3' untranslated region; or (iii) covered the major part of the coding region (656 bp) and 3' untranslated region (221 bp) of HvPIP1;6 (not shown). The former two probes were designed using primers 5'-CAGGATCCATGGAGGGCAAGGAGGAGG/5'-CTGGATCCTTAGGACTTGGTCTTGAAT and 5'-CTGCTGCTACAAA GAAAGATG/5'-ACTACACAACA ACTGAGCAC, respectively.

Probes for expression analysis of HvPIP2;1, actin and 18S rRNA were prepared as follows: total RNA was extracted from leaf 3, reverse-transcribed and the cDNA amplified by PCR using gene-specific primers [actin (accession No. U21907): 5'-CCCA GCATTGTAGGAAGGCC/5'-CCTCGGTGCGACACGGAGC; 18S rRNA (accession No. AY211905): 5'-AATATACGCTATT GGAGCTGG/5'-ATGGCTCATTAATCAGTTAT, HvPIP2;1 (accession No. AB009307): 5'-GCAAGCACCGTTCAAGCTAG/5'-GACACCTTCCTCGCCAGGA]. The PCR products were then purified (PCR purification kit, QiaGen) and used as probes.

In situ PCR

In situ PCR experiments were performed according to Popova et al. (2003) with modifications. Tissue sections of 12 µm thickness were prepared of paraffin-embedded leaf segments from the elongation zone, the non-elongation zone and the emerged blade, respectively, of leaf 3 with a microtome and mounted on aminoalkylsilane-coated microscopic slides (Sigma, Germany). The tissue was de-paraffinized with xylene, re-hydrated, and treated with proteinase K (Roche, Germany). cDNA was synthesized with oligo(dT) priming and MMLV reverse transcriptase [H-] (Promega, Germany). The following HvPIP1;6-specific sense and antisense oligonucleotide primers were used for PCR amplifications: 5'-CATCATCATGCAGTGCCT-3' and 5'-TCACTC AGAACACGCAGT-3'. The primers amplified a 518 bp fragment of HvPIP1;6 that includes 457 bp of the coding region and 61 bp of the 3'-non-coding region of the transcript. The following PCR cycle parameters were used: 94°C for 90 min in the first cycle followed by 1 min at 94°C, 1 min at 55°C, 2 min at 72°C with 32 cycles, and a final extension at 72°C for 10 min. Alexafluor 488-5-dUTP (Molecular Probes, The Netherlands) was used as a label. Negative control reactions were performed in parallel by omitting the gene-specific oligonucleotide primers to test the background fluorescence signals. Microscopic images were obtained with a cooled CCD camera coupled to an Axioskop fluorescence microscope using filter set 09 (Zeiss, Germany). The images show HvPIP1;6 transcripts with green fluorescence signals.

Real-time PCR analyses

Three independent harvests of plant material were carried out. During each harvest, the elongation zone, non-elongation zone and emerged blade of the developing leaf 3 was sampled from 5–6 plants, together with 30 mm of the tip of roots (encompassing both growing and non-growing root tissue). Material of a respective plant region was pooled, stored at -80°C and ground to powder in liquid nitrogen prior to extraction of RNA. About 100 mg fresh weight of powdered material was extracted using a Qiagen RNeasy kit (Qiagen, West Sussex, UK) following the manufacturer's instructions. Concentrations of RNA in samples were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, OH, USA), and 2 µg of total RNA was DNase treated in a 20 µl reaction mixture containing 2 µl of 10× buffer (Invitrogen, Carlsbad, CA, USA.), 2 µl of Invitrogen RNase-free DNase I, plant RNA and RNase-free water. The mixture was incubated for 15 min at 25°C and the reaction stopped by addition of 2 µl of 25 mM EDTA, followed by incubation at 65°C for 10 min. Mixtures were briefly chilled on ice, centrifuged and then used for synthesis of cDNA.

cDNA was synthesized in a 20 µl reaction mixture containing 10 µl of DNase-treated sample, 4 µl of 5× First strand buffer (Invitrogen), 2 µl of 0.1 M dithiothreitol (DTT; Invitrogen), 1 µl of dNTP mix (10 mM each, Invitrogen), 1.9 µl of sterile water and 0.1 µl of random primers (Promega). To this mixture was added

1 µl of either Superscript[®] II RNase H⁻ Reverse Transcriptase (Invitrogen) or sterile water (negative control, to check for residual contamination with DNA). Reaction mixtures were incubated at 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. Thereafter, 80 µl of sterile water was added to each sample, and these diluted cDNA samples were stored at -20°C.

Real-time PCR was carried out using the Rotor-Gene 3000 real time amplification (Corbett Life Science, Sydney, Australia) and Qiagen QuantiTect[®] SYBR Green system. Each 25 µl reaction mixture contained 12.5 µl of SYBR-Green master mix, 4.5 µl of RNase-free water, 1.5 µl each of gene-specific forward and reverse primer and 5 µl of diluted cDNA sample. Up to 36 samples were run in parallel. The Rotor-gene run set-up consisted of a hot start (15 min at 95°C), followed by 40 cycles of 95°C for 20 s, 59.5°C for 30 s and 72°C for 30 s. Product size and the specificity of primers were routinely checked by running PCR products on 1.5% agarose gels. All aquaporin primers, which were designed for the untranslated region of genes, gave only one band and at the expected (bp) size (170, 131, 189, 117 and 147 bp for HvPIP1;2, 1;3, 1;4, 1;5 and 1;6, respectively). The primer for ubiquitin (X04133), which was designed for the translated region of polyubiquitin, also gave one band, at about 230 bp. Sequences (5' to 3') of gene-specific forward (F) and reverse (R) primers were as follows: HvPIP1;2 (F-CAAGAGCCGCGACTAGTATAC, R-CGCTCGC TCAAGAACAGTAC), HvPIP1;3 (F-AATAGTTCTCAAATCT GCCGTCTC, R-TACTGGTACAATGCAAAAGGTAGG), HvPIP1;4 (F-CGACTGTGGATGGAGTCTTC, R-CCTTATAT GGGCACATAGCC), HvPIP1;5 (F-ATCGGAATAACGA GCCTGTTTC, R-ACCACAACACTGTCACACTGAATTAC), HvPIP1;6 (F-ACTGCGTGTCTGAGTGAATTTCT, R-CAT ACAAGTCTCTGTCTGGTTC), and barley polyubiquitin (F-AGGCGAAGATCCAGGACAAG, R-ACAACCAGACATG CTCCAACCT). During each run, a calibration curve using ubiquitin primers and bulked-up root cDNA was run, together with one complete set of samples; this consisted of three leaf zones and the root sample of one particular harvest being analyzed for expression of HvPIP1;2-1;6 and ubiquitin. Standard curves were also prepared for each set of primers (using bulked-up root cDNA) to check for 100% PCR efficiency corresponding to a slope of -3.3 (Marino et al. 2003); all primers yielded slopes of between -3.1 and -3.4. Expression data were analyzed by the $2^{-\Delta\Delta C_t}$ method (Pfaffl 2001). Normalization of expression data of PIP genes was achieved using ubiquitin as reference ('housekeeping') gene. Although we would have preferred to use several control genes (Vandesompele et al. 2002), we were unable to do so since alternative genes that are often used as control (e.g. actin, tubulin, elongation factor II) showed large differences in expression between growing and non-growing leaf tissues. To avoid any interference with comparison of expression between plant regions through slight differences in yield between PCR runs, expression ratios (PIP: ubiquitin) of leaf zones were related to the value for the root. For each plant harvest, leaf region and PIP1 gene, the average of three independent PCR runs was calculated and used to calculate the overall average for the three plant harvests.

Plasma membrane isolation

Plasma membranes were isolated from barley leaves following the procedure described by Kjellbom and Larsson (1984). Four replicate experiments were carried out. During each experiment, between 40 and 70 plants were harvested. The growing leaf 3 was freed of surrounding sheaths of older leaves, and the younger leaf 4, which developed inside leaf 3, was discarded. Leaf 3 was sectioned into three zones: (i) elongation zone (2-40 mm from the

leaf base); (ii) non-elongation zone (40 mm from the leaf base to the point of emergence from the sheath of leaf 2); and (iii) emerged portion of the blade (the non-elongation zone and emerged blade samples comprised more leaf tissue than for RNA extraction to ensure that enough plant material was available for plasma membrane isolation). It took 1-2 min to harvest one plant, and sections were stored in homogenization buffer (Kjellbom and Larsson 1984) on ice for up to 90 min prior to isolation of plasma membranes.

The protein concentration of samples was determined according to Bearden (1978).

SDS-PAGE and Western blot

Plasma membrane proteins were separated on SDS-acrylamide gels using a BioRad mini gel system. Between 7.5 and 15 µg of protein was loaded per lane (for details see figure legends). Gels were run at 160 V for 40-45 min. Following electrophoresis, gels were either stained for protein using Coomassie brilliant blue R250 or electroblotted onto Immobilon-P transfer (polyvinylidene fluoride) membranes (Millipore) at 100 V for 1 h using a BioRad mini-system (Correct, Hercules, CA, USA).

Membranes were blocked with a 3% solution of skimmed milk powder (BioRad) in phosphate-buffered saline (PBS) for 1 h at room temperature and then rinsed briefly with PBS and incubated with primary antibody on a shaker overnight at 4°C or for 75 min at room temperature. This was followed by a brief rinse with PBS and three washes, 10 min each, with PBS containing 0.05% Tween-20. Membranes were incubated with secondary antibody for 1 h at room temperature and washed twice, 10 min each, with PBS containing 0.05% Tween-20, followed by two 5 min washes with PBS. Antibody binding was visualized using ECL[™] detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Two different primary antibodies were used, one recognizing (barley) PIP1s and one recognizing HvPIP2;1. The anti-PIP1 antibody had been designed originally to recognize a conserved region at the N-terminus of spinach PIP1s (Epitope: RLGANKYSC, Innovagen, Lund, Sweden). This region is shared by barley PIP1s (Fig. 1, amino acid symbols in boxes). The second antibody was a kind gift of Professor Katsuhara (Okayama University, Kashiki, 710-0046 Japan) and had been raised to recognize HvPIP2;1, the only previously known water channel in barley.

Statistics

The statistical significance of differences in P_f between treatments was assessed by *t*-test (Excel). The number of replicates and the level of significance are given in the figure legends and text, respectively.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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